Development 130, 3821-3829

doi:10.1242/dev.00572

vhnf1 and Fgf signals synergize to specify rhombomere identity in the zebrafish hindbrain

Elizabeth L. Wiellette[1] and Hazel Sive[1,2,*]

[1]Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142, USA
[2]Massachusetts Institute of Technology, Cambridge MA, USA
*Author for correspondence (e-mail: sive@wi.mit.edu)

Accepted 1 May 2003

SUMMARY

Vertebrate hindbrain segmentation is a highly conserved process but the mechanism of rhombomere determination is not well understood. Recent work in the zebrafish has shown a requirement for fibroblast growth factor (Fgf) signaling and for the transcription factor *variant hepatocyte nuclear factor 1* (*vhnf1*) in specification of rhombomeres 5 and 6 (r5+r6). We show here that *vhnf1* functions in two ways to subdivide the zebrafish caudal hindbrain domain (r4-r7) into individual rhombomeres. First, *vhnf1* promotes r5+r6 identity through an obligate synergy with Fgf signals to activate *valentino* and *krox20* expression. Second, *vhnf1* functions independently of Fgf signals to repress *hoxb1a* expression. Although *vhnf1* is expressed in a broad posterior domain during gastrulation, it promotes the specification of individual rhombomeres. This is achieved in part because *vhnf1* gives cellular competence to respond to Fgf signals in a caudal hindbrain-specific manner.

Key words: Vhnf1, Fibroblast growth factor, Fgf3, Fgf8, Zebrafish, Hindbrain, Valentino, Krox20, Rhombomere, Neural patterning

INTRODUCTION

The vertebrate hindbrain is organized into segments along the anterior-posterior axis, and the process by which these segments are generated follows a sequence of progressive partitioning of the tissue. During and just after gastrulation, a broad domain of tissue is set aside as the future posterior hindbrain and this domain is then subdivided into protorhombomeres, lineage-restricted compartments that presage formation of rhombomeres. The seven or eight rhombomeres that appear as transient bulges along the anterior-posterior axis of the hindbrain have unique identities. These identities are the basis for development of specified neurons within the hindbrain and of specialized migrating cranial neural crest cells (Moens and Prince, 2002; Trainor and Krumlauf, 2001).

By late gastrula stages, cells that will contribute to the hindbrain are already committed to that fate (Woo and Fraser, 1998). At this time, a broad region of the presumptive posterior neurectoderm is distinguished by the expression of genes including *hoxa1*, *hoxb1* and *meis3* (Alexandre et al., 1996; Kolm and Sive, 1995; Murphy and Hill, 1991; Prince et al., 1998; Sagerstrom et al., 2001; Salzberg et al., 1999). Further, because the anterior boundary of *hoxA1* gene expression probably lies at the future r3-r4 break and only the posterior hindbrain tissue up to the r3-r4 boundary is dependent on retinoic acid (RA), the presumptive caudal hindbrain (r4-r8) appears to compose an early, distinct domain (Dupe and Lumsden, 2001; Gavalas and Krumlauf, 2000).

The posterior hindbrain is subsequently subdivided, as indicated by restricted gene expression, including *krox20* (*egr2* – Zebrafish Information Network) in r3 and r5, and *valentino* (*val*)/*kreisler*/*mafB* in r5 and r6. Both *krox20* and *val* functions are required for the correct expression of some of the rhombomere-specific Hox genes (Frohman et al., 1993; Giudicelli et al., 2003; Manzanares et al., 1999; Prince et al., 1998; Seitanidou et al., 1997). In turn, Hox gene expression domains delineate presumptive rhombomeres, and Hox gene function is required for the development of neurons and other cells produced within each rhombomere (Lumsden and Krumlauf, 1996; Moens and Prince, 2002; Trainor and Krumlauf, 2001). For instance, *hoxb1* is expressed in future rhombomere 4 (r4) and is sufficient to provide ectopic r4 neuronal morphology (Bell et al., 1999; Vlachakis et al., 2001). In combination with *hoxa1*, *hoxb1* is required for normal development of presumptive r4 in mice and zebrafish (Gavalas et al., 1998; McClintock et al., 2002; Rossel and Capecchi, 1999; Studer et al., 1998). Similarly, the Hox paralog group 3 genes are expressed in r5 and r6 and are required for formation of specific neurons and mesenchymal neural crest-derived structures (Manley and Capecchi, 1997).

