INTRODUCTION

Embryonic organizing centers, specialized groups of cells that are both necessary and sufficient to pattern a larger cell population to which they belong (Struhl and Basler, 1993; Spemann and Mangold, 1924), have been shown to play crucial roles in patterning the anteroposterior axis of the vertebrate central nervous system. Two examples of such organizers involved in local patterning within the CNS include the anterior neural ridge (or row 1 cells in zebrafish) of the forebrain (Shimamura and Rubenstein, 1997; Houart et al., 1998) and the midbrain-hindbrain boundary (reviewed by Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). These organizers were discovered to have forebrain- and midbrain/cerebellar-inducing properties, respectively, through transplantation experiments. Fibroblast growth factor (FGF) signals, in particular FGF8, are expressed in these particular organizers, and FGF8 is sufficient to mediate their inducing properties (Shimamura and Rubenstein, 1997; Crossley et al., 1996; Irving and Mason, 2000). Furthermore, mutational analyses of Fgf8 in mice and fgf8 in zebrafish demonstrate that FGF signaling is required for forebrain and midbrain/cerebellar development (Meyers et al., 1998; Shanmugalingam et al., 2000; Reifers et al., 1998). It is not clear to what extent such local organizers are used in patterning other regions of the CNS.

Segmentation is another mechanism that is used to promote patterning and regionalization along the anteroposterior axis of the CNS. This is particularly true in the vertebrate hindbrain, which becomes subdivided into segments, termed rhombomeres, each of which acquires distinct cellular and molecular characteristics (reviewed by Lumsden and Krumlauf, 1996). Rhombomeres function to order neuronal differentiation and cranial neural crest migration patterns that are crucial for the proper development and function of the vertebrate head. Several transcription factors have been identified that are expressed in conserved, rhombomere-specific patterns and are required for proper hindbrain development. These include krox-20 (egr2 – Zebrafish Information Network) kreisler/mafB/valentino and several Hox genes (reviewed by Schneider-Maunoury et al., 1998). While signals such as FGFs (Marín and Charnay, 2000a) and retinoic acid (reviewed by Gavalas and Krumlauf, 2000) have been proposed to play roles in activating the expression of these hindbrain segmentation genes, it is not clear when and from which tissues such signals might be acting. Evidence for interactions between rhombomeres, particularly in promoting krox-20 and val expression (Graham and Lumsden, 1996; Helmbacher et al., 1998; Marín and Charnay, 2000b), suggests that local organizing signals may play roles in patterning the hindbrain.

Using time lapse analyses of zebrafish hindbrain development, we find that r4 is the first rhombomere to form. Reticulospinal and motoneuron differentiation occurs earliest in r4. Two FGF signals, FGF3 and FGF8, are expressed early in r4, and are together required for the development of rhombomeres adjacent to r4, particularly r5 and r6. Transplantation of r4 cells can induce expression of r5/r6 markers, as can misexpression of either FGF3 or FGF8. Genetic mosaic analyses also support a role for FGF signaling acting from r4. Taken together, our findings demonstrate a crucial role for FGF-mediated inter-rhombomere signaling in promoting early hindbrain patterning and underscore the significance of organizing centers in patterning the vertebrate neural plate.

SUMMARY

The segmentation of the vertebrate hindbrain into rhombomeres is highly conserved, but how early hindbrain patterning is established is not well understood. We show that rhombomere 4 (r4) functions as an early-differentiating signaling center in the zebrafish hindbrain. Time-lapse analyses of zebrafish hindbrain development show that r4 forms first and hindbrain neuronal differentiation occurs first in r4. Two signaling molecules, FGF3 and FGF8, which are both expressed early in r4, are together required for the development of rhombomeres adjacent to r4, particularly r5 and r6. Transplantation of r4 cells can induce expression of r5/r6 markers, as can misexpression of either FGF3 or FGF8. Genetic mosaic analyses also support a role for FGF signaling acting from r4. Taken together, our findings demonstrate a crucial role for FGF-mediated inter-rhombomere signaling in promoting early hindbrain patterning and underscore the significance of organizing centers in patterning the vertebrate neural plate.

Key words: Hindbrain, Rhombomere, Organizer, FGF3, FGF8, acerebellar, valentino, krox-20, Zebrafish

FGF3 and FGF8 mediate a rhombomere 4 signaling activity in the zebrafish hindbrain

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acting from r4. Our findings thus demonstrate the existence of an FGF-mediated signaling center in r4. The early establishment of r4 as a signaling center promotes the development of adjacent rhombomeres and thus supports the propagation of hindbrain segmental patterning. Evidence from studies of other vertebrates suggests that this r4 signaling center is conserved.

