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The zebrafish *spiel-ohne-grenzen* (*spg*) gene encodes the POU domain protein Pou2 related to mammalian *Oct4* and is essential for formation of the midbrain and hindbrain, and for pre-gastrula morphogenesis

Shawn Burgess[1,*,‡], Gerlinde Reim[2,‡], Wenbiao Chen[1,†], Nancy Hopkins[1] and Michael Brand[2,§]

[1]Massachusetts Institute of Technology, Center for Cancer Research, E17-340, 77 Massachusetts Avenue, Cambridge, MA 02139, USA
[2]Max Planck Institute for Molecular, Cell Biology and Genetics, Dresden, Pfotenhauer Str. 108, 01307 Dresden, Germany
*Present address: Genome Technology Branch, National Human Genome Research, National Institutes of Health, Bethesda, MD 20892, USA
†Present address: Vollum Institute, Oregon Health Sciences University, Portland, OR 97201, USA
‡These authors contributed equally to this work
§Author for correspondence (e-mail: brand@mpi-cbg.de)

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SUMMARY

In early embryonic development, the brain is divided into three main regions along the anteroposterior axis: the forebrain, midbrain and hindbrain. Through retroviral insertional mutagenesis and chemical mutagenesis experiments in zebrafish, we have isolated mutations that cause abnormal hindbrain organization and a failure of the midbrain-hindbrain boundary (MHB) to form, a region that acts as an organizer for the adjacent brain regions. The mutations fail to complement the *spiel-ohne-grenzen* (*spg*) mutation, which causes a similar phenotype, but for which the affected gene is unknown. We show through genetic mapping, cloning of the proviral insertion site and allele sequencing that *spg* mutations disrupt *pou2*, a gene encoding the Pou2 transcription factor. Based on chromosomal synteny, phylogenetic sequence comparison, and expression and functional data, we suggest that *pou2* is the zebrafish ortholog of mouse Oct3/Oct4 and human POU5F1. For the mammalian genes, a function in brain development has so far not been described. In the absence of functional *pou2*, expression of markers for the midbrain, MHB and the hindbrain primordium (*pax2.1*, *wnt1*, *krox20*) are severely reduced, correlating with the neuroectoderm-specific expression phase of *pou2*. Injection of *pou2* mRNA restores these defects in *spg* mutant embryos, but does not activate these markers ectopically, demonstrating a permissive role for *pou2*. Injections of *pou2*-morpholinos phenocopy the *spg* phenotype at low concentration, further proving that *spg* encodes *pou2*. Two observations suggest that *pou2* has an additional earlier function: higher *pou2*-morpholino concentrations specifically cause a pre-gastrula arrest of cell division and morphogenesis, and expression of *pou2* mRNA itself is reduced in *spg*-homozygous embryos at this stage. These experiments suggest two roles for *pou2*. Initially, Pou2 functions during early proliferation and morphogenesis of the blastomeres, similar to Oct3/4 in mammals during formation of the inner cell mass. During zebrafish brain formation, Pou2 then functions a second time to activate gene expression in the midbrain and hindbrain primordium, which is reflected at later stages in the specific lack in *spg* embryos of the MHB and associated defects in the mid- and hindbrain.

Key words: *pou2*, Oct3/4, Pou5f1, Isthmus, MHB, Organizer, *pax2.1*, *spiel-ohne-grenzen*, Hindbrain, Zebrafish

INTRODUCTION

The mechanisms involved in the formation of the various subdivisions of the brain share many common elements among vertebrates, making it possible to generalize information learned in one organism to all vertebrates. Because zebrafish can be used effectively in large-scale classical genetics, there is great potential to use zebrafish to discover new factors involved in the early brain development of all vertebrates.

In vertebrates, regional identity within the neurectoderm is established during gastrulation. Originally it was believed that the dorsal gastrula organizer (Spemann, 1938; Nieuwkoop, 1973; Kodjabachian and Lemaire, 1998; Solnica-Krezel, 1999), which establishes dorsoventral (DV) patterning through BMP, Noggin and Chordin signaling (Hammerschmidt et al., 1996; Bauer et al., 1998; Fekany-Lee et al., 2000), is also responsible for the anteroposterior (AP) determination of the neurectoderm. However it has since been shown by studying several zebrafish mutants that the AP axis is not disrupted in the absence of many of these signals (Barth et al., 1999; Fekany-Lee et al., 2000). Thus, how the AP axis of the neurectoderm is established in the early embryo remains unknown.

Cell fates of the neurectoderm are in large part demarcated by a sequence of gene expression patterns that are established early in the developmental program. In zebrafish, *otx1* and *otx2* are first observed at about 65% epiboly in what will ultimately become the fore- and midbrain (Li et al., 1994; Mori et al., 1994). *fgf8, wnt1* and *pax2.1* (*pax2a* – Zebrafish Information Network) appear next, defining the mid-hindbrain region (Krauss et al., 1991a; Kelly and Moon, 1995; Lun and Brand, 1998; Reifers et al., 1998; Rhinn and Brand, 2001). Slightly later, *pax6* expression is evident in both the forebrain and hindbrain regions (Krauss et al., 1991b; Amirthalingam et al., 1995). Thus, the neuroepithelium is divided into a minimum of three domains with unique molecular identity before any brain structures are visible under the microscope. However, many aspects of how this regionalization is established remain elusive.

The boundary region that separates the midbrain and the hindbrain (MHB, also known as isthmus), serves as an organizer for midbrain and cerebellum development. Grafts of isthmic tissue induce ectopic midbrain and/or cerebellar tissue in chick embryos, depending on the location of the graft (Martinez et al., 1991; Puelles et al., 1996; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). In zebrafish, large-scale genetic screens have identified many mutations that affect early developmental stages (Driever et al., 1996; Haffter et al., 1996; Amsterdam et al., 1999; Talbot and Hopkins, 2000). Among the isolated mutations, three genes affect the MHB region in a specific manner when mutated. The *acerebellar* (*ace*) mutation inactivates the zebrafish *fgf8* gene (Brand et al., 1996; Reifers et al., 1998). *no isthmus* (*noi*) mutations are in the zebrafish *pax2.1* gene (Brand et al., 1996; Lun and Brand, 1998). Mutations in the third gene, *spiel-ohne-grenzen* (*spg*), cause lack of the cerebellum and the isthmus, with a variably enlarged tectum and ear defects (Schier et al., 1996; Driever et al., 1997), a phenotype resembling that of *acerebellar* mutants. We have identified the gene altered by *spg* mutations as *pou2*, which encodes a POU homeobox transcription factor.

POU homeobox transcription factors have been described in different species, and function during nervous system development. Four original transcription factors *Pit-1, Oct-1, Oct-2*, and *Unc-86* share a region of homology that defines a unique class with a bipartite DNA-binding domain that consists of a POU-specific domain (POU_S) and a homeodomain (POU_H) (for a review, see Ryan and Rosenfeld, 1997).

In two ongoing genetic screens, one using pseudotyped retroviruses as an insertional mutagen (Lin et al., 1994; Gaiano et al., 1996; Amsterdam et al., 1999; Talbot and Hopkins, 2000) and the second a chemical mutagenesis screen in haploid embryos (C. Klisa, N. Morita, G. R., W. Huttner and M. B., unpublished), mutations were isolated that mimicked the *spg* phenotype. We demonstrate here, by complementation testing and genetic mapping that the newly isolated mutations fail to complement each other and are allelic to *spg.* A chemically induced allele and the proviral integration in the insertional mutation map to and disrupt the *pou2* gene (Takeda et al., 1994; Hauptmann and Gerster, 1995), which encodes a class V homeodomain transcription factor with a POU domain (Ryan and Rosenfeld, 1997). Injection of antisense morpholinos phenocopy the *spg* phenotype. Based on the syntenic chromosomal position and phylogenetic sequence comparisons, we suggest that *pou2* is orthologous to the mammalian *Oct3/Oct4/Pou5f1* genes. We show that *pou2* expression is crucial to proper formation and development of the zebrafish midbrain, isthmus and hindbrain primordia, in keeping with its specific expression in these territories. We also demonstrate that the expression of *pou2* is essential for the early onset of the MHB, establishing *pou2* as a regulator upstream of *pax2.1* in formation of the mid-hindbrain boundary. In the accompanying paper, we also report a detailed phenotypic analysis of *spg* function in early brain development, leading us to propose that *spg*/*pou2* acts to confer regionally specific competence to respond to Fgf8 signaling during brain development (Reim and Brand, 2002).

