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

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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) kreasler/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; Marin 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 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

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.
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 acerebellar2 pan(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 pax2.1 expression (Brand et al., 1996). valentine337 (val; a null allele) (Moens et al., 1996; Moens et al., 1998) homozygous mutant embryos were scored by their loss of r5 krox-20 expression.

Time-lapse analyses
Embryos were incubated in a 200 μM solution of BODIPY FL C5-ceramide (Molecular Probes) from sphere stage to bud stage. Embryos were mounted between two coverslips in a mixture of 0.5% agar in EM and 3% methyl cellulose in EM. Images were collected every 6 minutes at 28.5°C from about the two-somite (s) stage to about the 16 s stage. Recordings were analyzed using 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.

RNA in situ hybridization
cDNA probes that detected the following genes were used: isl1 (Appel et al., 1995); krox-20 (Otxoby and Jovett, 1993); pax2a (Krauss et al., 1991); fgf3 (this paper); no tail (Schulte-Merker et al., 1992); fgf8 (Reifers et al., 1998); val (Moens et al., 1998); hoxb3a, hoxb1a, hoxb4a (Prince et al., 1998); efnb4, efnb2a (Cooke et al., 2001); and emr, pea3 (Münchberg et al., 1999). Probe syntheses and whole-mount in situ hybridization were performed as previously described (Jovett and Lettice, 1994; Hauptmann and Gerster, 1994). Embryos were de-yolked using tungsten needles, mounted in 90% glycerol in phosphate-buffered saline (PBS) and photographed using a Zeiss Axiophot 2 microscope.

fgf3 cDNA cloning and misexpression
Based on the published zebrafish fgf3 genomic DNA sequence (Kiefer et al., 1996), we screened a zebrafish 15-19 h cDNA library (Appel and Eisen, 1998) by PCR to isolate a full-length fgf3 cDNA. Full-length fgf3 cDNA was subcloned into pCS2+ (Rupp et al., 1994), yielding pCS2+ -fgf3. To make full-length fgf3 antisense RNA probe, pCS2+ -fgf3 was linearized with BamHI and transcribed with T7 polymerase. To tag fgf3 with a Myc epitope, we subcloned full-length fgf3 cDNA into pCS2+MT (Rupp et al., 1994), which contains a 6×Myc epitope, yielding pCS2+ -fgf3-MT. To make heat shock (HS)-fgf3, the CMV promoter of pCS2+MT was removed, and a zebrafish hsp70 promoter (Halloran et al., 2000) and full-length fgf3 cDNA were inserted into that plasmid. Sequencing confirmed proper fgf3 sequence in frame with the Myc tag at the 3′ end of fgf3. The Myc tag does not affect FGF3 activity in overexpression assays.

For misexpression experiments, HS- fgf3 or HS-fgf8 (Roehl and Nüsslein-Volhard, 2001) plasmid was injected into one- to four-cell-stage embryos at about 5 ng/μl. Uptake of the transgenes by early blastomeres is very mosaic, leading to small clones of fgf-expressing cells upon heat shock. Embryos were heat shocked at 37°C for 1 hour at about bud stage, then were fixed for staining at about the 6-8 s stage. Transgene-injected/non-heat-shocked control embryos and non-injected/heat-shocked control embryos show largely normal val and krox-20 expression.

To make FGFE8-soaked beads, 45 μm polystyrene beads (Polysciences) were rinsed in PBS, then were incubated in 5 mg/ml heparin for 1 hour at room temperature, then were incubated in 250 μg/ml [in 0.5% bovine serum albumin (BSA) in PBS] mouse FGFE8b (R&D Systems) for 2 hours at room temperature. Control beads were incubated in 0.5% BSA in PBS. Beads were then rinsed in PBS. Bead implants were carried out similar to transplantation experiments. To test efficacy of FGFE8 beads, embryos with implanted beads were stained for pea3, an FGF target gene (Roehl and Nüsslein-Volhard, 2001; Raible and Brand, 2001). Nine out of nine embryos showed a ring of pea3 expression around the FGFE8 bead.

