**Spiel ohne Grenzen/Pou2 is Required for Zebrafish Hindbrain Segmentation**

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**Summary**

Segmentation of the vertebrate hindbrain leads to the formation of a series of rhombomeres with distinct identities. In mouse, Krox20 and kreisler play important roles in specifying distinct rhombomeres and in controlling segmental identity by directly regulating rhombomere-specific expression of Hox genes. We show that *spiel ohne grenzen* (spg) zebrafish mutants develop rhombomeric territories that are abnormal in both size and shape. Rhombomere boundaries are malpositioned or absent and the segmental pattern of neuronal differentiation is perturbed. Segment-specific expression of *hoxa2*, *hoxb2* and *hoxb3* is severely affected during initial stages of hindbrain development in spg mutants and the establishment of *krx20* (*Krox20* ortholog) and *valentino* (*val*; kreisler ortholog) expression is impaired. spg mutants carry loss-of-function mutations in the *pou2* gene. *pou2* is expressed at high levels in the hindbrain primordium of wild-type embryos prior to activation of *krx20* and *val*. Widespread overexpression of Pou2 can rescue the segmental *krx20* and *val* domains in spg mutants, but does not induce ectopic expression of these genes. This suggests that *spg/pou2* acts in a permissive manner and is essential for normal expression of *krx20* and *val*. We propose that *spg/pou2* is an essential component of the regulatory cascade controlling hindbrain segmentation and acts before *krx20* and *val* in the establishment of rhombomere precursor territories.

Key words: Rhombomere, Hox genes, ephA4, kreisler, Krox20, krx20, rtk1, spg, valentino, Danio rerio

**Introduction**

The regionalization of the vertebrate hindbrain involves a segmentation process, well conserved through vertebrate evolution (Gilland and Baker, 1993). During early hindbrain development, a series of morphological units, termed rhombomeres (r), forms along the anteroposterior axis (Hanneman et al., 1988; Lumsden and Keynes, 1989). The rhombomeres serve to organize segmental patterns of neuronal differentiation (Lumsden and Keynes, 1989; Trevarrow et al., 1990; Clarke and Lumsden, 1993) and the ordered migration of cranial neural crest cells into specific branchial arches (Lumsden and Guthrie, 1991; Sechrist et al., 1993; Schilling and Kimmel, 1994). Cell-lineage studies in chick have demonstrated that each of r2-r6 constitutes a lineage-restricted compartment (Fraser et al., 1990; Birgbauer and Fraser, 1994).

Members of the vertebrate Hox gene family are expressed in partially overlapping domains in the embryonic hindbrain with anterior expression borders that coincide with distinct segmental boundaries (reviewed by McGinnis and Krumlauf, 1992). The results of targeted gene inactivation of individual Hox genes in mouse revealed that Hox genes are involved in conferring rhombomeric identity (Goddard et al., 1996; Studer et al., 1996), and suggested that at least some Hox genes may play a role in the segmentation process itself (Carpenter et al., 1993; Dollé et al., 1993; Mark et al., 1993; Gavalas et al., 1997).

Krox20 (Egr2) (Wilkinson et al., 1989) and kreisler (kr/Mafb) (Cordes and Barsh, 1994) have been shown to be direct transcriptional regulators of distinct Hox genes in the hindbrain. Krox20 is activated before morphologically visible segmentation in the r3 and r5 primordia (Wilkinson et al., 1989; Oxtoby and Jowett, 1993) and directly regulates the expression of *Hoxa2* (Nonchev et al., 1996) and *Hoxb2* (Sham et al., 1993) in these rhombomeres. Targeted inactivation of Krox20 leads to progressive disappearance of r3 and r5 during development (Schneider-Maunoury, 1993; Swiatek and Gridley, 1993; Schneider-Maunoury et al., 1997). kr is required for specification of r5 and r6 (Frohman et al., 1993; Cordes and Barsh, 1994; McKay et al., 1994) and has been shown to be a direct regulator of *Hoxa3* and *Hoxb3* in r5 (Manzanares et al., 1997; Manzanares et al., 1999). In contrast to our knowledge of *Hox* gene regulation, however, relatively little is known about the control of Krox20 and kr expression.