MATERIALS AND METHODS

Fish maintenance

Normal practices were used for fish rearing and mating (Westerfield, 1995; Brand et al., 2002). Mutant carriers were identified by PCR or random intercrosses. Mutant embryos were obtained by inbreeding heterozygous carriers. Morphological features and time of development at 28.5°C were used to stage embryos.

Gene cloning

Linker-mediated PCR was used to identify the integration responsible for the mutant phenotype and to isolate the adjacent genomic DNA. Briefly, DNA was isolated from either tail biopsy from the adult carrier fish or whole phenotypic embryos digested with *Mse*I and ligated to linkers made from annealed primers with the sequences 5′-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGG-GCTGGT-3′ and 5′-TAACCAGCCCAACTTCGAATTAAA-3′. PCR was performed using the oligonucleotides 5′-GTAATACGACT-CACTATAGGGC-3′ and 5′-CGTGTATCCAATAAACCCTCTTGC-3′. A nested PCR reaction was performed using the primers 5′-ACTATAGGGCACGCGTGGT-3′ and 5′-AACAAGGAAGTAGC-AAAG-CTGCTC-3′. The samples were run on a 7% non-denaturing polyacrylamide gel, stained with Ethidium Bromide and destained with distilled water. The single band common to all the carrier adults and all the mutant embryos was excised from the acrylamide gel, eluted re-amplified and sequenced. Further genomic sequence was obtained using linker-mediated PCR from wild-type genomic DNA using primers based on the sequence obtained from the first amplification.

Linkage of the proviral integration to the mutant phenotype was established using PCR. DNA from phenotypically mutant embryos or phenotypically wild-type embryos was isolated and PCR was performed using the oligos 5′-ACGTCTTCTTTTAAGG-AGACTCTGACTA-3′, 5′-ACTCACATCCTGAGGGTTCTCG-3′ and 5′-CGTGTATCCAATAAACCCTCTTGC-3′. These primers differentiated between homozygous wild type, heterozygous and homozygous mutants for the proviral locus. Inverse PCR was performed as described previously Becker et al. (Becker et al., 1998). Genetic linkage of the *spg* locus to the *pou2* locus was carried out by a PCR-based mapping method (Knapik et al., 1996) showing co-segregation of the *spg* phenotype with the SSR (simple sequence repeat) marker z13467. The mapping marker serves as a PCR primer. Wild-type and spg^{e713} DNA of haploid embryos was used. Haploid embryos were generated as described (Westerfield, 1995).

Sequencing of mutant alleles

cDNA from homozygous mutant *spg* and control wild-type sibling embryos was used to amplify the coding region of the mutant spg alleles, then subcloned and sequenced. Surprisingly, spg^{m216} and spg^{e713} showed the same base change, causing a Lys to Pro exchange in the POU-specific domain. The result was reproduced many times.

Both mutations were induced in different genetic backgrounds in different laboratories, but ultimately derive from a common Oregon AB background. We recovered *spg*^{e713} from a mutagenized strain that was genetically marked with the pigment mutation golden; moreover, *spg*^{e713} was initially linked to a second unrelated mutation that was induced on the same chromosome and that we subsequently removed by recombination. The same base change in *pou2* was found on the chromosomes before and after the recombination event. We therefore think it unlikely that *spg*^{e713} arose owing to contamination of our golden stock that was used in the mutagenesis. Because the two mutations carry the same molecular change, and also share a number of closely linked SNIPs, the mutation may have been present as a polymorphism in the AB wild-type population that was mutagenized; *spg*^{e713} and *spg*^{m216} would then be re-isolates of a background mutation that was present in the original AB wild-type population at a low frequency. Alternatively, a mutation in this position could generate a particularly strong, and hence easily detected, phenotype (for an example of two identical mutations of Pax2 arising in mouse and human) (Favor et al., 1996). Until this issue is resolved, we will treat the two mutations as separate alleles.

In situ hybridization and antibody staining

In situ hybridization and antibody detection were performed using protocols described previously. Probes used were for *pou2* (Takeda et al., 1994), *pax2.1* (previously *pax[zf-b]*) (Krauss et al., 1991a), *krox20* (*egr2* – Zebrafish Information Network) (Oxtoby and Jowett, 1993) and *pax6* (Li et al., 1994). Anti-acetylated tubulin antibody (Sigma) and 3A10 monoclonal antibody (Furley et al., 1990) were also used. Distribution of co-injected *lacZ* mRNA is visualized by staining with anti-β-gal antibody (Promega, 1:500) after in situ hybridization (Dornseifer et al., 1997).

RNA and morpholino injections

RNA was transcribed from plasmid pCS2+ (Rupp et al., 1994), using conventional methods, and injected as described previously (Reifers et al., 1998). Briefly, embryos were harvested from matings of wild-type fish 10 minutes after the appearance of the first fertilized eggs. Embryos were put in 20 ml of Holtfreter's solution (Westerfield, 1995) and several drops of 30 mg/ml pronase was added. At the first sign of chorion separation, the embryos were washed five to six times with Holtfreter's solution. Embryos were placed on a agarose ramp under Holtfreter's solution and RNA is injected into the cell cytoplasm from the one- or two-cell stage. Embryos were then moved to 24-well dishes filled with Holtfreter's solution and placed at 28.5°C for 8-24 hours. Morpholinos were designed and synthesized by Gene-Tools, LLC (Corvallis, OR). The sequence of the anti-*pou2* morpholino-1 is 5′-CGCTCTCTCCGTCATCTTTCCGCTA-3′ (underlined bases were exchanged to generate the 4 bp mismatch control morpholino, Mo1-ctrl), the morpholino-2 is 5′-TTCAAACAAGAAAGCGTAAA-GACTG-3′. The sequence of the control morpholino is 5′-CCT-CTTACCTCAGTTACAATTTATA-3′ (Gene Tools).

RESULTS

Mutagenesis screens yield new alleles of *spg*

In the course of a large-scale, insertional mutagenesis screen for mutations that affect embryonic development (Amsterdam et al., 1999), we isolated a mutant carrier family Hi349. The mutation has several different phenotypes that display significant variation in their expression. The earliest visible phenotype is a loss of the fold at the midbrain hindbrain boundary (MHB), which is clearly visible after the first 24 hours of development (Fig. 1). Phenotypes range from a reduction of the cerebellum to a complete deletion of the MHB area without visible change in the anterior tectum or the posterior hindbrain regions. Other phenotypes include mis-shapen and smaller otic vesicles [commonly containing only a single otolith (Fig. 1B)], a variable defect in the length of the tail (Fig. 1D) and a significant reduction in the escape reflex response to touch (not shown). Two additional alleles were isolated in a haploid ENU mutagenesis screen for mutants affecting development of the MHB organizer, as initially judged by loss of *fgf8* expression at the MHB (N. Morita, C. Klisa, G. R., W. Huttner and M. B., unpublished). Homozygotes for the mutation *e713*, which causes lack of *fgf8* expression, lack the MHB during pharyngula stages. A similar, albeit weaker disruption was found in the mutant *e68*, which retains a partially formed MHB (Fig. 1C). The similarity of the mutant phenotypes to the mutant *spg* isolated in an earlier chemical mutagenesis screen (Schier et al., 1996) suggested that we had isolated new alleles of this mutation. In complementation test crosses with the previously isolated MHB mutants *noi*, *ace* and *spg* (Brand et al., 1996; Schier et al., 1996), Hi349, *e713* and *e68* all failed to complement *spg*^{m216}, and are therefore new alleles of *spg*. From this point onwards, the mutations will be known as *spg*^{hi349}, *spg*^{e713} and *spg*^{e68}. For further phenotypic analysis we concentrated on the strong alleles *spg*^{hi349}or *spg*^{e713}, which give very similar phenotypes when homozygous.

