Development 129, 1645-1655 (2002)
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DEV2750

spiel ohne grenzen/pou2 is required for zebrafish hindbrain segmentation

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Accepted 26 December 2001

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; Spaniol 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 strong *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 (Westerfield, 1994). Two *spg* alleles with intermediate (*spg^{m216}*) (Schier et al., 1996) and strong phenotypes (*spg^{m793}*) (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 essentially as described previously (Moens et al., 1996). Three-dimensional images of tetra-methyl-rhodamine 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*/+ or +/+) 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 *hoxa1* (Alexandre et al., 1996) has recently been assigned to the *hoxbb* cluster (Amores et al., 1998), and is therefore termed *hoxb1b*. The gene previously termed *hoxb1* (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 microinjectors. 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^{m793}* 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^{m216}* (Schier et al., 1996) and *spg^{m793}* (Belting et al., 2001), to analyze the effects of loss of *pou2* function on hindbrain segmentation. *spg^{m793}* 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^{m216}* 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^{m216}* 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^{m793}* 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^{m216}* 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^{m793}* 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^{m216}* 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^{m793}* 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^{m793}* 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^{m793}* mutants by a Mauthner cell specific antibody (3A10; data not shown). Weak *spg^{m216}*

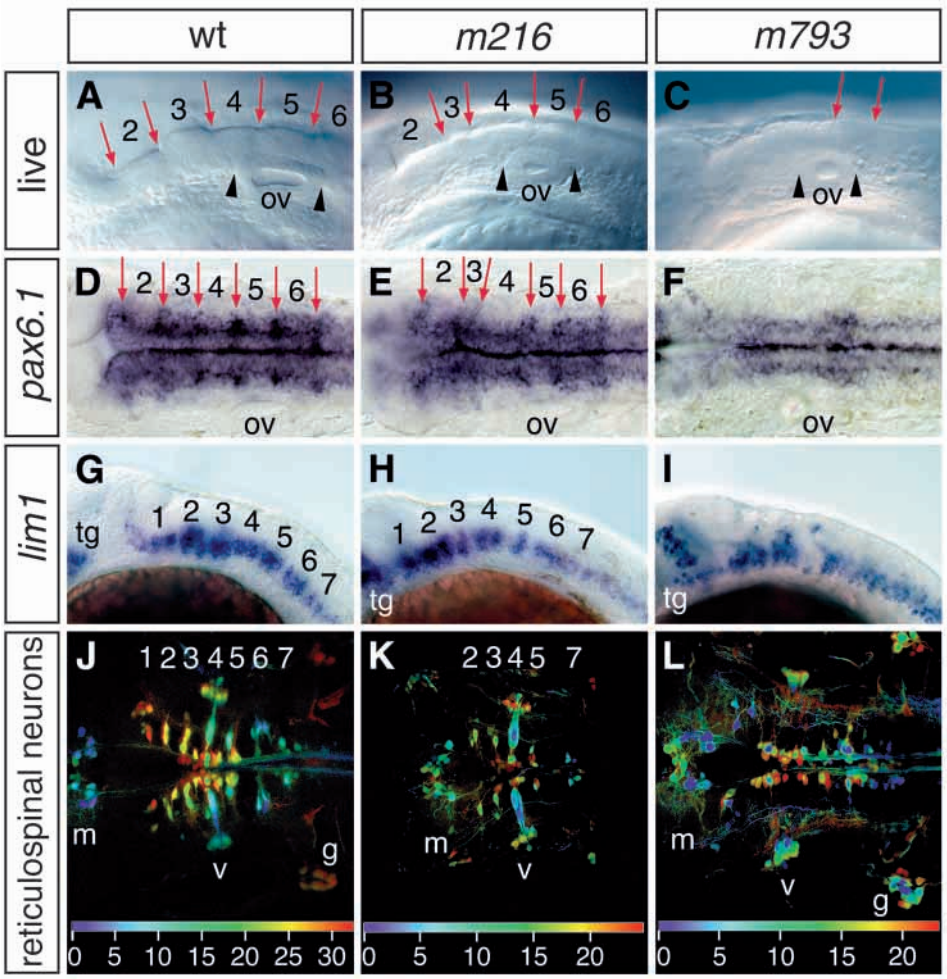