Another family of regulatory genes with members expressed in the developing hindbrain is the POU gene family (Ryan and Rosenfeld, 1997). POU genes are expressed in the zebrafish CNS in distinct but overlapping domains during embryonic development (Takeda et al., 1994; Hauptmann and Gerster, 1995; Hauptmann and Gerster, 1996; Śniapiol et al., 1996;
Hauptmann and Gerster, 2000a). Zebrafish spiel ohne grenzen (spg) mutants were isolated based on their failure to develop a normal mid/hindbrain boundary (Schier et al., 1996) and we have identified pou2 (Takeda et al., 1994; Hauptmann and Gerster, 1995) as the gene affected in these mutants (Belting et al., 2001). Here, we provide evidence for the involvement of spg/pou2 in regional patterning of the hindbrain primordium. Segment-specific expression of hox genes, krx20, ephA4 and val is altered in spg mutants, indicating reduction of r1, r3, r5 and r6 territories. Since both the size and shape of rhombomeric territories are altered in spg mutants from the beginning of segmentation, pou2 appears to function in an early step of hindbrain regionalization to ensure the establishment of normal rhombomere precursor territories. Our findings suggest that spg/pou2 acts before krx20 (egr2) and val in hindbrain segmentation.

MATERIALS AND METHODS

Zebrafish strains and embryo culture

Fish stocks were raised under standard laboratory conditions (Westfield, 1994). Two spg alleles with intermediate (spg<sup>m216</sup>) (Schier et al., 1996) and strong phenotypes (spg<sup>m793</sup>) (Belting et al., 2001) were analyzed. Embryos were incubated at 28.5°C and staged in hours postfertilization (hpf) and days postfertilization (dpf) according to Kimmel et al. (Kimmel et al., 1995). Some embryos were incubated with the addition of 0.2 mM phenylthiourea to prevent pigmentation. Embryos of the desired stage were manually dechorionated and anesthetized in 0.03% tricaine (Sigma). They were immobilized in 2% methyl cellulose and photographed using differential interference contrast optics.

Retrograde labeling of reticulospinal neurons

Retrograde labeling of reticulospinal neurons was performed as described previously (Moens et al., 1996). Three-dimensional images of tetra-methylrhodamine dextran-labeled reticulospinal neurons were reconstructed using a confocal Zeiss LSM 510 laser scanning microscope, and were depth-coded in color using the LSM 510 3D imaging software.

Whole-mount in situ hybridization and immunohistochemistry

Standard methods for one- and two-color whole-mount in situ hybridization (WISH) were used (Hauptmann and Gerster, 1994; Hauptmann and Gerster, 2000b). Cryosections were prepared following WISH. Control embryos, denoted wild-type in the figures, are phenotypically wild-type siblings (spg<sup>/+</sup> or /<sup>/+</sup>/) of mutant embryos shown in the same experiment.

The wild-type expression patterns of the genes analyzed in this study and the cDNAs used for generation of probes have been described in the references provided. The gene previously described as hoxal (Alexandre et al., 1996) has recently been assigned to the hoxba cluster (Amores et al., 1998), and is therefore termed hoxba1. The gene previously termed hoxbl (Prince et al., 1998) has been assigned to the hoxba cluster and is therefore termed hoxb1a. As there are no duplicates of the other hox genes used as probes in this study, the a or b assignment for duplicated clusters (Amores et al., 1998) has been omitted from their names. For identification of Mauthner cells, we used the monoclonal antibody 3A10 (Hatta, 1992).

Microinjection of mRNA

pou2 cDNA (Hauptmann and Gerster, 1995) was subcloned into the pCS2+ vector and transcribed using the Sp6 Message Machine kit (Ambion). About 20 pg in vitro synthesized mRNA was microinjected into one-cell stage embryos using pressure driven manual micropipets. In some experiments co-injection of lacZ mRNA was used to monitor the distribution of overexpressed proteins. After whole-mount in situ hybridization and photographic documentation, spg<sup>m793</sup> homozygous mutant embryos were identified by PCR-genotyping as described previously (Belting et al., 2001).

RESULTS

Abnormal hindbrain segmentation in spg mutants

We used two different spg alleles, spg<sup>m216</sup> (Schier et al., 1996) and spg<sup>m793</sup> (Belting et al., 2001), to analyze the effects of loss of pou2 function on hindbrain segmentation. spg<sup>m793</sup> is likely a null allele, since the open reading frame is truncated, removing both DNA-binding domains, the POU domain and the POU homeodomain. spg<sup>m216</sup> may be a hypomorph, caused by a leucine-to-proline substitution in the first helix of the POU homeodomain (Belting et al., 2001).

In wild-type embryos at the 22-somite stage, r2 through r6 are recognized as an anteroposterior series of similar-sized bulges, with the otic vesicle lying lateral to r5 (Fig. 1A). In spg<sup>m216</sup> embryos, the r3 and, to a lesser extent, the r5 bulges are reduced in size, while those of r2 and r4 are enlarged (Fig. 1B). In spg<sup>m793</sup> mutants, most rhombomere boundaries are not discernible (Fig. 1C). In addition, the otic vesicle is shorter anteroposteriorly, resulting in a circular rather than an oval shape (Fig. 1B,C).