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′-CATTTGTCGATCCGATGTCCG-3′; fgf3B, 5′-GTTCCATCAAAGAAGTTACCTTG-3′; fgf3C, 5′-TCTCGCTGGGATAGAAAGAGCTGGC-3′; and fgf3MMA, 5′-CAATGTCGATCGGACGGG-3′. 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.

Embryos were pressure injected, using a pulled glass micropipette and a microinjector (ASI), into the yolk at the one- to four-cell stage. We inject a 2-3 nl volume into each embryo.

To show that the MOs can block translation of FGF3, we co-injected fgf3A and fgf3C with pCS2+-fgf3-MT, which contains the target sequence for these MOs, and found that the MOs can block expression of FGF3-Myc protein; the MOs also block the effects of overexpression of an fgf3 cDNA containing the MO target sequence (not shown).

To reduce or eliminate non-specific MO side-effects, we determined the highest dose for each MO that still yielded ‘normal’ embryos and larvae (larvae that develop swim bladders) and used these doses in pair-wise combinations to achieve synergistic effects on fgf3 while minimizing non-specific effects. The three pair-wise combinations of the fgf3 MOs all cause the same phenotype: a reduced forebrain, a slightly reduced tail and small ears. We presume this phenotype corresponds to a severe reduction of fgf3 function. The four-base mismatch control MO (fgf3MMA), when injected (at the same dose used for fgf3A) alone or in combination with the others, generates normal embryos and larvae. To generate fgf3-MO; fgf8+ embryos, we injected the fgf3B (1.0 mg/ml) + fgf3C (0.25 mg/ml) combination into ace embryos. Each fgf3 MO (A, B, C) causes loss of r5 krox-20 expression when injected into ace. fgf3MMA does not.

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 to 7.5, and 0.1% Triton X-100) with 2% normal goat serum (NGS) for 30 minutes. Embryos were then
incubated overnight at 4°C in primary antibodies at the following dilutions in PBDTX with 2% NGS: RMO-44, 1:25 (Zymed); Islet 39.4D5, 1:100 (Ericson et al., 1992); zn-8, 1:1000 (Trevorrow et al., 1990); anti-Myc, 1:100 (Oncogene Research Products). After PBDTX rinses, embryos were incubated in secondary antibody goat anti-mouse Alexa Fluor 488 (Molecular Probes) at 1:200 dilution in PBDTX with 2% NGS for 5 hours at room temperature. Embryos were then rinsed in PBS and analyzed using a Leica MZ FLIII fluorescence stereomicroscope. Images were obtained using a Zeiss 310 confocal microscope.

**Transplantation and mosaic analysis**

Transplantation techniques were adapted from Moens et al. (Moens et al., 1996) and Woo and Fraser (Woo and Fraser, 1997). All embryos used for transplantations were raised in filter-sterilized EM supplemented with penicillin (5000 U/l)/streptomycin (100 mg/l; Sigma). Donor embryos were labeled at the one-cell stage with a spin-filtered mixture of 3% lysine-fixable fluorescein dextran (or 2% Alexa Fluor 488 dextran) and 3% lysine-fixable biotin dextran (10,000 M; Molecular Probes) in 0.2 M KCl. Dechorionated donor and host embryos were mounted in 2% methyl cellulose in Ringer’s (Westerfield, 1995) on a glass depression slide. Working with a Nikon SMZ-U fluorescence stereomicroscope and using a pulsed glass micropipette as a knife, donor tissue (about 50-100 cells) was excised and then inserted into a host embryo. The position of labeled donor tissue in the host was checked briefly with UV light. Embryos on the depression slide were then submerged in EM with pen/strep. Donor and host embryos were processed for in situ hybridization as described above. To detect donor-derived biotin-labeled cells, embryos were rinsed with water following the in situ, then were rinsed with PBDTX, then were incubated overnight at 4°C in streptavidin Alexa Fluor 488 (1:100 in PBDTX with 2% NGS; Molecular Probes). Embryos were then rinsed and analyzed as they were for immunocytochemistry.