MATERIALS AND METHODS

Zebrafish lines

Zebrafish (Danio rerio) were raised and staged as previously described (Westerfield, 1995; Kimmel et al., 1995). Time (h) refers to hours post-fertilization at 28.5°C. In some cases, embryos were raised overnight at 25°C. The wild-type line used was AB. The acerebellarti (ace) line, a strong hypomorph for fgf8, has been described previously (Brand et al., 1996; Reifers et al., 1998; Draper et al., 2001). Homozygous ace mutant embryos were scored by their loss of the cerebellum or loss of midbrain described previously (Brand et al., 1996; Reifers et al., 1998; Draper et al., 2001). Staging of embryos was also performed as described previously (Jowett and Lettice, 1994; Hauptmann and Gerster, 1994). Embryos were mounted between two coverslips in a mixture of 0.5% agar in seawater.

RNA in situ hybridization

cDNA probes that detected the following genes were used: isl1; pax2.1; val; pea3 (Münchberg et al., 1999). Probe syntheses and whole-mount in situ hybridization were performed as previously described (Krauss et al., 1991; Kimmel et al., 1996). RNA in situ hybridization was performed by NIH Image software (http://rsb.info.nih.gov/nih-image/index.html). The presence and identities of early boundaries were confirmed by analyzing recordings both retrospectively and prospectively.

fgf3 morpholinos and embryo injections

Based on the published sequence of zebrafish fgf3 (Kiefer et al., 1996) (we also confirmed fgf3 5'UTR sequence in our AB wild-type line), four antisense morpholino oligos (MOs) were ordered (Gene Tools): fgf3A; 5'-CAGTTGTTGAGCCGGAATGTCCG-3'; fgf3B; 5'-GGTCCCATATCAAGAAGTACATTTTG-3'; fgf3C; 5'-TCTCGCTGGGATGAAAGAAGCTGCC-3'; and fgf3MMA, 5'-CAATGTGCATGCGGGGTTTGGC-3'. Embryos were injected/heat-shocked control embryos show largely normal development of adjacent rhombomeres and thus supports the establishment of r4 as a signaling center promotes the propagation of hindbrain segmental patterning. Evidence from studies of other vertebrates suggests that this r4 signaling center is conserved.

Immunocytochemistry

Embryos were fixed in 4% paraformaldehyde in PBS for 2 hours at room temperature or, for RMO-44, in 2% trichloroacetic acid in water for 4 hours at room temperature. After fixation, embryos were rinsed with PBS, then rinsed with distilled water, permeabilized with acetone treatment at –20°C for 10 minutes, rinsed again with distilled water and then PBS, and then blocked in PBDTX (PBS with 1% bovine serum albumin, 1% DMSO, pH 7.5, and 0.1% Triton X-100) with 2% normal goat serum (NGS) for 30 minutes. Embryos were then...
Rhombomere 4 (r4) is the first rhombomere to differentiate in the zebrafish hindbrain

To investigate how early hindbrain patterning develops in zebrafish, we used time-lapse analyses to determine which rhombomeres form earliest. We generated movies of early hindbrain development in live embryos using confocal imaging of embryos stained with the fluorescent vital dye BODIPY-ceramide, which labels interstitial space in zebrafish embryos (Cooper et al., 1999) and thus allows detection of early rhombomere boundary formation (Moens et al., 1998). Recordings were made from a stage when no rhombomere boundaries are visible (about 10.5 hours post-fertilization (h)/two somites (s)) to a stage when rhombomeres can be identified based on their position relative to the otic vesicle (about 17 h/16 s) (Moens et al., 1998). The earliest rhombomere boundaries to form are the r3/4 and r4/5 boundaries (Fig. 1A,B). These boundaries first appear between 11.6 h/5 s and 12.5 h/7 s (Fig. 1A,B) (Moens et al., 1998). The remaining boundaries, between r1/2 and r6/7, form by about 14 h/10 s (Fig. 1C,D). We have generated similar movies using Nomarski imaging and find that the r3/4 and r4/5 boundaries are again the earliest to appear (not shown; a confocal movie showing rhombomere boundary formation can be viewed at http://www.neuro.uoregon.edu/kimmel/rhomform.html). These results reveal that r4 is the first rhombomere established with morphological boundaries in the zebrafish hindbrain.

We next investigated early neuronal differentiation in the hindbrain. Mendelson (Mendelson, 1986) analyzed the time of origin of reticulospinal interneurons using ³H-thymidine incorporation and found that the earliest-born cells of this group are the pair of Mauthner neurons in r4. Hatta (Hatta,
1992) found that Mauthner neurons are also the earliest reticulospinal cells to label with an anti-neurofilament antibody. To ask whether other types of hindbrain neurons also differentiate first in r4, we examined the early differentiation of hindbrain motoneurons using expression of islet1 (isl1), which encodes a LIM-homeodomain protein expressed in zebrafish cranial motoneurons (Chandrasekhar et al., 1997). isl1 first appears in the hindbrain in ventral clusters of cells in r4 at about 14.5 h/11 s (Fig. 1F), labeling the early facial (nVII) motor nucleus (Chandrasekhar et al., 1997). Shortly thereafter, isl1 also appears in ventral cell clusters in r2, labeling the early trigeminal (nV) motor nucleus (Fig. 1G). isl1 expression then extends into more caudal hindbrain segments, reflecting the caudal migration of the facial motoneurons (Fig. 1G,H) (Chandrasekhar et al., 1997; Higashijima et al., 2000). Thus, in addition to reticulospinal interneurons, differentiation of cranial motoneurons occurs earliest in r4. These results, in addition to the time-lapse analyses, reveal that r4 is the first rhombomere to differentiate in the zebrafish hindbrain.