Recent findings identify some additional factors required for posterior hindbrain segmentation. In the chick, fibroblast growth factor (Fgf) signals have been shown to be sufficient for ectopic induction of *krox20* and *mafB*/*kreisler* in caudal hindbrain neuroepithelium and neural crest, and drug-based inhibition of Fgf signaling results in inhibition of *krox20* and *mafB*/*kreisler* within their normal expression domains (Marin and Charnay, 2000). In the zebrafish, Fgf signals emanating from the anterior hindbrain are required to initiate expression

of posterior hindbrain gene expression, in particular *val*, *krox20* and *hoxb3*. Loss of both *fgf*3 and *fgf*8 functions together results in a loss of r5 and r6 identity (Maves et al., 2002; Walshe et al., 2002). Loss of function of the gene *variant hepatocyte nuclear factor 1* (*vhnf1*; *tcf2* – Zebrafish Information Network) in the zebrafish results in small ears and loss of *val* and *krox20* (r5) expression (Sun and Hopkins, 2001). Although *vhnf1* knockout mice have been made, the role of *vhnf1* in murine hindbrain development has not been studied (Barbacci et al., 1999; Coffinier et al., 1999).

It remains to be defined how the broad domain of gastrula stage posterior neuroectodermal gene expression is subdivided into individual rhombomeric domains. Partially explaining this, we show here that *vhnf1* is expressed in a broad domain during gastrulation and that it is required for differentiation of caudal hindbrain rhombomeres by two distinct mechanisms. Through an obligate synergy with Fgf signals, *vhnf1* promotes expression of *val*, thereby promoting r5 and r6 identity. In addition, *vhnf1* represses *hoxb1a* expression independently of Fgf function, thereby limiting r4 identity to the appropriate narrow domain.

MATERIALS AND METHODS

Fish lines and maintenance

Fish were raised and bred according to standard methods (Westerfield, 1995). Lines used were: AB, Tübingen Long Fin, *val*^{338b} (Moens et al., 1996). We recently identified a novel allele of *vhnf1*: *vhnf1*^{wi408} (E. Wiellette et al., unpublished). The molecular identity of our allele was confirmed by complementation crosses in which *vhnf1*^{wi408} failed to complement the day 3 kidney defects and the lethality of both *vhnf1*^{hi2169} (null) and *vhnf1*^{hi548} (hypomorph) (Sun and Hopkins, 2001). In addition, *vhnf1*^{wi408} fails to complement the ear defect of *vhnf1*^{hi2169}. Expression of *vhnf1* transcript is completely missing at gastrulation and tailbud stages in 1/4 of the embryos derived from *vhnf1*^{wi408} heterozygous parent crosses.

RNA injections

pCS2+ plasmids with cDNA containing *vhnf1* (Sun and Hopkins, 2001), *val* (Moens et al., 1998) or *lacZ* were linearized and transcribed using the mMessage mMachine kit (Ambion). Capped mRNA concentration was measured and RNAs were injected in the following final amounts: *vhnf1*, 25 pg; *lacZ*, 25 pg; *val*, 5 pg. β-Galactosidase (β-gal) was visualized after fixation of embryos overnight in BT fix (Westerfield, 1995) at 4°C by washing in PBT and then staining in β-gal stain buffer (1× PBS, 4 mM $MgCl_2$, 3 mM $K_4[Fe(CN)_6]$, 3 mM $K_3[Fe(CN)_6]$) + 0.2% X-gal at room temperature. Embryos were analysed first by β-gal stain, followed by dechorionation, dehydration in methanol overnight and then the standard in situ method (see below).

Morpholino oligo injections

To knock down the functions of the *fgf*3 and *fgf*8 genes, morpholino oligomers (MOs) targeted to the translation start sites (Raible and Brand, 2001) were injected into 1-2 cell embryos. The final concentrations used were: 2.5 ng of each MO (Fig. 3C,D) or 1 ng of each MO (Fig. 5). In each case, two controls were performed in which a double concentration of one oligo or the other was used and, in each case, the strongest effect was observed by injection of the combination of MOs directed against both *fgf* transcripts. For analysis of earlier staged embryos (Fig. 3E,F), 0.8 ng of each MO was injected, with an unrelated negative control MO used to make 1.6 ng total MO in the injections of either *fgf*3MO or *fgf*8MO alone.

In situ hybridization

Standard methods for hybridization and for single and two-color labeling were used and have been described elsewhere (Sagerstrom et al., 1996). Probes used were: *vhnf1* (Sun and Hopkins, 2001), *krox20* (Oxtoby and Jowett, 1993), *hoxb1a*, *hoxb3*, *hoxb4* (Prince et al., 1998), *myod* (Weinberg et al., 1996), *val* (Moens et al., 1998), *hoxb1b* (Alexandre et al., 1996), *fgf*8 (Reifers et al., 1998), *no tail* (Schulte-Merker et al., 1992).