We used antibodies against acetylated tubulin at 30 hours postfertilization (hpf) on whole mutant embryos homozygous for *spg*^{hi349}or *spg*^{e713} to examine the architecture of the axonal scaffold in the brain. The segmented appearance of the wild-type scaffold is severely disrupted in the hindbrain of mutant embryos (Fig. 1E,F) and the cerebellar commissure at the MHB is missing, probably because of the missing MHB, whereas other parts of the brain appear unaffected in the mutant embryos (not shown). Therefore, in addition to the MHB, overall hindbrain development is also disrupted in *spg* mutants. In wild-type embryos, monoclonal antibody 3A10 clearly stains the Mauthner neurons in whole-mount staining at 30 hpf. In the mutant embryos, the Mauthner neurons, which normally form in rhombomere 4 of the hindbrain, are completely absent (Fig. 1G,H), suggesting that very specific disruption of cell types also occurs in the hindbrain of *spg* mutants.

spg mutations are in the zebrafish *pou2* gene

Four out of 14 mating pairs from family had offspring that showed the mutant phenotype. Tail biopsies from each *spg*^{hi349} heterozygous carrier fish were taken and the genomic DNA was isolated. Using linker-mediated PCR and a primer specific to proviral sequences, we identified a single proviral insert that was present in both parents of each pair whose offspring showed the mutant phenotype, but was never present in both parents in pairs that did not display the phenotype (data not shown). The fragment containing the genomic DNA that was 3′ of the identified insertion site was excised from a polyacrylamide gel and sequenced. The genomic DNA showed that the provirus had integrated into an exon of the zebrafish *pou2* gene, a transcription factor identified previously (Takeda et al., 1994; Hauptmann and Gerster, 1995). Using Southern blot analysis and inverse PCR, the genomic DNA adjacent to the 5′ side of the virus was also isolated and it showed that the DNA on the other side of the provirus continued in the same *pou2* exon. The proviral integration was 875 nucleotides from

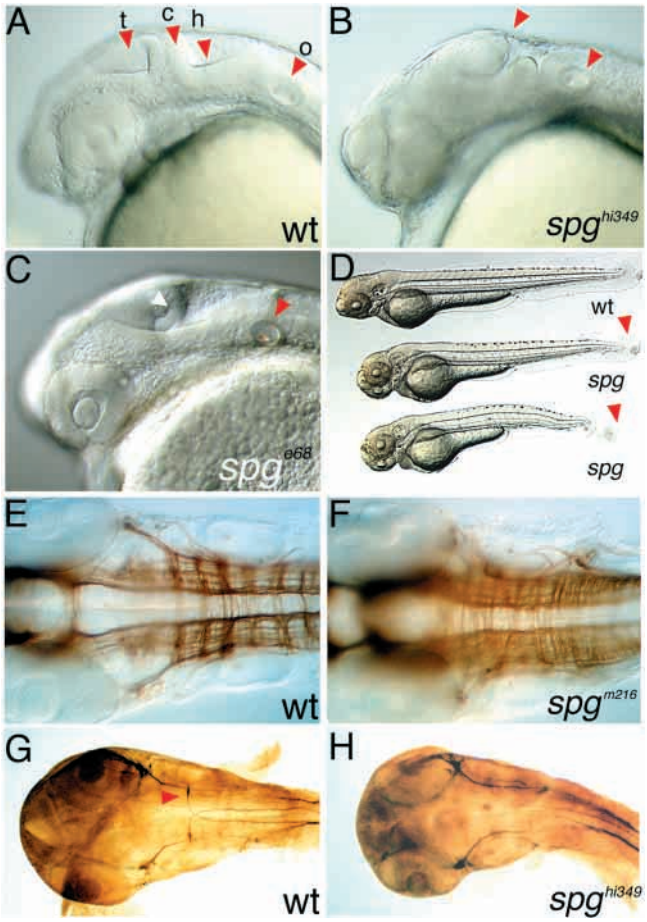

Fig. 1. Morphology of *spg* mutants. (A-D) Lateral views. (E-H) Dorsal views. (A) Wild-type embryo at 26 hours post fertilization. c, cerebellum; h, hindbrain; o, otic placode; t, tectum. (B) *spg*^{hi349} homozygous mutant embryo at 26 hpf. There is significant variability of the phenotype. The hindbrain displays disorganization (left arrowhead) and the otic placode is reduced in size, containing only one otolith (right arrowhead); note the proximity of the otolith to anterior brain structures when compared with wild type. (C) *spg*^{e68} homozygous embryos are less affected at the hindbrain than the insertional mutant (B). (B,C) In *spg* mutant embryos, no proper MHB structures are visible (red and white arrowheads, respectively). (D) The *spg*^{hi349} mutation causes a variable shortening and altered morphology of the tail. The embryo at the top is wild type, and the two beneath are both *spg*^{hi349}/*spg*^{hi349}. The effect varies from a very minor kink to major shortening and structural defects (see arrowheads). (E,F) α-acetylated tubulin staining recognizing the axonal scaffold in the developing brain at 28 hpf. (E) Wild type. Six bilateral transverse axon bundles mark the borders between single rhombomeres. (F) *spg* mutant embryo shows strong disorganization of the axonal scaffold within the hindbrain. (G,H) Staining with the monoclonal antibody 3A10 at 30 hpf. (G) The Mauthner neurons in the wild-type embryo are marked with a red arrowhead. (H) *spg*^{hi349}/*spg*^{hi349} mutant embryo showing complete absence of the Mauthner neurons.

the starting methionine in the cDNA sequence, 37 amino acids into the POU-specific domain (Fig. 2A). As this integration disrupts the POU-specific domain, and truncates the protein ahead of the entire POU homeodomain, it is likely that this integration generates a null allele.

Independently, we mapped one of the chemically induced alleles, *spg*^{e713}, and found it to be linked to the same chromosomal location as *pou2* (Fig. 2B,C) (Geisler et al., 1999). z13467 is a marker located on chromosome 21 at 32.4 cM, very close to *pou2*. This marker gives different size bands, depending on the genetic background, and we found no recombinants between this marker and the spg mutant phenotype among 39 haploid embryos obtained from a heterozygous carrier female (21 *spg* and 18 wild-type; Fig. 2B), showing that *spg* is linked genetically to *pou2*. To determine the molecular defect in the ENU alleles, we used *pou2*-specific primers to amplify the coding region of the ENU alleles. Sequencing revealed a single amino acid exchange in *spg*^{m216} and *spg*^{e713} from Lys to Pro in helix one of the POU homeodomain, which most probably disrupts the function of the protein. Both mutations were induced on a similar genetic background (AB), but were found in independent mutagenesis experiments with strains that carried different genetic markers, suggesting that this mutation may have been present in the AB background at a low frequency (see also Materials and Methods). No amino acid exchange was found in the coding region of the weak allele *spg*^{e68}, which we hypothesize might therefore affect the regulatory sequences of *pou2*.

Once the molecular nature of *spg* alleles had been identified, we designed primers that distinguished between homozygous wild-type, homozygous mutant and heterozygous embryos for the proviral insertion. Nineteen phenotypically mutant embryos and, 19 phenotypically wild-type embryos were tested with these primer sets, 19/19 mutant embryos were homozygous for the Pou2 insertion while 0/19 phenotypically wild-type embryos where homozygous at that locus (data not shown). In situ hybridization of embryos from *spg*^{hi349} carrier parents using *pou2* sequences as the probe showed 22 of 78 embryos with a severe reduction in staining starting at 40-50% epiboly (not shown); expression is totally abolished at the end of gastrulation (see below, Fig. 4E). A reduction of *pou2* mRNA is also observed in homozygous *spg*^{m216} and *spg* ^{e713} embryos, but slightly later from the beginning of somitogenesis onwards (not shown). These observations argue that Pou2 is involved in feedback regulation of its own expression, that it already functions before the onset of gastrulation in all embryonic cells and that *spg*^{hi349} is the strongest allele.