Fig. 1. Inter-rhombomeric boundaries and segmental neuronal differentiation are affected in *spg* mutants. (A-C) Lateral views of the hindbrain of live wild-type (A), *spg*^{m216} (B) and *spg*^{m793} (C) embryos at the 22-somite stage. Red arrows indicate the positions of rhombomere boundaries and black arrowheads mark the size of the otic vesicle (ov). (D-F) Dorsal views of the hindbrain of wild-type (D), *spg*^{m216} (E), and *spg*^{m793} (F) embryos at 1 dpf that were assayed for *pax6.1* expression by WISH. (G-I) Lateral views of wild-type (G), *spg*^{m216} (H), and *spg*^{m793} (I) embryos at 1 dpf that were assayed for *lim1* expression by WISH. (J-L) Confocal images of wild-type (J), *spg*^{m216} (K) and *spg*^{m793} (L) embryos at 5 dpf in which hindbrain reticulospinal neurons were back-filled with lysinated rhodamine-dextran. Stacks of confocal images were merged to produce a composite image in which the signal from each layer was depth-coded in color. A color-coded depth scale (in μm) is shown below each figure (dorsal-most blue to ventral-most red). In the *spg*^{m216} mutant embryo shown (K), reticulospinal neurons in r1 and r6 are not seen while the large Mauthner neurons in r4 are duplicated. In *spg*^{m793} mutant embryos (L), fully-differentiated primary reticulospinal neurons are not observed. Anterior is to the left in all panels. 1-7, rhombomeres 1-7; g, ganglia of the posterior lateral line neurons; m, nuclei of the medial longitudinal fascicles; tg, tegmentum; v, vestibular nuclei.

mutants show relatively normal primary reticulospinal neurons in rhombomeres 2-5 (Fig. 1K). Analysis of 15 *spg*^{m216} mutants revealed that expressivity of the phenotype ranges in severity from the embryo shown in Fig. 1K to neuronal disorganization similar to that of *spg*^{m793} mutants (Fig. 1L). Duplicated (Fig. 1K) or lost Mauthner cells are occasionally observed in *spg*^{m216} mutants (in 2 and 3, respectively, of 15 embryos). Our results show that disruption of hindbrain segmentation in *spg* mutants is apparent at the cellular level and affects various hindbrain neurons.

Altered *hox* gene expression in *spg* mutants

To investigate hindbrain segmental patterning in *spg* mutants, we assayed *hox* (Prince et al., 1998) and *krx20* (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. 2I,L). Both the *hoxb1a* domain (Fig. 2I1,I2) and the low-level expression domain of *hoxb2* (Fig. 2O1,O2), 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. 2I,O with 2C). Thus, r2 and r4 territories appear to directly juxtapose in *spg*^{m793} mutants.