Reticulospinal neuron labeling

Reticulospinal neurons were labeled in embryos fixed at 48 hours of development, using 1:50 dilution of the primary antibody RMO44 (anti-Neurofilament; Zymed Laboratories #13-0500), as described (Waskiewicz et al., 2001). Localization of RMO44 was visualized using a 1:50 dilution of FITC-α-mouse (Zymed). The brains were partially dissected and mounted for visualization by confocal microscopy.

RNA injection with bead implantation

Beads were prepared and coated in mouse Fgf8b protein (R&D Systems) [or bovine serrum albumen (BSA)] as described in Reifers et al. (Reifers et al., 2000). *vhnf1* or *val* mRNA was injected at the 2-cell stage and the embryos were allowed to grow until the shield stage. Injected embryos were placed in the lid of a small Petri dish lightly coated with 3% methylcellulose and covered with normal Ringer's solution (Westerfield, 1995). A needle prepared as for injection was used to tear a small hole in the ectoderm and sharp forceps were then used to pick up a single bead and push it into the incision. The needle was then used to push the bead farther under the ectoderm. When all embryos on a dish lid were treated, the lid was placed in a standard Petri plate, which was then flooded with Embryo Medium (Westerfield, 1995). Embryos were left untouched until the appropriate stage for fixation, at which point they were gently removed from the methylcellulose and transferred for fixation, β-gal staining and in situ hybridization (as above).

RESULTS

Transformation of posterior hindbrain to r4 identity in *vhnf1* mutants

Consistent with previously published data, we find that *vhnf1*^{wi408} mutant embryos show defects in the expression of genes normally expressed in rhombomeres 4, 5, 6 and 7 (r4-r7) (Sun and Hopkins, 2001). Expression of Hox genes that distinguish r2 and r3 are unaffected in the *vhnf1*^{wi408} mutant background (data not shown). However, expression of *hoxb1a*, which is normally limited to r4 (Fig. 1A), is expanded into the posterior hindbrain in mutant embryos (Fig. 1B) (Sun and Hopkins, 2001). *hoxb3* expression, which is normally limited to r5 and r6 (Fig. 1C), is completely missing in *vhnf1*^{wi408} mutants (Fig. 1D). Finally, *hoxB4* expression, which normally has its anterior boundary at the r6-r7 limit and extends into the spinal column (Fig. 1E), is misexpressed in *vhnf1*^{wi408} mutants such that the anterior boundary of expression is not sharp and is more posterior (Fig. 1F). *krox20* expression, which is normally present in r3 and r5, is missing in r5 in *vhnf1*^{wi408} mutants. Although the r5 phenotype is 100% penetrant, *vhnf1*^{wi408} mutants show variable levels of *krox20* expression in r5 (compare Fig. 1B,D,F).

The earliest defect that we have been able to identify in *vhnf1*^{wi408} mutants is a lack of *valentino* (*val*) expression, which normally begins at the end of gastrulation [10 hours post-fertilization (hpf)] in presumptive r5 and r6 (Fig. 1G,H).