Previous studies of *pou2* had not resolved the relationship between zebrafish *pou2* and mammalian Pou genes. Phylogenetic sequence comparison with the full-length sequence furthermore shows that these genes fall into the same class V subfamily of POU transcription factors (Fig. 2E). In the conserved POU domain, Pou2 is 74% identical and 89% similar to murine Oct3, and in the in the homeodomain 71% and 84%, respectively. Outside these functional domains there is little conservation, giving lower values over the entire amino acid sequence (30% identity, 40% similarity). Importantly, the chromosomal position of *pou2* shows that it is located in an area that is syntenic with mammalian chromosomes, and which contains the *Oct3*/*Oct4*/*Pou5f1* genes in mice and humans (Fig. 2D) (Woods et al., 2000). Furthermore, mouse *Oct3/Oct4* is expressed in the neural plate at the right time, and injection of mouse *Oct3/Oct4* rescues *pax2.1* expression in *spg* homozygotes (Reim and Brand, 2002). We therefore suggest that *pou2* is the ortholog of the mammalian *Oct3/Oct4/Pou5f1* genes.

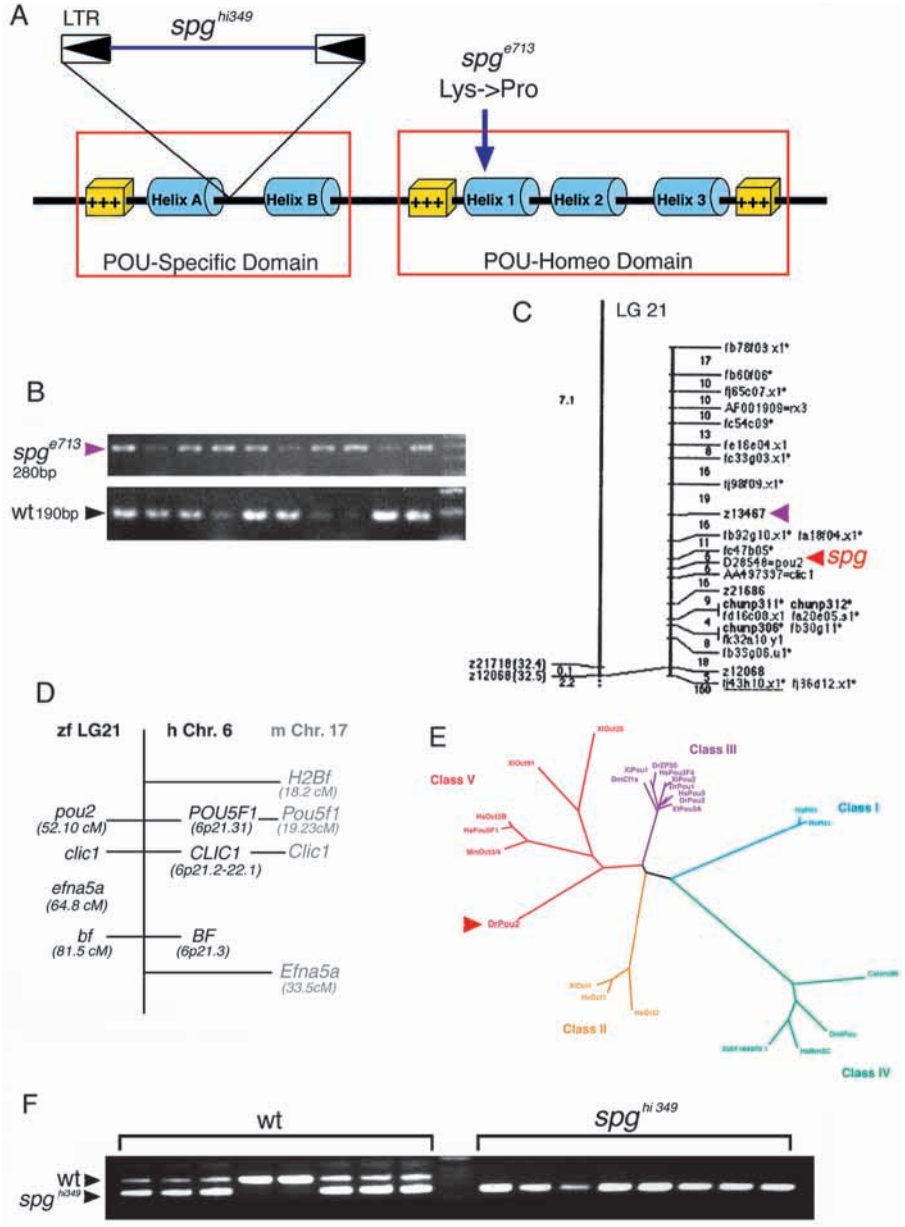

Fig. 2. *spg* mutations affect the *pou2* gene. (A) Part of the Pou2 protein containing the POU-specific and the POU-homeodomain. The insertional mutation *spg*^{hi349} and the point mutation *spg*^{e713} are indicated. The point mutation is based on a transition from T to C, leading to an amino acid exchange from leucine to proline. (B) *spg* is genetically linked to the SSR marker z13467 mapping on chromosome 21 (indicated by an purple arrow in C). z13467 was used as a diagnostic marker in PCR-based mapping of wild type and *spg*^{e713}, resulting in an amplification product of 280 bp for the mutant (purple arrow) and 190 bp for the wild type (black arrow). (C) Detail from chromosome 21. The red and the purple arrow indicate the position of the mapping marker z13467 and *pou2*/*spg*, respectively. (D) Syntenic relationship between the zebrafish linkage group 21, human chromosome 6 and mouse chromosome 17. Zebrafish *pou2*, *clic1*, *efna5a* and *bf* and their mammalian orthologs show conserved synteny (sources, OMIM and ZFIN). The different order of *efna5a* and *bf* may be due to smaller inversions that frequently occur in an overall syntenic area (Woods et al., 2000). (E) Zebrafish Pou2 (DrPou2, red arrowhead) and its mouse (MmOct3/4) and human (HsPou5F1) ortholog cluster within the ClassV POU domain protein subfamily in an unrooted phylogenetic tree, the closest mammalian members being in the Oct3/4/Pou5f1 subgroup. (F) PCR genotyping of eight normally expressing *pax2.1* embryos and eight embryos with impaired *pax2.1* expression showing all *pax2.1*-deficient embryos are also *spg*^{hi349}/*spg*^{hi349}. PCR product sizes for wild-type genomic DNA (WT) and *spg*^{hi349} are marked. Among eight embryos normally expressing *pax2.1* are *pax6* embryos that are heterozygous for *spg*, as indicated by the occurrence of both the wild-type- and the mutant-specific PCR products.

Pou2 is required for *pax2.1* and *krox20* expression

To analyze the *spg* phenotype further, we performed in situ hybridization on embryos collected from matings of two *spg*^{hi349} or *spg*^{e713} heterozygous adult carrier fish. Using *krox20* and *pax6*, we found disruptions in the normal organization of the hindbrain at very early stages of development. *Krox20* expression normally starts as a stripe of expression in the presumptive rhombomere 3 (Fig. 3A). Later, at the three-somite stage, two parallel bands of expression define the location for rhombomeres 3 and 5 (Fig. 3C). In *spg*^{hi349} homozygous embryos, the expression of *krox20* shows a marked reduction even at the very first sign of *krox20* staining, and in particular the shape of the expression domain becomes pointed and smaller close to the midline (Fig. 3B,D). *pax6* also shows alterations in expression. The normal regions of expression for *pax6* are in the forebrain and hindbrain separated by the MHB that is devoid of expression (Fig. 3E). In *spg*^{hi349} homozygous embryos, the forebrain domain of *pax6* expression expands posteriorly and nearly fuses with its hindbrain expression domain, creating one large domain of expression by the 10 somite stage (Fig. 3F).