**fgf3 and fgf8 are both expressed in early r4**

We hypothesized that the early development of r4 may indicate a possible function for r4 in promoting subsequent development of neighboring rhombomeres by acting as an early signaling center. In support of this hypothesis, we find that an FGF signaling gene, fgf3, is expressed early in presumptive r4. Zebrafish fgf3 (Kiefer et al., 1996) is expressed in the presumptive hindbrain beginning at 9 h/90% epiboly (Fig. 2A) in a transverse stripe, which, relative to the midbrain-hindbrain boundary (MHB) marker pax2a (Krauss et al., 1991), is in the center of the early hindbrain (arrow in Fig. 2A). At 10 h/bud stage, when krox-20 expression first appears in presumptive r3 (Oxtoby and Jowett, 1993), the fgf3 stripe is just caudal to r3 (and possibly some expression in r3, Fig. 2B). From 10.3-12 h/1-6 s, we find strong fgf3 expression in presumptive r4, as well as some weaker expression in r3, r5 and r6 (Fig. 2C,D). Beginning at about 13 h/8 s, fgf3 expression in the hindbrain is restricted to r4 (Fig. 2E), and fgf3 continues to be expressed in r4 until about 18 h/18 s, at which time hindbrain expression of fgf3 becomes undetectable (not shown). During gastrulation and somitogenesis stages, fgf3 also has other domains of expression, some of which have been described by Fürthauer et al. (Fürthauer et al., 2001) and Phillips et al. (Phillips et al., 2001), including the margin during gastrulation (not shown), the forebrain (Fig. 2C), the MHB (Fig. 2E), the tail bud (Fig. 2C) and the head periphery (Fig. 2E). Zebrafish fgf3 is thus expressed in several domains that are similar to those observed for fgf3 expression in other vertebrates (Wilkinson et al., 1988; Tannahill et al., 1992; Mahmood et al., 1995; Mahmood et al., 1996) (see Discussion).

A second fgf gene, fgf8, is also expressed in the early hindbrain, beginning at about 7.5 h/70% epiboly (Reifers et al., 1998). Until about 10.3 h/1 s, fgf8 is expressed in the anterior half of the hindbrain (Fig. 2F) and subsequently begins to resolve into domains at the MHB/r1, ventral r2 and r4 (Reifers et al., 1998) (Fig. 2G). We find that the r4 domain of fgf8 persists until about 13 h/8 s (not shown). To show that fgf3 and fgf8 are co-expressed early in r4, we performed double in situ hybridization on 10 h/bud-stage embryos and find overlap between the two signals in presumptive r4 (Fig. 2H).

**fgf3 and fgf8**

fgf3 and fgf8 are two of the earliest genes known to be expressed in the zebrafish hindbrain primordium, and their overlapping expression in presumptive r4, beginning with the initiation of fgf3 expression at 9 h/90% epiboly, precedes that of krox-20 in r3 and r5 (late bud stage) (Oxtoby and Jowett, 1993), val in r5 and r6 (bud stage) (Moens et al., 1998) and rhombomere-specific expression of the Hox genes (Prince et al., 1998). fgf3 and fgf8 are thus present at the proper time and place to mediate a potential early signaling activity of r4 and regulate the expression of hindbrain segmentation genes.

**FGF3 and FGF8 are required for r5 and r6 development**

We next addressed the requirements for FGF3 and FGF8 in hindbrain patterning. Although a mutant line exists [acerbellar\textsuperscript{282a} (ace)] that is a strong hypomorph for fgf8 (Reifers et al., 1998; Draper et al., 2001), an fgf3 mutant line has not yet been isolated. To knock down the function of FGF3, we used antisense morpholino oligos (MOs), which have been shown to block translation and function of target genes when injected into zebrafish embryos (Nasevicius and Ekker, 2000). We performed several controls to demonstrate that the fgf3 MOs can indeed block translation of FGF3 and also act specifically on fgf3 (see Materials and Methods). As we will
document in the following sections, we do not see severe defects in hindbrain patterning in fgf3 morphant (fgf3-MO) embryos. Similarly, fgf8 mutant, or fgf8\(^{-}\), embryos, aside from loss of the cerebellum (Reifers et al., 1998), show only subtle defects in hindbrain patterning (Roehl and Nüsslein-Volhard, 2001) (this paper). Thus, severe reduction of either FGF3 or FGF8 has little effect on hindbrain patterning.