In wild-type embryos, expression of pax6.1 (Püschel et al., 1992) is enriched along inter-rhombomeric boundaries generating a pattern of six equally spaced stripes of strong pax6.1 expression (Fig. 1D) (Xu et al., 1995). In spg<sup>m216</sup> embryos, the second and third stripe, corresponding to the r2/r3 and r3/r4 boundaries, and, to a lesser extent, the fourth and fifth stripe, corresponding to the r4/r5 and r5/r6 boundaries, are closer together (Fig. 1E). This pattern indicates reduced r3 and r5 territories and enlargement of r2 and r4. In strongly affected spg<sup>m793</sup> mutants, enriched pax6.1 expression along inter-rhombomeric boundaries is not observed (Fig. 1F).

To determine whether the segmental pattern of neuronal differentiation in the hindbrain is also altered in spg mutants, we assayed the segmental distribution of specific subsets of hindbrain neurons. In wild-type embryos at 1 dpf, lim1-positive neurons are arranged in two transverse stripes in each rhombomere (Fig. 1G) (Toyama and Dawid, 1997). In spg<sup>m216</sup> mutants, only a single lim1-positive domain is detected in each of r1, r3, and r5 (Fig. 1H), consistent with reduction of these rhombomeres. In spg<sup>m793</sup> mutants, individual lim1 expression domains cannot easily be assigned to specific segments. In addition, ectopic lim1-positive neurons are found in aberrant dorsal locations within the preotic hindbrain (Fig. 1I).

In wild-type embryos at 5 dpf, a series of defined reticulospinal neurons can be visualized by retrograde labeling and each neuron recognized based on cell size, position, and projections (Fig. 1J) (Kimmel et al., 1982). In spg<sup>m793</sup> mutants, reticulospinal neurons cannot be visually identified (Fig. 1L). Spherical neuronal cell bodies are observed, but these exhibit no segment-specific morphology. Accordingly, Mauthner cells, primary reticulospinal neurons that form in r4 (Kimmel et al., 1981), are not detected in spg<sup>m793</sup> mutants by a Mauthner cell specific antibody (3A10; data not shown). Weak spg<sup>m216</sup>
Altered hox gene expression in spg mutants.

Expression of hox genes is apparent at the cellular level and affects various hindbrain regions in spg mutants (in 2 and 3, respectively, of 15 embryos). Our results show that disruption of hindbrain segmentation in spg m216 mutants is similar to that of spg m793 mutants (Prince et al., 1998) and hox genes (Oxtoby and Jowett, 1993) expression at the 10-somite stage. As each rhombomere is characterized by a distinct combination of hox gene and krx20 expression, we were able to identify r2 to r7 individually in wild-type, spg m216 and spg m793 embryos (Fig. 2A-W). Our results are summarized in schematic drawings (Fig. 2X,Y,Z).

In spg m216 embryos, we made three major observations concerning hox gene expression. First, the anterior limit of hoxa2 expression is shifted rostrally on the ventral side, indicating an oblique orientation of the r1/r2 boundary and a rostral expansion of r2 (Fig. 2A,B,D,E). Second, the r3 and, to a lesser extent, the r5 domains of krx20 are smaller along the anteroposterior axis when compared to wild-type embryos, while the expression domains of hoxb1a and hoxb2 in r4 are larger (Fig. 2G,H,J,K,M,N). These alterations are consistent with an anteroposterior enlargement of the r4 territory at the expense of r3 and r5. Third, expression of hoxb3 (Fig. 2P,Q), hoxd3 (Fig. 2S, data not shown) and hoxb4 (Fig. 2U,V) is essentially unaltered in spg m216 embryos, indicating normal patterning of r6 and r7.

In spg m793 mutants, the krx20 expression domains in r3 and r5 are even smaller than in spg m216 mutants, and are split into bilateral patches that are displaced laterally and develop at variable dorsoventral positions (Fig. 2L). Both the hoxb1a domain (Fig. 2I,J) and the low-level expression domain of hoxb2 (Fig. 2O,P), corresponding to r4, are variably enlarged, and extend medially between the lateralized krx20 domains. These changes in gene expression indicate that medial regions normally fated to form r3 and r5 may have acquired some aspects of r4 identity. The lateral displacement of r3 and r5, as assayed by krx20 expression, appears to lead to the medial juxtaposition of r2, r4, and r6 (see schematic drawing in Fig. 2Z). In spg m793 mutants, high-level hoxa2 expression indicative of r2 directly juxtaposes low-level hoxa2 expression corresponding to r4 (Fig. 2C). Similarly, hoxb1a and hoxb2 expression marking r4 abuts strong hoxa2 expression in r2 (compare Fig. 2J,O with 2C). Thus, r2 and r4 territories appear to directly juxtapose in spg m793 mutants.
Elevated \textit{hoxb3} expression between the lateralized r5 patches likely indicates r6 identity (Fig. 2R1), and directly adjoins the posterior side of r4 (compare Fig. 2R1 and 2I1/2O1). Similarly, the anterior expression limit of \textit{hoxd3}, which normally correlates with the r5/r6 boundary (Fig. 2S), extends anteriorly between the lateralized r5 patches (Fig. 2T), reaching the r4 domain (compare Fig. 2T with 2I1/2O1). Thus, r4 and r6 are also juxtaposed in \textit{spgm793} mutants. In the most strongly affected \textit{spgm793} mutants, elevated \textit{hoxb3} expression is seen only laterally, indicating medial disruption of both r5 and r6 (Fig. 2R2). Expression of \textit{hoxb4}, which has an anterior boundary within r7 at this stage, is not obviously altered in \textit{spgm793} mutant embryos (Fig. 2W).