Using *pax2.1*, a marker for the presumptive midbrain and MHB, showed that one quarter of the embryos had a strong reduction of *pax2.1* expression (Fig. 3H), as reported previously (Schier et al., 1996). Because of the slight variability associated with the *spg* phenotype, we wanted to ascertain that the early defective gene expression is specific to homozygous *spg* embryos. We collected individual embryos either lacking *pax2.1* expression or with wild-type expression and isolated their genomic DNA. PCR analysis confirmed that the embryos without *pax2.1* expression were homozygous for the *spg*^{hi349} integration (Fig. 2F). Induction of *pax2.1* was unaffected in other areas such as the otic placode (not shown).

To understand the mechanism by which Pou2 activates

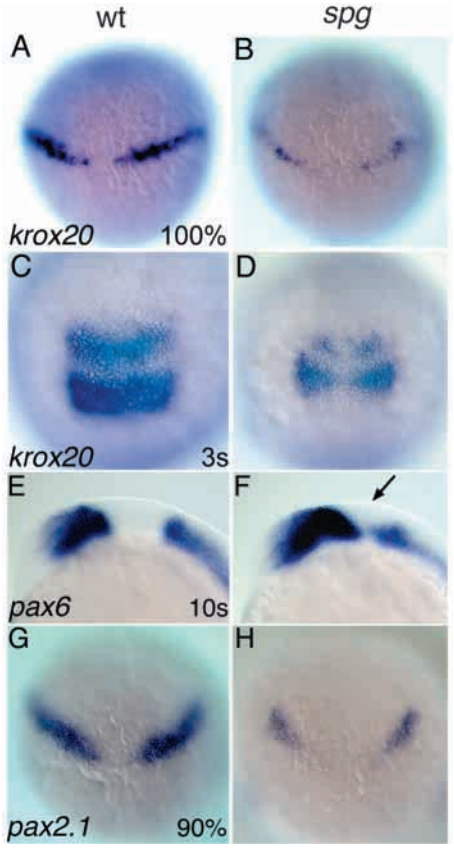

Fig. 3. Abnormal development of the hindbrain- and the MHB primordium in spg mutants. (A-D) *krox20* is not properly initiated in r3 (B,D) and r5 (D); in *spg*^{hi349} mutant embryos, *krox20* staining often fails to fuse at the midline in mutant embryos (D). (E) *pax6* is expressed within the forebrain and the hindbrain, but spared at the MHB in wild-type embryos. (F) The forebrain expression domain of *pax6* invades into the prospective MHB region (arrow). (G) *pax2.1* expression is initiated between 80 and 90% of epiboly in wild-type embryos at the MHB. (H) In *spg*^{hi349} mutant embryos, *pax2.1* is downregulated from its onset of expression at the MHB.

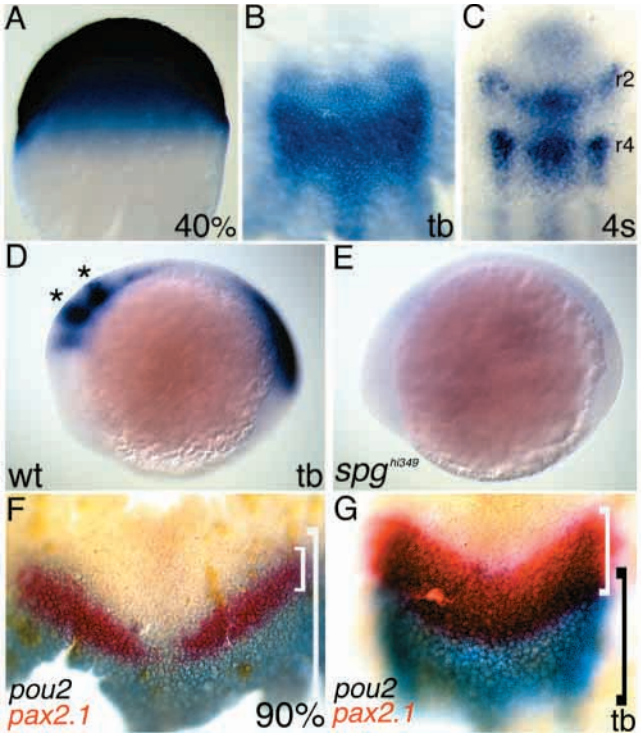

Fig. 4. *pou2* expression during embryogenesis. Shown are wild-type (A-D,F-G) and *spg*^{hi349}/*spg*^{hi349} mutant embryos (E). (A) Animal pole upwards. (B,C,F,G) Dorsal views, anterior is upwards; (D,E) Lateral view, anterior towards the left. (A) *pou2* is ubiquitously expressed before gastrulation. (B) *pou2* expression refines within the neuroectoderm at the end of gastrulation. (C) During early somitogenesis, *pou2* expression becomes restricted to the hindbrain within r2 and r4, and to a ventromedial patch at the MHB. (D) Wild-type embryo at the tailbud stage. Expression in rhombomeres 2 and 4 is indicated by asterisks. Beside the expression within the brain, *pou2* is also expressed within the posterior spinal cord. *pou2* vanishes during gastrulation in *spg*^{hi349} embryos (not shown) and at tailbud stage (E), no *pou2* expression can be detected anymore throughout the embryo. (F,G) Double in situ hybridization with *pou2* (blue, long brackets) and *pax2.1* (red, short brackets). At 90% epiboly, MHB expression of *pax2.1* is completely contained within the anterior *pou2* expression domain (F) but slightly later, at tb stage, expression domains of *pax2.1* and *pou2* start to separate from each other (G).

pax2.1 transcription, we examined the relative distribution of these genes during formation of the midbrain and hindbrain primordia by whole-mount double ISH. Both Takeda et al. (Takeda et al., 1994) and Hauptmann and Gerster (Hauptmann and Gerster, 1995) reported that at tailbud- to two-somite stages these genes occupy non-overlapping expression domains in the midbrain and anterior hindbrain primordium, respectively, but earlier stages of neural development were not examined. We find that at 80%-90% epiboly, the *Pou2* domain is initially broader and encompasses the *pax2.1* domain at its anterior end (Fig. 4F). Pou2 is therefore available to regulate *pax2.1* in the same cells at this stage, which may explain why the midbrain is affected in *spg* mutants. As development of the embryo progresses, the overlap of expression reduces in size, until at the tailbud stage, the majority of the *pou2* expression lies in the anterior hindbrain primordium (Fig. 4G), posterior to the *pax2.1* domain. As described above, *pou2* is required at this stage for expression of *krox20*. *otx2* and *gbx1* are involved in forebrain, midbrain and hindbrain development, respectively, and determine the site of MHB formation, including *pax2.1* expression (K. Lun and M. B., unpublished). Expression of *otx2* and *gbx1* is initially normal in *spg*^{hi349} homozygotes (Reim and Brand, 2002), arguing that *spg* specifically affects activation of *pax2.1* and *krox20* expression, without disrupting earlier neuroectodermal gene expression.