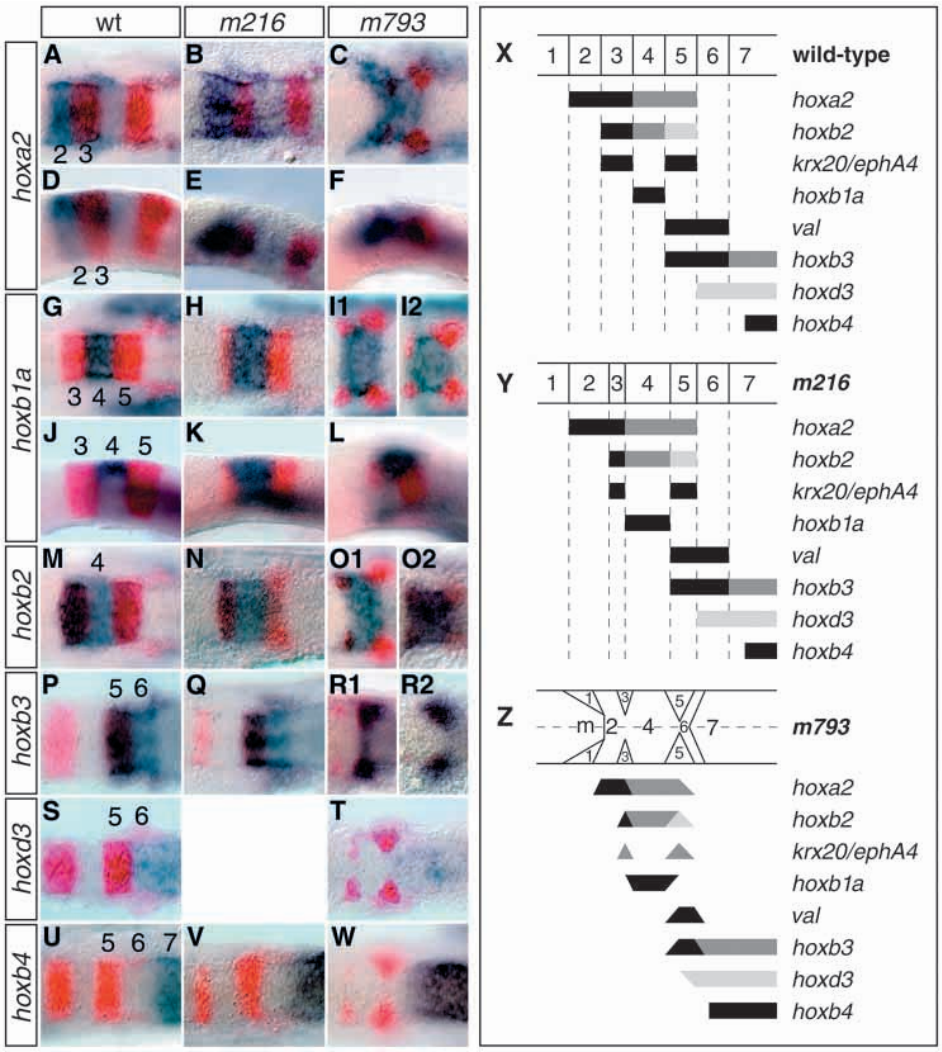

Fig. 2. Abnormal *hox* gene expression in *spg* mutants at the 10-somite stage. Dorsal (A-C,G-I,M-W) and lateral (D-F,J-L) views of the hindbrain of wild-type (left column), *spg*^{m216} (middle column) and *spg*^{m793} (right column) embryos at the 10-somite stage. Embryos have been assayed for expression of hox genes (blue) by WISH as indicated at left. *krx20* expression (red) in r3 and r5 was also detected. Schematic summary of *hox*, *krx20*, *ephA4* and *val* expression in wild-type (X), *spg*^{m216} (Y) and *spg*^{m793} (Z) embryos at the 10-somite stage. The diagrams do not exactly represent the proportional sizes of rhombomeres. The shape of r1 in (Z) is deduced from *ephA4* expression at 1 dpf (Fig. 5G,I). In all photographs, anterior is to the left. In D-F,J-L, dorsal is up. 1-7, rhombomeres 1-7.

Elevated *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 *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 *spg*^{m793} mutants. In the most strongly affected *spg*^{m793} mutants, elevated *hoxb3* expression is seen only laterally, indicating medial disruption of both r5 and r6 (Fig. 2R2). Expression of *hoxb4*, which has an anterior boundary within r7 at this stage, is not obviously altered in *spg*^{m793} mutant embryos (Fig. 2W).