To address whether fgf3 and fgf8 interact or function redundantly in hindbrain patterning, we injected fgf3 morpholinos into fgf8- embryos, thus generating fgf3-MO; fgf8- embryos. We examined the expression of several hindbrain patterning genes at the 18-19 h/18-20 s stage, a period when these genes show well-defined rhombomere-specific expression. We find that, although krox-20 (Oxtoby and Jowett, 1993) expression appears fairly normal in fgf3-MO embryos and fgf8\(^{-}\) embryos (although rhombomeres often appear reduced in width; Fig. 3A, parts a and c), the r5 stripe of krox-20 expression is completely lost in fgf3-MO; fgf8\(^{-}\) embryos and the r3 stripe appears reduced (Fig. 3A, part d). fgf3-MO; fgf8\(^{-}\) embryos also show complete loss of val (Moens et al., 1998) expression in r5 and r6 (Fig. 3A, part n) and low levels of hoxb3 (Prince et al., 1998) in r5 and r6 (Fig. 3A, part l). Furthermore, we find that expression of hoxb4, which normally has a rostral boundary at the r6/r7 boundary (Prince et al., 1998) (Fig. 3A, part q), is contiguous with hoxb1a in fgf3-MO; fgf8\(^{-}\) embryos (Fig. 3A, part p). Staining of fgf3-MO; fgf8\(^{-}\) embryos with hoxb4 (without hoxb1a) reveals a space corresponding to the width of r4 between the anterior hoxb4
We next asked whether the loss of r5 and r6 in fgf3-MO; fgf8- embryos was evident at the level of neuronal development. We analyzed the reticulospinal interneurons of the hindbrain using the anti-neurofilament antibody RMO-44 (Pleasure et al., 1989). The reticulospinal neurons show rhombomere-specific cell body shapes and axonal projection patterns (Metcalfe et al., 1986) (Fig. 4A). For example, at 48 h, RMO-44-labeled reticulospinal cells in r2, r4 and r6 have axons that cross the midline (Fig. 4A). In addition, the T interneurons, which are present in r7 and extend into the rostral spinal cord, have characteristically large, round cell bodies and T-shaped axonal branching patterns (Kimmel et al., 1985) (Fig. 4A). The reticulospinal pattern appears largely normal in fgf3-MO embryos and fgf8- embryos (Fig. 4B,C). However, fgf3-MO; fgf8- embryos have T cells just caudal to the pair of Mauthner cells in r4 (Fig. 4D) and no r5 or r6 cells can be identified, providing further support for the loss of r5 and r6.

We also analyzed the development of motoneurons in fgf3-MO; fgf8- embryos. The anti-Isl antibody 39.4D5 labels rhombomere-specific branchiomotor nuclei (nV, nVII, nIX and nX) and the abducens motor nucleus (nVI) at 48 h (Chandrasekhar et al., 1997) (Fig. 4E). We find that the motoneuron pattern appears largely normal in fgf3-MO embryos and fgf8- embryos (Fig. 4F,G). However, the motoneuron pattern in fgf3-MO; fgf8- embryos is quite disrupted: nV neurons in r2 and r3 appear less organized and nX neurons are situated much more rostrally relative to the nV neurons (Fig. 4H). We suspect that the Isl-positive cells found between nV and nX in fgf3-MO; fgf8- embryos are nVII neurons for two reasons. First, the zn-8 antibody specifically labels nV and nX neurons [similar to zn-5 (Chandrasekhar et al., 1997)] in fgf3-MO; fgf8- embryos is quite disrupted: nV neurons in r2 and r3 appear less organized and nX neurons are situated much more rostrally relative to the nV neurons. Second, we find that the nVII neurons, analyzed during somitogenesis for isl1, as in Fig. 1, arise in r4 in fgf3-MO; fgf8- embryos as in wild-type embryos (not shown). These results show that motor nuclei that are thought to either arise in r5 and r6 (nVI) or be dependent on r5/6 for their development (nX) (Chandrasekhar et al., 1997) are lost in fgf3-MO; fgf8- embryos. Taken together, the results of our rhombomere-marker and neuronal-marker analyses show that severe reduction of FGFR3 and FGFR8 leads to a reduction of rostral rhombomeres 1-3, maintenance of r4 and r7, and complete loss of r5 and r6.