Rescue of gene expression at the MHB

Previous reports have suggested that misexpression of *pou2* had no effect on the developing embryo (Takeda et al., 1994). We studied *pou2* function further by injecting 120 pg *pou2* mRNA into one side of two-cell stage embryos from a clutch of eggs derived from *spg*^{hi349} or *spg*^{m216} heterozygous parents. Before fixation, the injected embryos looked morphologically normal. The embryos were fixed at either the tailbud or the three-somite stage and stained for *pax2.1*. Injection of *pou2* mRNA rescued the reduced *pax2.1* expression at the MHB of mutant embryos in all embryos (Fig. 5B,D,F,H; *n*=447). In addition, *pax2.1* expression was slightly upregulated and

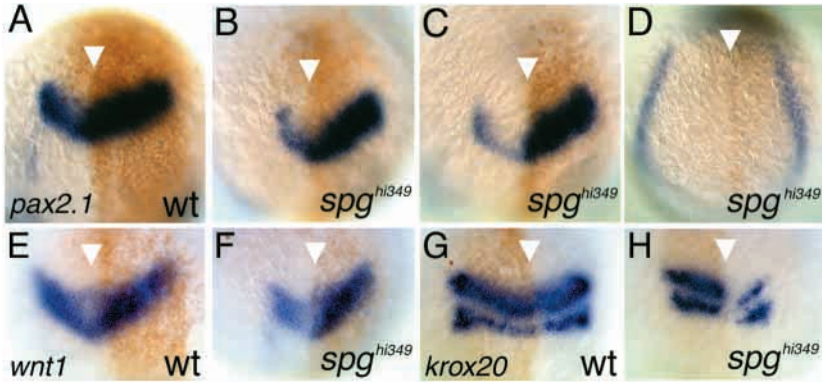

Fig. 5. *pou2* mRNA injection rescues *spg* mutants. (A-H) Injections of *pou2* mRNA and *lacZ* mRNA. The dorsal midline of the injected embryos is indicated by a white arrowhead, to show the unilateral injection of *lacZ*/*pou2*. (A-D) *pax2.1* staining. Wild-type embryo showing laterally expanded *pax2.1* expression on the injected side. (B-D) spg^{hi349}/spg^{hi349} mutant embryos injected into one cell of a two-cell stage embryo with *pou2* and *lacZ* mRNA and fixed at the three-somite stage. *pax2.1* staining is purple, *lacZ* staining in brown. (B,C) *pax2.1* expression at the MHB is clearly rescued on the injected side of the embryo. (D) Dorsoposterior view of a mutant embryo, showing *pax2.1* staining in the intermediate mesoderm. *pax2.1* expression is not affected in this tissue. (E,F) Lateral expansion of *wnt1* after *pou2* mRNA injection in the wild-type embryo (E) and rescue of expression in the mutant embryo (F). (G,H) Rescue of *krox20* expression after *pou2* mRNA injection.

broadened in its dorsoventral extent in both the wild-type and *spg* embryos on the injected side of the embryos (Fig. 5A-C), perhaps owing to a slight shortening of the injected embryos. In a similar fashion, we were able to rescue the expression of the MHB markers *wnt1* and *her5*, as well as the rhombomere marker *krox20*, by *pou2* overexpression (Fig. 5E-H; data not shown). This demonstrates that *pou2* injection rescues the loss of *pax2.1* expression and of the other markers, which are normally reduced in *spg* mutants, adding further proof that the loss of *pou2* is what is causing the *spg* mutant phenotypes. Importantly, *pax2.1*, *wnt1* and *krox20* are not expressed outside their normal domain of expression on the injected side in either mutant or wild-type embryos, nor is *pax2.1* expression in the intermediate mesoderm affected in these embryos (Fig. 5A-H). This suggests that the induction of these genes by *pou2* is specific to the midbrain and hindbrain region, and that *pou2* is necessary but not sufficient for their expression. In addition to rescuing expression of MHB- and hindbrain markers, higher doses of injected *pou2* mRNA cause a slight overall morphologically aberrant development; the basis for this phenotype currently not clear. The morphogenetic defects may be due to nonspecific effects from higher than normal early expression, specific effects on cell migration and axis formation during gastrulation, a slight dorsalizing potency or a combination of all.

Morpholino-induced knock-down of *pou2* function

pou2 mRNA is initially maternally supplied to the embryo and then supplemented with zygotic expression (Fig. 4) (Takeda et al., 1994; Hauptmann and Gerster, 1995). Recently, antisense inhibition using oligos with morpholino chemistry has proven to be extremely effective in eliminating protein translation in zebrafish embryos (Nasevicius and Ekker, 2000). To determine if maternally contributed *pou2* message has a role, we injected two different *pou2* antisense morpholinos into wild-type embryos (Table 1) (morpholino 2 required a higher concentration to work than morpholino 1). At low doses (2-5 ng, depending on the morpholino), the injections phenocopy the *spg* phenotype. Injected embryos displayed a loss of cerebellar structures and a disorganization of the hindbrain, as

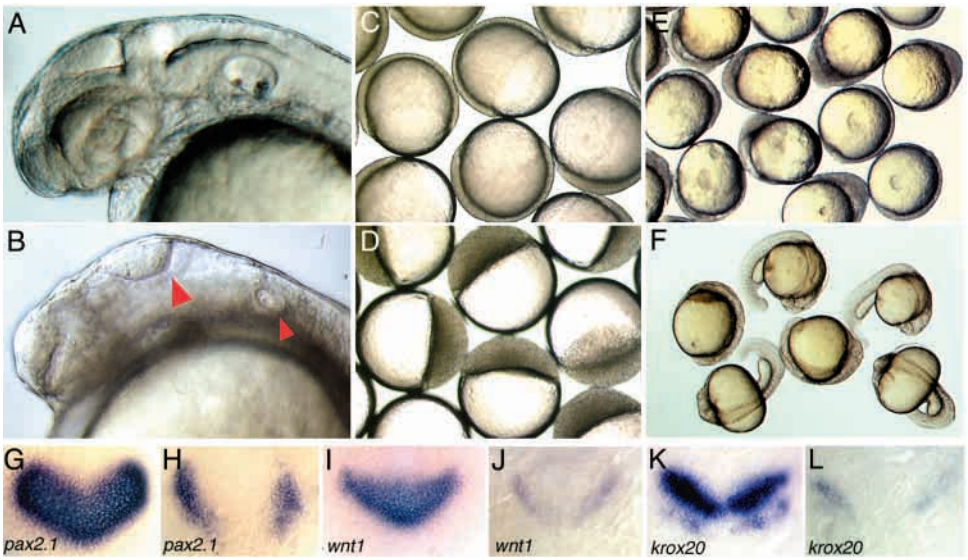

Fig. 6. Morpholinos phenocopy the *spg* mutant phenotype. (A-L) Results of morpholino experiments where *pou2* antisense morpholinos (B,D,E,F,H,J,L) or control morpholinos (A,C,G,I,K) were injected at the one-cell stage. (A) Control embryo 26 hours after injection. (B) Embryos injected with lower dose of morpholinos phenocopy the phenotype of *spg* 26 hours after injection. Loss of the MHB and reduced size of otic placode are indicated by red arrowheads. (C) Control embryos at 80% of epiboly. (D) Morpholinos were injected at high concentration at the one-cell stage. Cells arrest development at the sphere-dome stage. Embryos were photographed at the same timepoint after injection as Control embryos in C. (E) Embryos continue development after co-injecting morpholino 1 (E) or morpholino 2 (F) with a non-inhibitable *pou2* mRNA(-5′UTR), showing that the observed blastula arrest after morpholino injection is specific. (G-L) Molecular defects of morpholino-injected embryos. *pax2.1*, *wnt1* and *krox20* expression at the end of gastrulation is strongly reduced in morpholino-injected embryos, as seen in *spg* mutants (H,J,K).

Table 1. Summary of *pou2* morpholino antisense inhibition studies

Injected *pou2* morpholino	Effect	Total number
Mo1 2 ng	11% phenocopy of *spg*	36
Mo2 5 ng	8% phenocopy of *spg*	48
Mo2 6 ng	22% phenocopy of *spg*	9
Mo1 4 ng	100% arrest during gastrulation	205
Mo1 6 ng	100% arrest at dome-sphere stage	143
Mo2 8 ng	35% arrest during gastrulation	60
Mo2 12 ng	100% arrest at dome-sphere stage	120
Mo1-control 5 ng	100% no effect	120
Mo1-control 8 ng	100% no effect	73
Mo1 4 ng + *pou2* mRNA-5′UTR 60 pg	80% reversion of blastula arrest	200
Mo2 12 ng + *pou2* mRNA-5′UTR 60 pg	100% reversion of blastula arrest	200

Loss-of-function studies were carried out using injection of two independent morpholinos against *pou2*. The treatment phenocopies the *spg* phenotype at low doses, whereas higher doses result in arrest of development at pre-gastrula stages. Mo1 seems to work consistently at somewhat lower a concentration than Mo2. Mo1-control is the same as Mo1 except for a four-base mismatch.

well as an ear and tail phenotype that is similar to *spg* mutant embryos (compare Fig. 6B with Fig. 1B,C). This effect is highly specific, because control morpholinos with randomized or a four bp mismatch sequence had no effect, and similar phenotypes were not observed with other morpholinos in our laboratory. Moreover, embryos injected with morpholinos show a strong reduction of *pax2.1*, *wnt1* and *krox20* at the end of gastrulation (Fig. 6G-L), whereas expression of *otx2* and *gbx1* at 60-70% of epiboly and the tailbud stage is never affected by morpholinos (not shown), thus reproducing also other phenotypic traits of *spg* homozygous mutants.