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

spg is required for formation of normal *krx20* and *val* expression domains

Krox-20/*krx20* (Oxtoby and Jowett, 1993; Schneider-Maunoury, 1993; Swiatek and Gridley, 1993) and *kr*/*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, *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, *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 *krx20* (Fig. 3E,I) and *val* (Fig. 3G,K) fuse at the midline during early somitogenesis stages. In *spg*^{m793} mutants, the initial activation of *krx20* (Fig. 3B1, and slightly later Fig. 3B2) and *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 *krx20*- and *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 *krx20*- and *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). *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 *spg/pou2* is required for two aspects of the

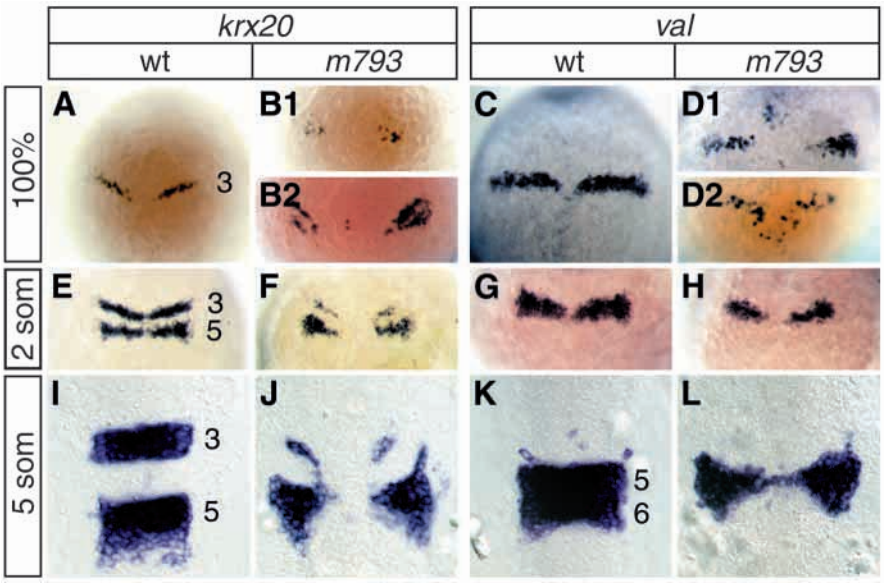

Fig. 3. Early expression of *krx20* and *val* is severely reduced in *spg*^{m793} mutants. Dorsal views of wild-type embryos (A,C,E,G,I,K) and *spg*^{m793} mutants (B,D,F,H,J,L) at 100% epiboly (A-D), 2-somite (E-H), and 5-somite (I-L) stages showing detection of *krx20* (A,B,E,F,I,J) or *val* (C,D,G,H,K,L) expression by WISH. Anterior is to the top. 3, 5, 6; rhombomeres 3, 5 and 6.

establishment of normal *krx20* and *val* expression. First, the initial expression of these genes is reduced in *spg*^{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 *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*^{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*^{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 *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*^{m216} mutants (Fig. 5B), while

absent medially in *spg*^{m793} 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).