**Transplantation and mosaic analyses reveal r4 signaling activity**

The loss of r5 and r6 upon reduction of FGFR3 and FGFR8 in r4, which normally promote the development of r5 and r6. To test for signaling activity from r4, we heterotopically transplanted r4 cells at bud/1-s stage, when we propose FGFR signaling from r4 is promoting r5/6 development, to see if they could non-autonomously induce r5/6 fates. Seven out of 10 control transplants, from r4 to r4, incorporated into r4 (Fig. 5A,B,B’); however, donor cells could additionally be found in other rhombomeres (from r3-r6). The remaining three out of ten transplants incorporated into r5/6. The presence of donor cells outside of r4 may in part be due to dispersal; when such transplants are fixed at an earlier stage (4 s), 16/17 were in r4 (and overlapped with r3 or r5 in most cases; not shown). These controls, with the expression of r4 markers in donor
cells (see Materials and Methods), demonstrate that we are largely targeting r4 in our transplants. Such isochronic transplants showed no non-autonomous induction of krox-20 expression either within the host hindbrain or if transplants were placed in other regions (not shown), so we heterochronically transplanted to the naïve ventral ectoderm of shield-stage hosts (Woo and Fraser, 1998) (Fig. 5A). r4 transplants in these hosts remain clumped together, reside in the ventral or lateral surface ectoderm on the yolk, and most express r4/5 markers (Fig. 5C-G). Five out of 21 r4 transplants induced non-autonomous expression of krox-20 and val (Fig. 5D-G). When hosts carrying r4 transplants are allowed to develop further, we find (in five out of 20 cases) non-autonomously-induced differentiated tissue that is normally associated with the r5/6 region, including pigment and ears (not shown). These transplants reveal that r4 cells have signaling activity outside of the hindbrain. To show that this signaling activity is restricted to the r4 region and is not a general activity of early hindbrain tissue, we transplanted cells from the r1 region to shield-stage hosts and found no expression of val either in the transplanted cells or induced by the transplant (n=8). We also transplanted cells from the r6 region and found that even though many of these transplants expressed val (5/8; Fig. 5H-H'), none showed non-autonomous induction of val. Taken together, these transplant experiments reveal a signaling activity that is strongly associated with, or centered on, r4.

As the above experiments show that r4 cells are sufficient to induce r5/6 development, we next addressed whether r4 cells are required for r5/6 development. Embryos with partial removal of r4, as in the above donors, show normal krox-20 expression (n=22; not shown), because sufficient signaling probably remains, but larger ablations cause too much damage. We attempted to target fgf3-MO; fgf8− cells to r4 of wild-type host embryos but found that cells with the optimum dose of fgf3 morpholino do not survive in a wild-type background (not shown). We therefore performed the experiment shown in Fig. 6A and found that wild-type cells in an fgf3-MO; fgf8− hindbrain can partially rescue expression of krox-20 in r5 (Fig. 6C). We observe several characteristics of these rescued mosaic embryos that support a requirement for r4 signaling. First, we find that the rescue can occur non-cell-autonomously: fgf3-MO; fgf8− host cells are induced to express krox-20 (Fig. 6C). Second, some, but not all, wild-type donor cells show krox-20 expression in the rescued r5 region of fgf3-MO; fgf8− hosts (Fig. 6C), suggesting that these wild-type cells require a signal (presumably from other wild-type cells in r4) to activate krox-20. Third, when wild-type donor cells contribute to r4, rescue of r5 krox-20 expression is always observed (n=23). However, when wild-type donor cells do not contribute

Fig. 4. FGF3 and FGF8 are required for neuronal development in r5 and r6. Confocal images of 48 h hindbrains stained with RMO-44, labeling reticulospinal neurons (A-D), anti-Islet, labeling motoneurons (E-H) or zn-8, labeling nVI and nX motoneurons (I-L). Ventral views show anterior towards the top (A-H) or towards the left (I-L). Arrows in A,D indicate T interneurons. The large Mauthner neurons (M in A) are in r4. Arrows in E-H point to the rostral extent of nX neurons. n≥10 for each marker for each ‘genotype’. Scale bar: 50 μm. r2-r7, rhombomeres 2-7; V, nV neurons; VI, nVI neurons; VII, nVII neurons; IX, nIX neurons; X, nX neurons.
to r4, but instead are rostral or caudal to r4, no rescue is observed (n=10; Fig. 6D-D’). Fourth, rescue of r5 krox-20 expression occurs only on the side of the host that received the transplant (Fig. 6C’; 18/18 right-side-rescued hosts had right-side transplants, 5/5 left-side-rescued hosts had left-side transplants). Wild-type donor cells remain unilateral in the hindbrain until about 12.5 h/7 s, as the cell divisions that will cross the midline in the hindbrain will generally not occur until later in somitogenesis (Moens et al., 1996; Kimmel et al., 1994). We can detect rescue of r5 krox-20 expression as early as about 10.6 h/2 s, when donor cells are still unilateral (Fig. 6E-E’). To summarize, these findings are consistent with a requirement for wild-type cells in r4, which should express functional FGF signals, to promote r5 krox-20 expression in fgf3-MO: fgf8- hosts.

If r4 signaling acts upstream of val in r5 and r6, then val- cells in r4 should non-autonomously rescue r5 krox-20 expression in an fgf3-MO: fgf8- host. Although val is cell-autonomously required for r5 krox-20 expression (Moens et al., 1996), fgf3 and fgf8 are still expressed in r4 in val- embryos (not shown). We find that when val- donor cells contribute to r4 in fgf3-MO; fgf8-MO embryos, non-autonomous rescue of r5 krox-20 expression is always observed (n=4; Fig. 6F-F’; no rescue is observed if val- donor cells do not contribute to r4, n=5). This result strongly supports r4 signaling acting upstream of val function and, because val- cells cannot normally contribute to r5 and r6 (Moens et al., 1996) (see Fig. 6F’’), supports the specificity of r4 signaling in promoting r5 krox-20 expression. Taken together, our transplantation and rescue analyses demonstrate a necessary and sufficient role for r4 signaling in promoting r5 and r6 development.