Taken together, our analysis shows that in all \textit{spg} mutants, each rhombomere expresses the normal complement of \textit{hox} genes, indicating normal segmental identity. Differences in the spatial extent of individual \textit{hox} and \textit{krx20} expression domains, however, show that hindbrain segmentation is altered.

\textit{spg} is required for formation of normal \textit{krx20} and \textit{val} expression domains

\textit{Krox20}/\textit{krx20} (Oxtoby and Jowett, 1993; Schneider-Maunoury, 1993; Swiatek and Gridley, 1993) and \textit{kr}/\textit{val} (Cordes and Barsh, 1994; Moens et al., 1998) are thought to be required for development of r3/r5 and r5/r6, respectively. In wild-type embryos, \textit{krx20} expression is first detected during late gastrula stages (100% epiboly) in bilateral stripes in the r3 primordium (Fig. 3A) and slightly later in that of r5 (Oxtoby and Jowett, 1993). At about the same time, \textit{val} expression begins in bilateral stripes corresponding to the r5 and r6 primordia (Fig. 3C) (Moens et al., 1998). The initially bilateral expression domains of \textit{krx20} (Fig. 3E,I) and \textit{val} (Fig. 3G,K) fuse at the midline during early somitogenesis stages. In \textit{spgm793} mutants, the initial activation of \textit{krx20} (Fig. 3B1, and slightly later Fig. 3B2) and \textit{val} (Fig. 3D1,D2) is affected and occurs only in reduced bilateral expression domains, each composed of dispersed groups of cells. These bilateral domains do not properly extend towards the midline. During early segmentation stages, the \textit{krx20}- and \textit{val}-positive cells form lateral cell clusters (Fig. 3F,H,J,L), which may be connected by a very thin band of labeled cells (Fig. 3L). The \textit{krx20}- and \textit{val}-positive cell clusters in r5 and r5/r6, respectively, increase in size over time (compare Fig. 3F,H with 3J,L), but still remain smaller than the wild-type domains of the corresponding stage (Fig. 2J,L). \textit{krx20} expression in r3 is more severely affected and remains restricted to very tiny cell clusters that only slightly increase in size (Fig. 3F,J).Taken together, these data show that \textit{spg/pou2} is required for two aspects of the
establishment of normal krx20 and val expression. First, the initial expression of these genes is reduced in spg\textsuperscript{m793} mutants, and second, the condensation of the initially bilateral expression domains across the midline is impaired.

**pou2 expression in the hindbrain precedes krx20 and val expression**

To further investigate possible regulatory links between pou2, krx20, and val, we compared their expression in wild-type embryos. At about 80% epiboly, early ubiquitous pou2 expression evolves into a diffuse transverse domain in the midbrain and hindbrain primordia (Fig. 4A). Sagittal and cross sections show that pou2 expression is confined to the epiblast, and no expression is found in the hypoblast (Fig. 4G,H) (Takeda et al., 1994). pou2 is expressed in the forming neural plate but not in the axial mesendoderm (Fig. 4G) that is marked by expression of \textit{no tail} (ntl) (Schulte-Merker et al., 1992). pou2 is also expressed in medial and lateral cell columns extending to the posterior end of the neural plate (Fig. 4A-F) (Hauptmann and Gerster, 1995). The medial cell columns are located adjacent to the midline of the neural plate above the ntl-expressing axial mesoderm (Fig. 4C,I,J). Initial pou2 expression in the midbrain and hindbrain primordia is relatively widespread with diffuse anterior and posterior borders (Fig. 4A). During late gastrulation, pou2 expression sharpens, and resolves into a high-level expression domain corresponding to prospective r2 through r4 by tailbud stage (Fig. 4B,C) (Hauptmann and Gerster, 1995). Anterior and posterior to the high-level domain, weaker expression extends into the midbrain and posterior hindbrain, respectively. Thus, krx20 expression in r3 is activated within the high-level pou2 hindbrain expression domain at 100% epiboly (compare Fig. 4B and Fig. 3A) (Oxtoby and Jowett, 1993). In contrast, krx20 expression in prospective r5 and val expression in prospective r5/r6 is activated in a region with weaker pou2 expression (Fig. 4B-D). At the 2-somite stage, pou2 expression is split into two subdomains of high-level expression, corresponding to r2 and r4 (Hauptmann and Gerster, 1995), and displays a pattern complementary to that of krx20 and val (Fig. 4E,F). Only the medial and lateral pou2-positive anteroposterior cell columns extend through the krx20 and val expression domains. Both the earlier activation of pou2 in the hindbrain primordium and the spatial overlap of pou2 expression with the initial krx20 and val expression domains are consistent with pou2 involvement in the initial establishment of the segmental expression domains of these genes.