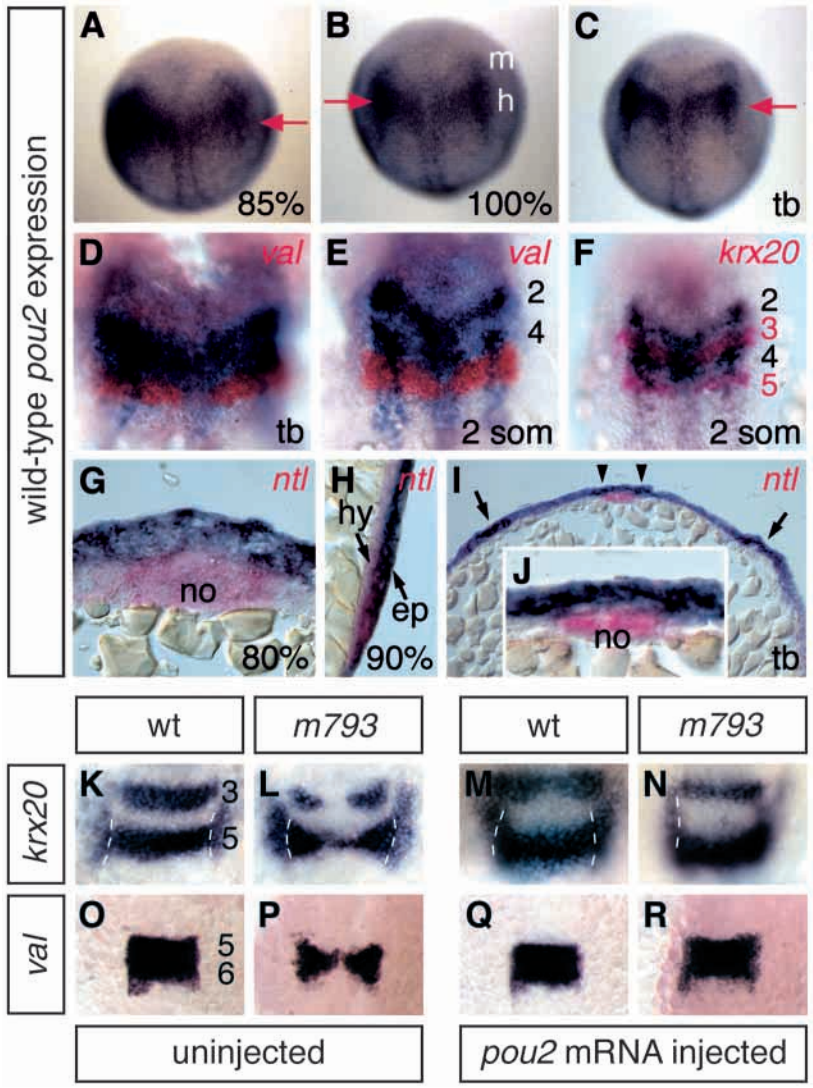

Fig. 4. Injection of *pou2* mRNA in *spg*^{m793} mutants can rescue segmental expression of *krx20* and *val*. (A-F) Dorsal views of *pou2* expression (purple/blue) during late gastrula to early somitogenesis stages in comparison with that of *val* in r5/r6 (red; D,E) and *krx20* in r3/r5 (red; F). (A-C) High-level *pou2* expression in the hindbrain primordium is marked by a red arrow. (G-J) Restriction of *pou2* expression to the epiblast. Sagittal (H) and transverse (G,I,J) sections through the neural plate and notochord of wild-type embryos stained for *pou2* (blue) and *ntl* (red). (I) Arrows and arrowheads mark strong lateral and medial *pou2* expression in the hindbrain primordium, respectively. Embryonic stages are indicated. (K-R) Dorsal views of the hindbrain primordia of uninjected and *pou2* mRNA-injected embryos at the 5- to 6-somite stage. The genotypes of the embryos are wild-type or *spg*^{m793}, as indicated at the top. Genotypes were determined by an allele-specific restriction polymorphism detected in genomic DNA amplified from the embryos shown. Expression of *krx20* (K-N) and *val* (O-R) was detected by WISH. *pax2.1* expression was also assayed (K-N) to compare rescue of hindbrain gene expression in *spg*^{m793} mutants with that of the MHB. While *pax2.1* expression in the MHB is not shown, *pax2.1* in the otic placodes is visible on the images (K-N; lateral edges between hindbrain and otic placodes highlighted by dotted white lines). Embryos in O and Q were determined to be +/+ for *spg*. Anterior is up in all whole-mount embryos shown. 2-6, rhombomeres 2-6; ep, epiblast; h, hindbrain; hy, hypoblast; m, midbrain; no, notochord; tb, tailbud stage.

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 *spg*^{m793} 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 *spg*^{m793} 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 *spg*^{m793} 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 *spg*^{m793} 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/ndr2* (Rebagliati et al., 1998; Sampath et al., 1998), *taram-a* (Renucci et al., 1996), *lefty1/antivin* (Bisgrove et al., 1999; Thisse and Thisse, 1999), *shh* (Krauss et al., 1993), *twhh* (Ekker et al., 1995), *ehh* (Currie and Ingham, 1996), *chordin* (Schulte-Merker et al.,