**Misexpression of either fgf3 or fgf8 can induce ectopic expression of val and krox-20**

To test whether FGF3 and FGF8 are sufficient to promote the development of r5 and r6, we asked whether misexpression of fgf3 or fgf8 is sufficient to induce expression of val and krox-20. To mimic the r4 transplantation experiments, we implanted beads coated with FGF8 protein in the ventral ectoderm of shield-stage embryos. At a low frequency (three out of 26 embryos), ectopic val and krox-20 expression was induced in non-neural ectoderm next to the FGF8 bead (Fig. 7A,B). When placed near the presumptive hindbrain in shield-stage embryos, FGF8 beads show strong induction (20/28 embryos) of val and krox-20 expression in the caudal hindbrain, anterior spinal cord and associated neural crest (Fig. 7C). To show that FGFs can signal in the

![Fig. 5. R4 transplants can induce expression of val and krox-20. (A) Diagrams of the transplantations. Putative r4 cells (positioned midway between the first somite boundary and the tip of the notochord) are excited from fluorescein-labeled donor embryos at early 1-somite stage and transplanted into either the r4 region of early 1-somite-stage hosts (left arrow) or ventral ectoderm of shield-stage hosts (right arrow). (B-B’’) Host embryo (16 s) showing donor cells (B), krox-20 expression (B’’), and merge of the two labels (B’’). Most donor cells are in r4, some are in r5. (C) Host embryo (10 s) with r4 transplant in lateral ectoderm (arrow) stained for val (blue), krox-20 (red). (D-E’’) Magnified view of transplant in C. (D) val (blue) and krox-20 (red) expression largely overlap (the krox-20-expressing cells express val, but the blue val is partially obscured). Donor cells (E) and krox-20 expression (E’’) partially overlap (arrowhead in a merge (E’’), showing induction of krox-20 (and val, compare with D) in the host (arrow). 12/12 transplants showed some val and krox-20 expression in donor cells; three out of 12 showed some val and krox-20 expression in host cells. (F-G’) R4 transplant (in the ventral ectoderm of a 10 s host embryo) showing hoxb1a (blue) and krox-20 (red) (F), donor cells (G), krox-20 (G’’) and merge (G’’). hoxb1a expression is found in donor cells (arrows) and in host cells. krox-20 expression is found in donor cells and host cells (arrowheads). Six out of eight transplants showed some hoxb1a and krox-20 expression in donor cells; two of these cases also showed hoxb1a and krox-20 expression in host cells; two out of transplants showed no hoxb1a or krox-20 expression associated with the transplant. (H-H’’) R6 transplant (in the ventral ectoderm of a 10 s host embryo) showing donor cells (H) and val (H’’). An overlay shows val expression within the transplant (H’’). Anterior is towards the left. Scale bars: in B, 50 μm for B-B’’; in C, 100 μm for C; in D, 50 μm for D-H’’.
neuroepithelium, we used a heat shock promoter to activate either fgf3 or fgf8 at 10 h/bud stage, a stage when we propose these FGFs are normally signaling from r4. We find that only fgf-expressing cells in the caudal hindbrain (Fig. 7D,E) or in surface ectoderm above the caudal hindbrain (Fig. 7,E,G-G") are able to induce ectopic val and krox-20 expression in neighboring cells within the caudal hindbrain; fgf-expressing cells elsewhere in the embryo, including the rostral hindbrain (Fig. 7F), do not induce val or krox-20. These results show that FGF signaling is sufficient to promote val and krox-20 expression, but it appears that only the caudal hindbrain is competent to activate val and krox-20 expression in response to FGF signaling at bud stage.

**DISCUSSION**

We have shown here that rhombomere 4 (r4) is an early-differentiating segment in the zebrafish hindbrain, that r4 tissue has signaling activity, and that FGF signals are both necessary and sufficient to promote the development of r5 and r6. Several lines of evidence suggest that this signaling activity of r4 is conserved across vertebrates.

**r4 as an early-differentiating rhombomere**

The results of our time-lapse imaging, in combination with the finding that isl1-positive cranial motoneurons (this work) and reticulospinal neurons (Mendelson, 1986; Hatta, 1992) differentiate earliest in r4, support r4 as an early-differentiating rhombomere in zebrafish. Studies of rhombomere boundary development in chick and alligator (Vaage, 1969; Pritz, 1999), including time-lapse analysis in chick (Kulesa and Fraser, 1998), show that the r5/6 and r3/4 boundaries develop earliest, suggesting that an r4-5 pro-rhombomere is established earliest in these animals. However, differentiation of hindbrain motor and reticular neurons occurs earliest in r4 in chick and mouse (Lumsden and Keynes, 1989; Layer and Alber, 1990; Sechrist and Bronner-Fraser, 1991; Nardelli et al., 1999; Pata et al., 1999). Thus, there is support for the conserved early differentiation of r4, and further analyses of rhombomere formation and hindbrain neuronal differentiation in different vertebrates should reveal the extent of the conservation.