When injected with intermediate doses (4-5 ng and 8 ng, respectively, depending on the morpholino) of *pou2* antisense morpholinos, embryos arrested between 50% and 70% of epiboly. At high doses of *pou2* antisense morpholino (6 ng), all embryos stopped cell division and morphogenesis during blastula stages (sphere-dome stage; Fig. 6D; Table 1), which we assume also reflects inactivation of the maternally supplied mRNA. The cells remained large and failed to migrate around the yolk normally, even 10 hours post-injection. By 12 hours, the blastoderm detaches from the yolk and none of the embryos survived until the next day. Embryos injected with a control morpholino displayed none of this behavior (Fig. 6C). This effect is similar to the effect caused by the injection of an RNA encoding a truncated version of the protein (Takeda et al., 1994).

To determine the specificity of the morpholinos, we attempted to rescue the developmental arrest by co-injecting 16 ng morpholino 2 at the one-cell stage together with 100 pg *pou2* mRNA(-5′UTR), which is devoid of the 5′UTR and hence is not recognizable by morpholino 2 (Fig. 6F). In contrast to morpholino 2, which exclusively interferes with sequences within the 5′UTR, morpholino 1 can also bind within the coding region of *pou2* mRNA. Embryos co-injected with 8 ng morpholino 1 and 100 pg *pou2* mRNA(-5′UTR) are released from the pre-gastrula arrest, but the rescue is less efficient compared with the experiment carried out with morpholino 2 (Fig. 6E). The reversion of the morpholino induced phenotype by *pou2* mRNA confirms that the pre-gastrula arrest is a specific consequence of the 'knockdown' of maternal and early zygotic *pou2* message. Further analysis of this phenotype is currently under way.

DISCUSSION

We have demonstrated that *spiel-ohne-grenzen* mutations affect the gene for the POU domain transcription factor Pou2, and have presented evidence that *pou2* is orthologous to the mammalian *Oct3*/*Oct4*/*Pou5f1* genes. We have examined *pou2* function through mutant analysis, morpholino-inhibition and *pou2* mRNA injections. *pou2* expression is essential for proper development of the midbrain, hindbrain, ear and body axis. The normal organization of the midbrain and hindbrain, including correct onset of *pax2.1* and *krox20* expression, is significantly disrupted in *spg* mutants, and may cause secondarily the defects in ear development. Many of the structures in the MHB are reduced or absent. Our data suggest two main periods of requirement for *spg* function. The first requirement is during the early cleavage period, and may be similar to the known function of the orthologous mammalian genes during differentiation and subsequent morphogenesis of the inner cell mass. Analysis of the second, tissue-specific period reveals a novel function of this gene during early brain regionalization in development of the midbrain-hindbrain organizer and the hindbrain, where it functions as a regulator of *pax2.1* and *krox20* expression. In the accompanying study, we examine the brain phenotype of *spg* mutants further and present evidence that *pou2* exerts these effects through regulating competence to respond to Fgf8 signaling (Reim and Brand, 2002).

Nature of the *spg* alleles

We have focussed our phenotypic analysis on two strong alleles, spg^{hi349} and spg^{e713}, which give essentially indistinguishable phenotypes. Two pieces of evidence point to the spg^{hi349} allele as being a null allele. First, the integration disrupts an exon with a 6000 base-pair proviral integration. The disruption is located in the middle of the POU_S domain, essentially disrupting both DNA binding domains. Second, in situ hybridization with *pou2* antisense RNA probes on mutant embryos displayed a strong reduction in signal of gene expression, both for the insertion allele and for spg^{e713}. Pou2 may therefore be involved in positive-feedback regulation of its own expression. An alternative possibility, that the insertion or mutation destabilizes *pou2* mRNA, is less likely to be correct, because *pou2* expression in tail somites at 26 hours is normal in homozygous spg^{hi349} mutants (data not shown). The loss of ubiquitous *pou2* expression in spg^{hi349} (see Fig. 4D,E) is already apparent before onset of gastrulation, around the time of normal onset of zygotic transcription. This has two important consequences for interpretation of *pou2* function: (1) prior to onset of gastrulation, *pou2* apparently functions in all cells of the embryo, raising the question whether, and if so how, this function contributes to the brain phenotype of the mutants; (2) homozygotes for the spg^{hi349} allele most probably lack all zygotic *pou2* function, but should still have normal maternal contribution. Thus, the phenotype of *spg* mutants is likely to be the result of the embryo using the substantial maternal wild-type *pou2* RNA/protein during the early stages of development,

and only after the embryo switches to zygotic expression can the effects of the mutation be seen.

The role of *pou2* in early embryonic development

The evidence presented suggests that there are two different functions for *pou2* in the developing embryo, an early general role, and later in development, a distinct and neuroectoderm-specific role. Maternal *pou2* message is localized to the oocyte cortex, and is restricted to the animal pole in the freshly laid oocyte (Howley and Ho, 2000). In the zygote, the message then becomes restricted to the deep layer cells that will become the actual zebrafish embryo (Hauptmann and Gerster, 1995). In situ hybridization data suggest a transition from maternal to zygotic *pou2* expression between 30% and 40% of epiboly. During gastrulation, *pou2* mRNA is still present throughout the epiblast, but becomes gradually restricted towards the end of gastrulation. We found that between 80% and 100% epiboly, the expression domain comprises both the midbrain- and hindbrain primordium, and only later does it become restricted to the hindbrain primordium (Fig. 4B,C) (Takeda et al., 1994).

Based on the syntenic chromosomal position and the phylogenetic sequence comparison (Fig. 2), we have argued that zebrafish *pou2* and murine *Oct3/Oct4* are orthologous. Several additional arguments support orthology, demonstrating a similar role for zebrafish *pou2* and murine *Oct3/Oct4*:

(1) Both our morpholino results and the overexpression of the truncated *t-pou2* by Takeda et al. (Takeda et al., 1994) suggest that the early role of *spg*/*pou2* may be to maintain the cells in an undifferentiated, rapidly dividing state. In the absence of functional *pou2* protein, either through antisense inhibition or through squelching with the truncated protein, the cells stop dividing and gastrulation is arrested. This role is very reminiscent of the orthologous murine POU transcription factor Oct4 (also known as Oct3 and Pou5f1) which is thought to maintain cells of the inner cell mass in a pluripotent state (Schöler et al., 1989; Okazawa et al., 1991; Nichols et al., 1998).

(2) Precursor cells of the enveloping layer of the zebrafish embryo are the first lineage to differentiate during early cleavage stages (Kimmel et al., 1995), and as they do so, they loose *pou2* expression (Hauptmann and Gerster, 1995). Murine Oct4 similarly acts during an early differentiation step: Oct4 expression is restricted to the pluripotent inner cell mass (ICM) of the developing mouse blastocyst, but is excluded from the extra-embryonic cells, and serves to maintain ICM in an undifferentiated state. Cells that lose Oct4 expression become trophectoderm, and ICM cells require Oct4 to maintain Fgf4 expression (Nichols et al., 1998).