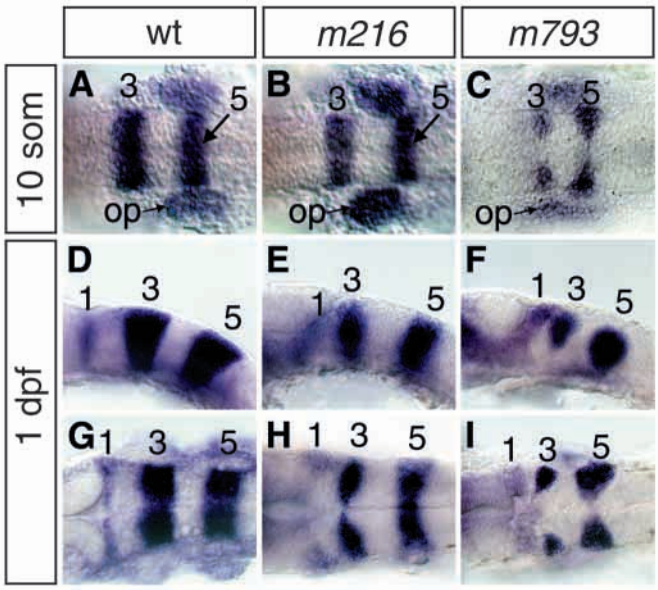

Fig. 5. Hindbrain expression of *ephA4* is altered in *spg* mutants. Dorsal (A-C,G-I) and lateral (D-F) hindbrain views of wild-type (A,D,G), *spg*^{m216} (B,E,H) and *spg*^{m793} (C,F,I) mutant embryos at the 10-somite stage (A-C) and at 1 dpf (D-I). Expression of *ephA4* is visualized by WISH. In all panels, anterior is to the left. In D-F, dorsal at top. 1, 3, 5, rhombomeres 1, 3and 5; op, otic placode.

1997), *noggin1* (Fürthauer et al., 1999), *ntl* (Schulte-Merker et al., 1992), *flh* (Talbot et al., 1995), *fkd1/axial* (Strähle et al., 1993), *fkd2* and *fkd4* (Odenthal and Nüsslein-Volhard, 1998) was present in all analyzed *spg*^{m793} mutant embryos (each gene analyzed at one or two time points; data not shown). At the beginning of somitogenesis, the expression levels of *twhh* in the prechordal plate appeared more variable in *spg*^{m793} mutants than in wild-type embryos. However, the *twhh* domain is anterior to the segmentation defects observed in the hindbrain. Taken together, this suggests that *pou2* is not required for normal activation of midline expression of these genes in or beneath the hindbrain primordium.

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*^{m216} 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 may play an essential role in defining the anteroposterior spatial extents of individual rhombomeric domains.In addition to abnormal rhombomere sizes, in *spg*^{m793} mutants r1, r3, r5, and in strongest mutants also r6 are disrupted in the midline of the neural plate (Fig. 2Z). The medial disruptions of rhombomere precursor territories in *spg*^{m793} 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 juxtapose. 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*^{m793} 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*^{m793} mutants (Fig. 2I1,I2,L).

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*^{m793} 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*^{m793} 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*^{m793} 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*^{m793} 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*^{m793} mutants in the mid-/hindbrain region during gastrulation. Increased cell death scattered throughout the neural keel in *spg*^{m793} mutants was detected from the 2-somite stage onwards (data not shown). This, however, could not cause

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).