**Redundant functions of FGFs in hindbrain patterning**

We find that reduction of either fgf3 or fgf8 alone has little effect on hindbrain segmental patterning. However, severe

Fig. 6. Wild-type, or val–, cells can partially rescue r5 krox-20 expression in the fgf3-MO: fgf8– hindbrain. (A) The transplantation technique used for the mosaic analyses. Naïve animal pole cells are excised from fluorescein-labeled donor embryos at shield stage and transplanted into the presumptive hindbrain region (Woo and Fraser, 1997) of shield stage fgf3-MO; fgf8– embryos. In some cases, fgf3-MO; fgf8-MO embryos were used as hosts. 100% (n=45) of the fgf3-MO; fgf8– or fgf3-MO; fgf8-MO control embryos (not receiving donor cells) showed no r5 krox-20 expression. (B) fgf3-MO; fgf8– control embryo (18 s) showing only r3 krox-20 expression. (C–C") A host fgf3-MO: fgf8– embryo (18 s) showing wild-type donor cells (C), krox-20 expression (C') and an overlay (C") of wild-type donor cells (green) and krox-20 expression (red). Yellow r5 cells (arrow, C") are wild-type donor cells expressing krox-20. Yellow r5 cells (arrow, C") are wild-type donor cells expressing krox-20. (D–D") A host fgf3-MO: fgf8– embryo (18 s) showing krox-20 expression (D) and a merge (D") showing wild-type donor cells (green) and krox-20 expression (red). Donor cells populate the caudal hindbrain and anterior spinal cord and no rescue of r5 krox-20 is observed. (E–E") A host fgf3-MO; fgf8-MO embryo (2 s) showing krox-20 expression (E) and an overlay (E") of wild-type donor cells (green) and krox-20 expression (black) (E’). Rescue of r5 ege2 expression (arrowhead) has occurred adjacent to unilateral donor cells in r4 (arrow). Four out of nine embryos showed similar rescue at about 2 s. (F–F") A host fgf3-MO: fgf8-MO embryo (18 s) showing val– donor cells (F), krox-20 expression (F") and merge of the two labels (F"’). val– cells are excluded from the rescued r5 region on the right side of the hindbrain (arrow). val– cells are not excluded from the ‘r5’ level on the left, non-rescued side (arrowhead). Dorsal views show anterior towards the left. Scale bars: in B, 50 μm for B-D'; F-F’’; in E, 50 μm for E-E’.
reduction of both signals leads to loss of the entire r5/6 domain. Expression of either fgf3 or fgf8 does not depend on the other, and both transcripts are still expressed in the hindbrain of fgf3-MO; fgf8-embryos (L. M., unpublished). We therefore suspect that these FGFs function redundantly in the hindbrain. Although some functional FGF3 and FGF8 may remain in fgf3-MO; fgf8-embryos, increasing doses of fgf3 and fgf8 MOs together show no heightened hindbrain effects (L. M., unpublished). The complete loss of hindbrain expression of both FGF target genes, *erm* and *pea3*, shows that fgf3 and fgf8 are the critical FGFs used in zebrafish hindbrain patterning. We are not aware of another FGF expressed in the zebrafish hindbrain, nor is there any evidence for duplicate fgf3 or fgf8 genes in zebrafish (B. Draper, personal communication).

The expression of fgf3 in other vertebrates is consistent with a conserved role for fgf3 in r4 signaling. In frogs (Tannahill et al., 1992; Lombardo et al., 1998), chicks (Mahmood et al., 1995) and mice (Mahmood et al., 1996; McKay et al., 1996), fgf3 is expressed broadly in the middle of the early hindbrain, including strong expression in r4, and this expression precedes that of *mafB* in r5/6 (Cordes and Barsh, 1994; Eichmann et al., 1997) and krox-20 in r5 (Nieto et al., 1991). In chicks and mice, fgf3 expression resolves into its more recognized r5/6 expression domain, which was initially observed in mice (Wilkinson et al., 1988). However, in frogs, fgf3 becomes restricted to r4 (Lombardo et al., 1998). If the r4-restricted expression of zebrafish fgf3 (although we do observe some weak r5/6 and possibly r3 expression during late gastrulation and early somitogenesis) is indicative of fgf3 expression in more primitive vertebrates, then this would provide even more support for a conserved role for r4 FGF signaling in the vertebrate hindbrain. The fgf3 knockout in mice has little effect on hindbrain patterning (Mansour et al., 1993), similar to fgf3-MO zebrafish embryos (this work). fgf8 has not been found to be expressed in r4 in other vertebrates as in zebrafish; however, fgf4 is expressed in the chick hindbrain before initiation of *mafB* expression in r5/6 (Shamim and Mason, 1999). We expect that in other vertebrates, additional FGFs to fgf3 will show early hindbrain expression, and we predict that loss of two or more of these FGFs will lead to dramatic defects in hindbrain patterning.