(3) Mouse ES cells overexpressing Oct4 produce primitive endoderm and mesoderm (Niwa et al., 2001). We find that loss of *pou2* function causes an early failure of endoderm differentiation, as seen with the marker *sox17*, which suggests that *pou2*/*Oct4* could act more generally as a lineage switch in endoderm formation (Reim and Brand, 2002).

(4) *Oct4*$^{-/-}$ mouse embryos arrest at the expanded blastocyst stage (Nichols et al., 1998), a stage that may be analogous to the arrest shown in the morpholino injected zebrafish embryos, and controls activity of the adhesion modulator osteopontin at pre-implantation stages, which is thought to be crucial for controlling migration of mouse hypoblast cells at blastocyst stages (Botquin et al., 1998). Speculatively, at the pre-gastrula stage Pou2 may act in a similar way to Oct4, by preventing the dividing cells from restrictions in fate and hence, migration, allowing the cell number to expand enough for the embryo to develop normally. Using the *spg* mutants and *pou2* morpholino inhibition, we currently examine these issues in more detail.

(5) During early brain development, *Oct4* is expressed in the murine neural plate, and injection of murine *Oct4* mRNA into *spg* mutants rescues the *spg* mutant phenotype (Reim and Brand, 2002).

The role of *pou2* in AP patterning of the neuroectoderm

The nature of the *spg* phenotype suggests that *pou2* also has a more specific function in later embryonic development. One would not anticipate the specific deletion of a brain structure if the role of *pou2* were simply to maintain pluripotency throughout the embryo; in mice a brain-specific role has not been reported. However, Oct4 is also expressed at the appropriate early neural plate stages in mice, and injection of mouse Oct4 rescues the loss of *pax2.1* expression normally seen in *spg* mutant embryos (Reim and Brand, 2002), suggesting that Oct4 may have a similar function in mice. In zebrafish, the refinement in expression during the transition from 80% epiboly to 100% epiboly is key to the observed phenotype. By the completion of epiboly, *pou2* expression is restricted to a T-shaped region. Our analysis shows that this region of expression actually corresponds initially to the presumptive midbrain and hindbrain (Fig. 4F,G), and only later to the presumptive hindbrain and the developing neural tube. As development progresses, the expression of *pou2* becomes more and more restricted, first to the second and fourth rhombomeres and the end of the tail, then by the seven-somite stage, expression is seen only in the tip of the tail (Fig. 4C,D) (Hauptmann and Gerster, 1995). Diffuse and slight expression in the di-, mes- and metencephalon can still be seen at 28 hpf, and expression in the tail is detected until 34 hpf (Hauptmann and Gerster, 1995). This pattern of expression directly correlates with many of the observed phenotypes. At 30 hpf, the hindbrain architecture is severely affected, and our evidence suggests that the defects originate at much earlier stages. Indeed, already during activation of the first hindbrain marker genes, such as *krox20* and *pou2* itself, it is evident that the hindbrain primordium is not properly specified. The segmentation and organization of the seven rhombomeres becomes less distinct and often it is not possible to recognize all of them. A particularly interesting observation is that the Mauthner neurons are absent in *spg*^{hi349} homozygous embryos. The cell body for the Mauthner neuron resides in r4 and in situ hybridization shows a concentration of *pou2* signal in r4 at the three to five somite stage (Fig. 4C,D) which suggests *pou2* may have important cell autonomous functions in establishing specific cell fates in r4. A perhaps similar situation has been observed in the determination of the NB4-2 neuroblast lineage in *Drosophila* (Yang et al., 1993; Bhat and Schedl, 1994). Pdm-1 and Pdm-2 are POU domain proteins expressed at high levels in the ganglion mother cell (GMC-1). As the level of Pdm-1 and Pdm-2 drops, the GMC-1 cell divides to form an RP2 motoneuron and a sibling cell. When Pdm-1 or Pdm-2 is overexpressed, the GMC-1 cell divides without a drop in Pdm level which generates two new GMC-1 cells. The level of Pdm

then drops, and a duplication of the RP2 motoneurons results. The absence of both Pdm-1 and Pdm-2 in mutants completely prevents the formation of RP2 motoneurons.

The most interesting observed phenotype of *spg* mutants is the absence of the MHB. As previously shown for the slightly weaker allele spg^{m216} (Schier et al., 1996), we have shown that for spg^{hi349} and spg^{e713}, in the absence of the *pou2* gene, the expression of *pax2.1* at the MHB is severely affected. The first observed stage of regionally specific *pou2* expression directly correlates with the first observed expression of *pax2.1* (Krauss et al., 1991a; Lun and Brand, 1998). In zebrafish embryos, *pax2.1* transcripts are first seen during late gastrulation. *Pou2* expression initially overlaps *pax2.1* expression and transplantation results suggest that this induction is cell-autonomous (Reim and Brand, 2002). Importantly, *otx2* and *gbx1* staining are not affected in *spg* mutant embryos, showing that the MHB patterning process upstream of *pax2.1* is not affected in a general way (Reim and Brand, 2002). In combination with the injection results presented here, this suggests that the absence of *pax2.1* in *spg* mutants is a direct result of the loss of *pou2* and not a secondary defect from a general disorganization of the neuroectoderm, and that *pou2* is an important positive upstream regulator of *pax2.1*. This is also consistent with our observation that injection of *pou2* mRNA into the zygote can rescue the expression of *pax2.1* staining in the MHB. We also observe that the absence of *pou2* expression negatively affects the expression of *wnt1* and *krox20*, suggesting that the role of *pou2* in neurectoderm patterning is not exclusively to induce *pax2.1* expression, but extends to activating gene expression in the hindbrain. Further experiments addressing the function of *spg/pou2*, as well as the similarity of the brain phenotype of *spg* and *acerebellar/fgf8* mutant embryos (Reifers et al., 1998), demonstrate an important role for Pou2 in mediating competence to respond to Fgf8 signaling (Reim and Brand, 2002).

Injection at higher levels of *pou2* also has other effects on embryonic development. High levels of *pou2* mis-expression slightly upregulate and expand *pax2.1* expression slightly in the dorsoventral direction at the MHB. However, *pax2.1* staining is not seen in other areas of *pou2* mis-expression and the endogenous expression of *pax2.1* in other parts of the embryo is unresponsive to mis-expression of *pou2*, showing that *pou2* is necessary but not sufficient to induce *pax2.1* expression.

pax6 expression in early zebrafish embryos normally first appears at approximately 10 hpf in the presumptive forebrain region. A second region of expression in the hindbrain appears shortly thereafter. *pax6* expression is normally completely excluded from the midbrain and MHB. In *spg* mutants, forebrain expression of *pax6* expands into where the MHB should be and actually fuses with the posterior domain of expression forming one large *pax6* domain (Schier et al., 1996; Reim and Brand, 2002). This suggests that in the wild-type environment, the midbrain or MHB is inhibiting the expansion of *pax6* expression, an observation also made in mouse double knockouts of *Pax2/Pax5*. Additionally, in both mice and chicken, *pax6* can inhibit *pax2* expression when overexpressed (Mastick et al., 1997; Schwarz et al., 1999; Matsunaga et al., 2000), making it likely that inhibitory signals from each domain are reinforcing the sharpness of the boundaries.

The role of *pou2* in ear development

spg mutant embryos typically show a slightly smaller otic vesicle. However, unlike the situation in the MHB, *pax2.1* expression in the otic placode appears unaffected in the mutant embryos. It is therefore unlikely that the ear defect is a result of a failure to induce proper expression of *pax2.1* in the ear. There are several examples of zebrafish mutations that affect the hindbrain initially and as a consequence of that hindbrain defect, display defects in ear development (Moens et al., 1996; Mendonsa and Riley, 1999). Several studies have also implicated signals emanating from the hindbrain in the induction of the otic placode (Gallagher et al., 1996; Mahmood et al., 1996; Groves and Bronner-Fraser, 2000; S. Léger and M. B., unpublished). Therefore it is likely that the defects in the ear seen in the *spg* mutation are a result of the primary defects in the hindbrain.

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