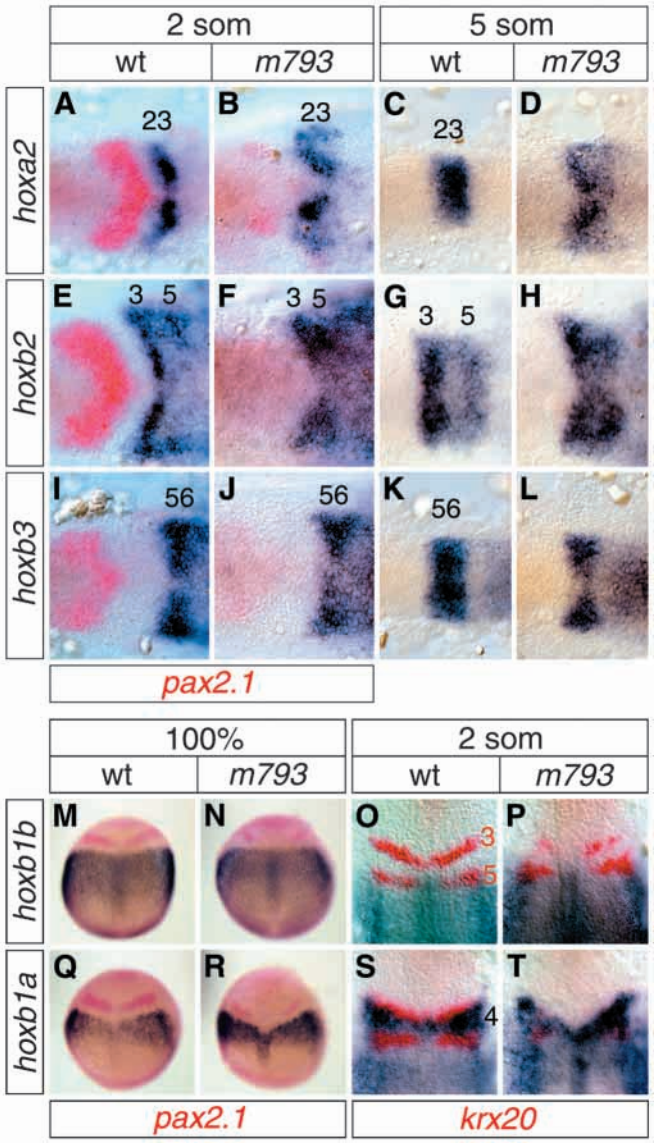

Fig. 6. Early segmental expression of *hoxa2*, *hoxb2* and *hoxb3* is altered in *spg*^{m793} mutants. Wild-type (A,C,E,G,I,K,M,O,Q,S) and *spg*^{m793} mutant embryos (B,D,F,H,J,L,N,P,R,T) at the 100% epiboly (M,N,Q,R), 2-somite (A,B,E,F,I,J,O,P,S,T) and 5-somite (C,D,G,H,K,L) stages showing expression of *hoxa2* (A-D), *hoxb2* (E-H), *hoxb3* (I-L), *hoxb1b* (M-P), and *hoxb1a* (Q-T) (WISH; purple/blue). To identify *spg* mutants, *pax2.1* (A,B,E,F,I,J,M,N,Q,R) or *krx20* (O,P,S,T) expression (red) was also assayed. All panels show dorsal views, anterior is to the left (A-L) or up (M-T). 2-6, rhombomeres 2-6.

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,I,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 *spg*^{m793} 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 *spg*^{m793} 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 *spg*^{m793} 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). *spg*^{m793} 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 *spg*^{m793} 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 *spg*^{m793} 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 *spg*^{m793} mutants is likely a consequence of the early disruption of *krx20* expression in r3 and r5, and *val* expression in r5 and r6.

Thanks to Jon Clarke for communication regarding effective reticulospinal neuron labeling, to Zoltan Varga for assistance with confocal microscopy, and to Judith Eisen for productive discussion concerning the neuronal deficits observed in *spg* mutants. We also thank T. Jowett, C. Moens, I. Dawid, A. Fjose, A. Püschel, S. Wilson and T. Gerster for providing cDNA plasmids for in situ hybridization. Thanks to Gerlinde Wussler and Sabine Götter for expert animal care, and to Roswitha Koppa for cryosectioning. This work was supported by a grant of the Deutsche Forschungsgemeinschaft (DR362 1/1), the Landesforschungsschwerpunktprogramm 'Entwicklungsmechanismen' and NIH/NIMH RO1 MH56552 to W. D.; G. H. was supported by a 'Forschungsstipendium' in the programme 'Evolution Entwicklungsbiologischer Prozesse' of the Deutsche Forschungsgemeinschaft (Ha 2706/1-1).

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