**Overexpression of Pou2 can rescue krx20 and val expression in spg mutants**

To test whether overexpression of Pou2 is sufficient to rescue krx20 and val expression in spg\textsuperscript{m793} mutants, we injected in vitro transcribed pou2 mRNA into one-cell stage embryos, and assayed expression of krx20 and val at the 5-somite stage (Fig. 4K-R). We found that amounts of pou2 mRNA (20 pg per embryo) that were able to rescue krx20 and val expression in almost all spg\textsuperscript{m793} homozygous mutants (16/17) did not induce specific developmental defects in most embryos (Fig. 4N,R). Only a few rescued embryos were developmentally delayed and showed broadening of the neural plate (4/17). Although injection of pou2 mRNA into single-cell embryos led to widespread expression throughout the embryo (data not shown), we did not observe ectopic krx20 or val expression in any pou2-injected embryo (86/86 wild-type and mutant embryos; Fig. 4M,N,Q,R, data not shown). The absence of ectopic krx20 and val expression in all injected embryos indicates that pou2 is not sufficient to induce ectopic expression of these genes.

**Segmental expression of ephA4 is altered in spg mutants**

In mouse, Krox20 has been shown to directly activate the expression of \textit{Epha4} in r3 and r5 (Theil et al., 1998). We therefore examined the expression of ephA4 (efna4/rtk1) (Xu et al., 1995) in spg mutant embryos. Shortly after the onset of krx20 expression, ephA4 expression begins in r3 (from tailbud stage) and r5 (from the 3-somite stage onwards; Fig. 5A) (Xu et al., 1995). In spg mutants, alterations in ephA4 expression are evident during early somitogenesis. By the 10-somite stage, ephA4 expression in r3 and r5 is slightly reduced in anteroposterior extent in spg\textsuperscript{m216} mutants (Fig. 5B), while
absent medially in spgm\textsuperscript{793} embryos (Fig. 5C). At 1 dpf, ephA4 is also expressed in a thin domain in r1 (Fig. 5D-I). In spg mutants, in addition to persistent alterations in r3 and r5, ephA4 expression is also absent medially in r1 (Fig. 5H,I). The r1 ephA4 domain is at an oblique angle, indicating a reduction of r1 on the ventral side in spg mutants (Fig. 5E,F).

**Early segment-specific expression of hoxa2, hoxb2 and hoxb3 is affected in spg mutants**

We looked for alterations in the initial expression of hox genes that may directly result from the abnormal krx20 and val expression seen in spg mutants (Fig. 6). In wild-type embryos at the 1- to 2-somite stage, expression of hoxa2, hoxb2, and hoxb3 is initiated in strong bilateral segmental domains corresponding to prospective r2/r3, r3 and r5, and r5/r6, respectively (Fig. 6A,E,I) (Prince et al., 1998). At the 5-somite stage, expression in these rhombomeres increases and bridges the midline (Fig. 6C,G,K). In spgm\textsuperscript{793} mutants, strong segmental expression of hoxb2 and hoxb3 is restricted to lateral territories (Fig. 6F,H,J,L). Similarly, expression of hoxa2 does not properly extend towards the midline at the 2-somite stage (Fig. 6B), but at the 5-somite stage, a low level of hoxa2 expression is found across the midline (Fig. 6D).

In contrast, initial expression of hoxb1b (Alexandre et al., 1996) and hoxb1a (Prince et al., 1998) in the hindbrain primordium is not altered in spgm\textsuperscript{793} embryos. During gastrula and early somitogenesis stages, hoxb1b and hoxb1a are normally expressed in broad domains with sharp anterior boundaries within the prospective hindbrain region (Fig. 6M,O,Q). By the 2-somite stage, hoxb1a expression is increased in r4 (Fig. 6S). In spgm\textsuperscript{793} embryos, hoxb1b and hoxb1a expression is initiated appropriately in the hindbrain (Fig. 6N,R). At the 2-somite stage, expression of hoxb1b is unaltered and the hoxb1a r4 domain is only slightly misshapen, while the neighboring r3 and r5 domains of krx20 are severely disrupted (Fig. 6P,T).