To test the identity of r4 transplants, we fixed shield-stage host embryos within an hour after transplantation and stained for rhombomere markers: 8/10 transplants expressed hoxb1 (r4), 9/10 transplants expressed fgf3 (r4), and 5/10 transplants expressed val (r5/6). To assess whether we were transplanting any paraxial head mesoderm along with the r4 cells, we stained for follistatin (Bauer et al., 1998): six out of nine transplants were follistatin-negative; three out of nine showed only two to three follistatin-expressing cells.

In cases where fgf3-MO; fgf8-MO embryos were used as hosts, the morpholinos were co-injected at the one-cell stage. The fgf8 morpholinos E212 and E313 (Draper et al., 2001) were used at 0.5 mg/ml each.

**RESULTS**

**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 and find that the earliest-born cells of this group are the pair of Mauthner neurons in r4. Hatta (Hatta, 1996) and Nomarski imaging and find that the r3/4 and r4/5 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.

**Fig. 1.** Rhombomere 4 differentiates earliest in the zebrafish hindbrain. (A-D) Single optical sections from a confocal time-lapse recording showing early rhombomere boundary formation. Images show dorsal views with anterior towards the left. Rhombomere boundaries are marked with arrowheads, and the r4/5 boundary arrowheads in B show dorsal views with anterior towards the left. Somite stages are indicated (lower right-hand corners). Scale bars: in A, 50 µm in A-D; in E, 50 µm in E-H.
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 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 [acerebellar<sup>282a</sup> (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 \textit{fgf3} morphant (\textit{fgf3-MO}) embryos. Similarly, \textit{fgf8} mutant, or \textit{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 \textit{fgf3} and \textit{fgf8} interact or function redundantly in hindbrain patterning, we injected \textit{fgf3} morpholinos into \textit{fgf8}− embryos, thus generating \textit{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 \textit{krox-20} (Oxtoby and Jowett, 1993) expression appears fairly normal in \textit{fgf3-MO} embryos and \textit{fgf8}− embryos (although rhombomeres often appear reduced in width; Fig. 3A, parts a and c), the r5 stripe of \textit{krox-20} expression is completely lost in \textit{fgf3-MO; fgf8}− embryos and the r3 stripe appears reduced (Fig. 3A, part d). \textit{fgf3-MO; fgf8}− embryos also show complete loss of \textit{val} (Moens et al., 1998) expression in r5 and r6 (Fig. 3A, part h) and loss of high levels of \textit{hoxb3} (Prince et al., 1998) in r5 and r6 (Fig. 3A, part l). Furthermore, we find that expression of \textit{hoxb4}, which normally has a rostral boundary at the r6/7 boundary (Prince et al., 1998) (Fig. 3A, part p) in \textit{fgf8}− embryos is contiguous with \textit{hoxb1a} in \textit{fgf3-MO; fgf8}− embryos (Fig. 3A, part t). Staining of \textit{fgf3-MO; fgf8}− embryos with \textit{hoxb4} (without \textit{hoxb1a}) reveals a space corresponding to the width of r4 between the anterior \textit{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 (Metcalf 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 nVI and nX neurons [similar to zn-5 (Chandrasekhar et al., 1997)] (Fig. 4I), and we find these neurons to be absent, or in some cases severely reduced, in fgf3-MO; fgf8−/− embryos (Fig. 4L). 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 (nV) and nX) and the abducens motor nucleus (nVI) at 48 h (Chandrasekhar et al., 1997) (Fig. 4E).