**r4 as a conserved signaling center**

Signaling from r4 has previously been implicated in hindbrain patterning. Transplantation studies in chick have shown that signaling from r4 regulates neural crest cell death in r3 and r5 (Graham et al., 1993), and krox-20 and follistatin expression in r3 (Graham and Lumsden, 1996). Mice deficient in *Hoxa1*, which is not known to be expressed rostral to r4, show defects in the development of r3, including patchy loss of krox-20 expression (Helmbach et al., 1998). However, in these studies, the identity of the r4 signal(s) is unknown. Chick embryo transplantations have also implicated signaling from the r2-r6 region in promoting expression of krox-20 in r5 and *mafB* in r5 and r6 (Marin and Charnay, 2000b). FGFs have been proposed to mediate this signaling because FGF-soaked beads can induce ectopic expression of krox-20 and *mafB*, and application of an FGF receptor-inhibitor drug inhibits krox-20 and *mafB* expression (Marin and Charnay, 2000a). However, in these studies, it was not determined when and from which tissue (neuroectoderm or adjacent mesoderm) these signals acted and the FGFs required were not identified. In light of our findings, these studies all support a conserved role for FGF signaling from r4 in promoting the development of adjacent rhombomeres.

If r4 is truly a signaling center in the hindbrain, then r4 tissue should be both necessary and sufficient to promote development of r5 and r6. Our transplantation and mosaic analyses demonstrate that such signaling activity is centered in...
FGF signaling from rhombomere 4

Although our transplants express r4 markers such as "hoxb1a", the transplants are not exclusively r4 tissue and we cannot rule out the possibility that cells from other rhombomeres contribute to the signaling activity. Our gain- and loss-of-function studies with fgf3 and fgf8 show that these FGF signals mediate the signaling activity of r4. It is possible that other signals participate as well, as FGFR8 beads promote val and krox-20 expression with low frequency outside of the hindbrain. Another factor that may contribute to the ability of both r4 transplants and FGF misexpression to induce r5/6 markers is that competence to respond to r4/FGF signaling may be tightly spatially and temporally regulated. Indeed, misexpression of fgf3 or fgf8 at bud stage is only able to induce val and krox-20 in the caudal hindbrain (this work). This apparent localized competence to respond to FGFs may be necessary because FGFs are used in many tissues for diverse functions. It is not clear how such a localized response is achieved, although the transcription factor POU2 (Reim and Brand, 2002) or the different FGF receptors (Thissie et al., 1995; Skeptsova-Friedrich et al., 2001) (S. Solinsky and L. M., unpublished) may play a role. The fact that the r4 transplants and the FGFR8 beads can induce hindbrain markers in ventral ectoderm shows that these signals have neural inducing activity, which has previously been attributed to FGFs (Lamb and Harland, 1995; Streit et al., 2000; Wilson et al., 2000). Intriguingly, FGFs themselves have been proposed to impart a differential competence to neural inducers in the zebrafish ectoderm (Koshida et al., 1998).

Another conserved signaling center, the MHB, promotes patterning of the rostral hindbrain via FGFRs (Irving and Mason, 2000). FGF signaling from the MHB has been proposed to interact with, or antagonize, signaling by retinoic acid (RA) from more posterior tissues to define the r1 territory (Irving and Mason, 2000; Gavalas and Krumlauf, 2000). We propose that a second, conserved, FGF-mediated signaling center, r4, be incorporated into this model of signals involved in early hindbrain patterning (Fig. 8). FGFR3 and FGFR8 signaling from r4 promote the development of r5 and r6, at least in part by promoting expression of val and krox-20. FGFR3 and FGFR8 from r4 may also promote the development of rostral rhombomeres 1-3, possibly acting with FGFs from the MHB. RA has been implicated in the regulation of val and krox-20 expression (reviewed by Gavalas and Krumlauf, 2000). It is likely that FGF signaling from r4 and RA signaling interact in regulating r5/6 development, and val may be a key target on which these two signals converge.

Although val is likely to be a critical target of FGF signaling in promoting r5/6 development, the defects observed upon loss of FGFR3 and FGFR8 signaling are more severe than those observed because of loss of val function. val−/− embryos retain a region, termed rX, which exhibits some characteristics of r5/6, including some r5 krox-20 expression and the r5- and r6-associated reticulospinal neurons (Moens et al., 1996), whereas fgf3-MO; fgf8−/− embryos show complete loss of all r5/6 markers, including r5/6 val expression. This reveals that some unknown factor(s) must normally contribute to r5/6 identity, possibly in parallel to val. vhlf1 (tcf2 – Zebrafish Information Network) (Sun and Hopkins, 2001), which is expressed in r5 and r6, may be one of these factors.

Recently, fgf3 and fgf8 have been shown to be redundantly required for otic placode induction in zebrafish (Phillips et al., 2001; Maroon et al., 2002). Otic placodes develop adjacent to r5, and fgf3 has previously been implicated in vertebrate otic placode induction (Represa et al., 1991; Vendrell et al., 2000). We have found that fgf3 and fgf8 are also required for other head periphery structures associated with the r5/6 region, including pharyngeal cartilages and cranial ganglia (L. M. and C. B. K., unpublished). Further studies should reveal whether FGF signaling from r4 acts directly on structures in the head periphery.

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