**Midline gene expression in spg mutants appears normal**

In spgm\textsuperscript{793} mutants, alterations in mid- and hindbrain gene expression are most severe in the medial neural plate. In this region, other genes expressed in wild-type axial mesendoderm and/or ventral CNS may mediate the spg mutant phenotype. We therefore investigated whether alterations in midline gene expression can be detected between 80% epiboly and the 2-somite stage, the developmental time period when defects in the mid- and hindbrain primordia of spg mutants are first observed. Midline expression of cyclops/znr1/mdr2 (Rebagliati et al., 1998; Sampath et al., 1998), taram-a (Renucci et al., 1996), lefty1/antivin (Bisgrove et al., 1999; Currie and Ingham, 1996), chordin (Schulte-Merker et al., 1996), cyclops (Ekker et al., 1995), ehh (Krauss et al., 1999), shh (Krauss et al., 1993), twhh (Ekker et al., 1995), ehh (Currie and Ingham, 1996), chordin (Schulte-Merker et al., 1996), and lefty1/antivin (Bisgrove et al., 1999; Currie and Ingham, 1996) is unaltered in spg mutants (Fig. 7A-G).
may play an essential role in defining the anteroposterior spatial extents of individual rhombomeric domains. In addition to abnormal rhombomere sizes, in spg<sup>m793</sup> mutants r1, r3, r5, and in strongest mutants also r6 are disrupted in the midline of the neural plate (Fig. 22). The medial disruptions of rhombomere precursor territories in spg<sup>m793</sup> mutants may result from enhanced deficiencies in anteroposterior patterning. These may cause increased thinning of prospective r3 and r5 territories, such that the bilateral rhombomeric primordia fail to fuse across the midline of the neural plate, allowing even-numbered rhombomeres to juxatapose. Since cells of the same parity can mix with each other relatively freely (Guthrie and Lumsden, 1991; Guthrie et al., 1993), the juxtaposition of r2, r4, and r6 in spg<sup>m793</sup> mutants could lead to irreversible fusion of these even-numbered segments. As a consequence, the krx20-expressing cell patches of r3 and r5 identity may be excluded from the fused r2/r4/r6 territory and pushed towards the lateral edges of the hindbrain. Consistent with this idea, lateral protrusions of r3 and r5 from the neural keel are often observed in spg<sup>m793</sup> mutants (Fig. 21I,12L).

Alternatively, the medial rhombomeric disruptions may point to a specific requirement for spg/pou2 along the midline of the neural plate. Likewise, expression of pax2.1 in the prospective posterior midbrain is also most strongly affected in the medial neural plate of spg<sup>m793</sup> mutants (Fig. 6B) (Belting et al., 2001). This raises the question of whether spg/pou2 may be involved in mediolateral patterning of both the midbrain and hindbrain primordia. Vertical signaling from the axial mesendoderm has previously been implicated in patterning the medial (later ventral) neural primordium (Placzek et al., 1993). However, expression of ntl (Schulte-Merker et al., 1992) and flh (Talbot et al., 1995), both essential for zebrafish notochord development (Halpern et al., 1993; Talbot et al., 1995), is not altered in spg<sup>m793</sup> mutants, suggesting normal specification of the notochord precursor underlying the hindbrain primordium. Further, pou2 is not expressed in the axial hypoblast and mesendoderm. Thus, if pou2 acts in mediolateral patterning, it acts only within the neuroectoderm. For example, pou2 may be involved in interpreting mesendoderm derived midline signals. Nodal and Sonic hedgehog signals have been implicated in dorsoventral patterning of the neural tube at all axial levels (Rubenstein and Beachy, 1998; Schier and Shen, 2000). In spg<sup>m793</sup> mutants, we found some variability in the expression of twhh, while the expression of other Hh homologs and Nodal pathway genes was not altered. However, homozygous cyclops mutants display normal expression of pax2.1 and krx20 in the mid- and hindbrain primordia, respectively, despite elimination of twhh and reduction of shh midline expression (Krauss et al., 1993; Ekker et al., 1995; Sirotkin et al., 2000). This suggests that the mediolateral abnormalities in mid- and hindbrain gene expression in spg<sup>m793</sup> mutants are probably not caused by defective Hh or Nodal midline signaling.