Transplantation and mosaic analyses reveal r4 signaling activity

The loss of r5 and r6 upon reduction of fgf3 and fgf8 suggests that fgf3 and fgf8, from their overlapping domain in r4, 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 FGF signaling for signaling activity from r4, we heterotopically transplanted r4 cells at bud/1-s stage, when we propose FGF 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
FGF signaling from rhombomere 4

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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. 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.

wild type  fgf3-MO  fgf8-  fgf3-MO; fgf8-
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 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.](image)
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. 7E,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 pr-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

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**Figure 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 fluorescent-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 merge of the two labels (C''). Red r5 cells (arrow, C'') are rescued fgf3-MO; fgf8-- host cells expressing krox-20. Yellow r5 cells (arrowhead, 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 FGFR3 and FGFR8 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 two 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 (Helmbacher 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

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**Fig. 7.** fgf8 or fgf3 can induce expression of val and krox-20. (A) Embryo (14 s) with FGFR8 bead (arrow). val (blue) and krox-20 (red) are induced in ectoderm adjacent to the bead, shown magnified in B. (C) Embryo (12 s) with FGFR8 bead next to the caudal hindbrain, showing ectopic val (blue) and krox-20 (red). (D) Embryo (6 s) with fgf8-expressing cells (red, arrowhead) in r5. Ectopic krox-20 expression (blue, arrow) is induced in r6-r7. Seven out of 43 heat-shocked embryos showed ectopic krox-20 expression in the caudal hindbrain with nearby fgf8-expressing cells. (E) Embryo (6 s) with fgf8-expressing cells (red, arrowheads) in r6 and in ectoderm overlying r6-r7. Ectopic val (blue, arrow) spreads caudally into r7. Twelve out of 34 heat-shocked embryos showed ectopic val expression in the caudal hindbrain with nearby fgf8-expressing cells. (F) Embryo (6 s) with fgf8-expressing cells (red, arrowheads) in the midbrain and rostral hindbrain. val expression is not affected (blue). (G-G‘) Embryo (8 s) with FGFR3-Myc-expressing cell (arrowhead in G) in ectoderm overlying r5. Ectopic val spreads caudally into r7 (arrow in G) and ectopic krox-20 spreads caudally into r6 (arrow in G‘). Ten out of 36 heat-shocked embryos showed ectopic val and krox-20 expression in the caudal hindbrain with nearby FGFR3-expressing cells. Dorsal views show anterior towards the left. Scale bars: in A, 100 μm for A; in B, 50 μm for B-G-G‘; in C, 50 μm for C; in D, 50 μm for D-F.
r4. Although our transplants express r4 markers such as hoxb1a, the transplants are not exclusively r4 tissue and we can not 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 FGF8 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 (Thisise et al., 1995; Sleptsova-Friedrich et al., 2001) (S. Solinsky and L. M., unpublished) may play a role. The fact that the r4 transplants and the FGF8 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 FGFs (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). FGF3 and FGF8 signaling from r4 promote the development of r5 and r6, at least in part by promoting expression of val and krox-20. FGF3 and FGF8 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 Krumsauf, 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 FGF3 and FGF8 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. vhnf1 (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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**Fig. 8.** Incorporation of a new r4 signaling center in promoting hindbrain development. A zebrafish hindbrain showing seven rhombomeres (r1-r7). Many FGFs are expressed at the midbrain-hindbrain boundary, and zebrafish fgf8 is required for cerebellar/r1 development (reviewed by Rhinn and Brand, 2001). Retinoic acid likely signals from more posterior tissues (neural and/or mesodermal) (reviewed by Gavalas and Krumlauf, 2000). fgf3 and fgf8 expression overlap in r4 and promote the development of r5 and r6. FGF3 and FGF8 are required for otic development, but it is not clear how directly the FGF/r4 signals act on the head periphery. FGF3 and FGF8 from r4 may also have an influence on the development of more rostral rhombomeres (such as r3).
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