Other developmental mechanisms could also account for the reduction of rhombomere precursor territories in spg mutants. Examination of cell death using the terminal nuclear transferase assay did not reveal specific patterns of dying cells in spg<sup>m793</sup> mutants in the mid-/hindbrain region during gastrulation. Increased cell death scattered throughout the neural keel in spg<sup>m793</sup> mutants was detected from the 2-somite stage onwards (data not shown). This, however, could not cause

**DISCUSSION**

**Roles of spg/pou2 in anteroposterior and mediolateral hindbrain patterning**

To date, only a few genes such as Krox20, kreisler, Gbx2, and Hoxa1 have been categorized as hindbrain segmentation genes (Schneider-Maunoury et al., 1998). Inactivation of each of these genes leads to size modification or elimination of rhombomeric territories (Schneider-Maunoury et al., 1998). In spg mutants, based on expression of hox genes and krx20 (Fig. 2X-Z), rhombomere identities develop in normal anteroposterior order, but the size and shape of rhombomeric territories are altered. This adds spg/pou2 to the small group of genes required for proper hindbrain segmentation.

In spg<sup>m216</sup> mutants, the even-numbered rhombomeres, r2 and r4 are expanded along the anteroposterior axis, whereas odd-numbered ones, r1, r3 and, to a lesser extent, r5 are shortened (Figs 2Y, 5E,H). This suggests that spg/pou2 is required for normal anteroposterior hindbrain patterning and
the earlier and selective reduction of distinct rhombomere precursor territories observed. Between 70% epiboly (7.7 hpf) and the 3-somite stage (11 hpf), deep cells that form all embryonic lineages undergo only one cell cycle (cycle 14, around 8.2 hpf) (Kane et al., 1992), so changes in cell proliferation could not cause the observed patterning defects. The reduction of odd-numbered rhombomeres may result from reduced recruitment of progenitor cells into the hindbrain territory. However, the expression domains of gbx1 and fgf8, which prefigure the anterior hindbrain territory including prospective r3, are of similar size in wild-type and spg m793 mutant embryos during late gastrula stages (Belting et al., 2001).

### Impaired hindbrain segmental boundary formation in spg mutants

Transplantation experiments in chick have shown that segmental boundaries do not usually form between even-numbered rhombomeres. The results from these experiments suggest that boundary formation in the hindbrain requires the juxtaposition of alternating odd- and even-numbered rhombomeres as these may have distinct cell adhesion properties (Guthrie and Lumsden, 1991; Guthrie et al., 1993). Recent studies in zebrafish suggest that the differences in adhesive and repulsive properties between odd- and even-numbered rhombomeres may be mediated through the bi-directional signaling between EphA4 receptors (expressed in r3 and r5) and EphrinB proteins (located in r2, r4, and r6) (Xu et al., 1995; Mellitzer et al., 1999; Xu et al., 1999; Cooke et al., 2001). In spg m793 mutants, subsequent to alterations in krx20 expression in r3 and r5, ephA4 expression is absent medially and restricted to lateral cell clusters. Thus, interactions of EphA4 receptors with Ephrin B proteins cannot occur in the medial hindbrain. Medial disruption of r3 and r5 territories brings even-numbered rhombomeres 2, 4 and 6 into direct opposition. The observed alterations might then prevent the formation of segmental boundaries. Consistent with this idea, spg m793 mutants have no obvious morphological inter-rhombomeric boundaries. Accordingly, pax6.1 expression, normally enriched along rhombomere boundaries, is not observed in spg m793 mutants. In contrast, rhombomere boundaries are formed in spg m216 mutants, as r3 and r5 territories, albeit reduced, separate even-numbered rhombomeres.

With the juxtaposition of even-numbered rhombomeres seen in strong spg m793 mutants, loss of the normal restriction of cell movement between segmental compartments might be expected (Fraser et al., 1990; Guthrie et al., 1993). In contrast, we observed that hox gene expression borders at the r2/r4 and r4/r6 interfaces are still relatively sharp in spg m793 mutants (Fig. 2C,1,O,R1). This could be due to reprogramming of hox expression when cells of adjacent segments intermingle. Alternatively, this could indicate that residual r2/r4 and r4/r6 boundaries may have formed or been maintained that are sufficient to restrict extensive intermingling between cells of adjacent segments. In support of this idea, Krox20 mutant mice show signs of segmental boundary formation in the ventral hindbrain despite the complete disappearance of r3 and r5 during development (Schneider-Maunoury et al., 1997).

### spg/pou2 is required in a permissive manner for segmental krx20 and val expression

Krox20 and kreisler play important roles in hindbrain segmentation (Schneider-Maunoury, 1993; Swiatek and Gridley, 1993; Cordes and Barsh, 1994; Schneider-Maunoury et al., 1997). The regulatory pathways that lead to their restricted expression in specific rhombomeres are, however, still elusive. We have shown that pou2 expression in the hindbrain primordium precedes the activation of krx20 and val, and overlaps with their expression domains. In spg mutants, the expression of krx20 and val is reduced from the earliest time of detection, while injection of pou2 mRNA can
restore normal expression. These data indicate that pou2 is required for the initial establishment of normal segmental expression of krx20 and val in the zebrafish hindbrain primordium. As widespread Pou2 overexpression is not sufficient to induce ectopic krx20 or val expression, Pou2 protein need not be strictly localized to limit its activity, suggesting that positional information is provided by other sources. Thus, Pou2 may function as an essential but permissive cofactor that, directly or indirectly, enables other transcription factors to activate krx20 and val. However, pou2-independent activation of krx20 and val may occur in the lateral neural plate, as both genes are still activated at lateral positions in spgm793 mutants. Further, pou2 appears not to be required for later maintenance of krx20 and val expression, as hindbrain expression of pou2 ceases during early somitogenesis (Hauptmann and Gerster, 1995).

**pou2 is an essential component of the regulatory cascade controlling hindbrain segmentation**

A pre-segmental stage of hindbrain regionalization likely involves the retinoid pathway (Gale et al., 1999; Gavalas and Krumlauf, 2000; Niederreither et al., 2000). During this stage, the hindbrain primordium is subdivided into an anterior (r1-r3) and a posterior (r4-r7) domain, which requires retinoid signaling to develop posterior characteristics. It has been shown that pou2 hindbrain expression can be influenced by application of retinoic acid in a similar manner as other segmentation genes such as krx20 (Hauptmann and Gerster, 1995). We suggest that pou2 may function in subdividing the pre-defined anterior and posterior hindbrain domains into single rhombomere territories.

Our data show that the establishment of normal segmental krx20 and val domains is affected in spg mutants. This suggests that pou2 is required for the initial specification of r3, r5, and r6. In contrast, analysis of Krox20 mouse mutants revealed that Krox20 is required for maintenance, but not for establishment, of r3 and r5 (Schneider-Maunoury, 1993; Swiatek and Gridley, 1993; Schneider-Maunoury et al., 1997). Analysis of zebrafish val mutants suggests that val functions in the later subdivision of an r5/r6 protosegment into definitive r5 and r6 (Moens et al., 1996; Moens et al., 1998). Thus, pou2 is required for the initial specification of distinct segmental territories, while krx20 and val appear to be required only subsequently. Taken together, this strongly suggests that pou2 acts before krx20 and val during hindbrain segmentation.

Similar to that of krx20 and val, the initial expression of hoxa2 in r2 and r3, hoxb2 in r3 and r5, and hoxb3 in r5 and r6 is affected in spgm793 mutants by the 1- to 2-somite stage. Thus, pou2 appears to be required for the proper establishment of the initial segmental expression domains of hoxa2, hoxb2 and hoxb3. In mouse, Krox20 directly regulates the expression of Hoxa2 and Hoxb2 in r3 and r5 (Sham et al., 1993; Nonchev et al., 1996), and Hoxb3 is a direct target of kreisler in r5 (Manzanares et al., 1997). Therefore, the alterations in hox gene expression in zebrafish spgm793 mutants may be secondary to those in krx20 and val in r3/r5 and r5/r6, respectively. Accordingly, hoxb3 is down-regulated in r5 and r6 in zebrafish val mutants (Prince et al., 1998). spgm793 mutants may also have krx20 and val independent alterations in hox gene expression as initial hoxa2 expression is disrupted in r2. In contrast, the initial hindbrain expression of early-acting hoxb1b and hoxb1a is normal in spg mutants, suggesting that these genes are activated independently of spg/pou2.

In addition to regulating Hox gene expression, Krox20 also directly activates the expression of EphA4 in r3 and r5 in mouse (Theil et al., 1998). In spgm793 mutants, we observed that ephA4 expression in r3 and r5 is restricted to the reduced krx20 expression domains. Thus, the abnormal rhombomere boundaries may be caused by disturbance of a regulatory pathway in which pou2 is required directly or indirectly for krx20 expression, which in turn controls ephA4 expression. Furthermore, the disruption of val expression in r5 and r6 of spgm793 mutants may also contribute to defective rhombomere boundary formation, since inactivation of val leads to lack of rhombomere boundaries caudal to the r3/r4 boundary (Moens et al., 1996; Moens et al., 1998) and disruption of normal Eph/Ephrin signaling (Cooke et al., 2001). Taken together, the loss of morphological rhombomere boundaries in spgm793 mutants is likely a consequence of the early disruption of krx20 expression in r3 and r5, and val expression in r5 and r6.

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