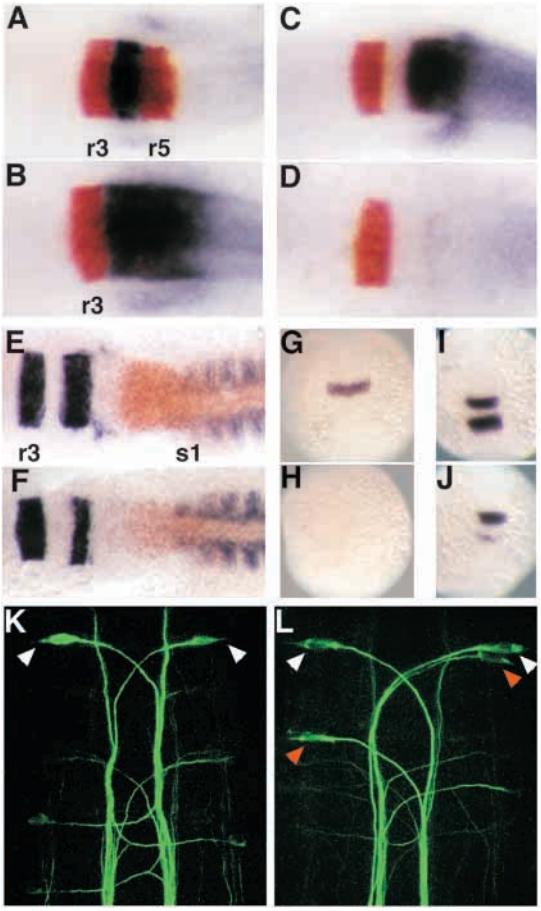

Fig. 1. Loss of *vhnf1* function results in transformation of posterior hindbrain to r4 identity. (A-F) 14-somite stage embryos. (A,B) *hoxb1a* gene expression (purple) is limited to the future r4 domain in a wild-type background and *krox20* expression (orange) is present in presumptive r3 and r5 (A). *hoxb1a* transcripts are expanded throughout the posterior hindbrain in *vhnf1* mutants, whereas r5-specific *krox20* expression is reduced or absent in *vhnf1* mutants (B). (C,D) *hoxb3* expression (purple) is present in future r5 and r6 in the wild-type background (C), but is not expressed in *vhnf1* mutants (D). (E,F) *hoxB4* expression (orange) has an anterior boundary of expression at the future r6/r7 boundary and occurs throughout the anterior spinal column in wild-type (E); *myod* expression (posterior purple stain) identifies the mesoderm underlying the spinal column (s1=somite 1) and *krox20* shows r3 and r5 (purple). In *vhnf1* mutants, the anterior boundary of *hoxB4* expression is indistinct and posteriorized (F). (G-J) One-somite-stage embryos. (G,H) *valentino* expression in presumptive r5 and r6 in wild-type embryos (G) is missing in *vhnf1* mutants (H). (I,J) The r5 stripe of *krox20* expression is present in wild-type embryos (I) but is severely reduced in *vhnf1* mutants (J). (K,L) Reticulospinal neurons visualized in 48 hour embryos using anti-neurofilament (RMO44) antibody. Mauthner neurons (arrowheads) are limited to the single r4-derived pair in wild-type embryos (K) but appear in additional, posterior locations in *vhnf1* mutants (red arrowheads) (L). (A-F) Dorsal view, anterior to the left. (G-L) Dorsal view, anterior to the top.

In addition, by the beginning of somitogenesis, it is apparent that the r5 stripe of *krox20* expression is severely reduced in *vhnf1*^{wi408} mutants (Fig. 1I,J).

Morphological defects are consistent with the observed pattern formation defects. The reticulospinal neurons that develop in the hindbrain are visible by 48 hours of development, including the large Mauthner neurons, which are derived from r4 (Fig. 1K). *vhnf1*^{wi408} mutants develop excess Mauthner neurons in parallel with loss of r5+r6-derived neurons (Fig. 1L). Although the normal r4-derived Mauthner neuron pair is always present in the correct location, the extra Mauthner neurons in *vhnf1*^{wi408} mutants are routinely observed both within the normal locale for Mauthner neurons and in more posterior locations. In addition to the neuron identity changes, the otic vesicle is small and round (data not shown) (Sun and Hopkins, 2001). Together with published data, the gene misexpression and morphological defect data are consistent with a transformation of rhombomere identity from r5+r6 to r4 during late gastrula and tailbud stages.

vhnf1 is expressed during gastrulation in the caudal hindbrain

It has been shown that *vhnf1* expression is present in the hindbrain by tailbud stage and that the anterior boundary of *vhnf1* expression lies within the presumptive r5 domain by the four-somite stage (Sun and Hopkins, 2001). Because *vhnf1* regulates *val* expression before tailbud stage, we wanted to generate an accurate description of the timing and initial localization of *vhnf1* expression. An excellent marker of relative position in the gastrulating embryo is *hoxb1b* expression, which appears at early gastrula stages, and which has a sharp anterior border (zebrafish *hoxb1b* is the same as mouse *hoxa1*) (Alexandre et al., 1996). It is not clear what region of the hindbrain will be derived from this early domain of Hoxb1a expression, but it is likely to map at or near the future r3-r4 boundary. In situ hybridization of *hoxb1b* and *vhnf1* shows that localized hindbrain expression of *vhnf1* begins after (not shown) and with a more posterior boundary than that of *hoxb1b* (Fig. 2A). Co-localization of *fgf*8 and *vhnf1* transcripts shows that *vhnf1* expression begins before that of *fgf*8 in the hindbrain (not shown) and that *fgf*8 expression is more anterior than that of *vhnf1* (Fig. 2B). There is a persistent gap of two or three cells between these *fgf8* and *vhnf1* expression domains. *fgf*8 expression abuts *hoxb1b* expression throughout late gastrula and tailbud stages (Fig. 2C).

Expression of *krox20* begins in r5 at the beginning of somitogenesis (Oxtoby and Jowett, 1993). At this stage, *vhnf1* expression overlaps the posterior half of the r5 *krox20* stripe (Fig. 2D), a relative position that is maintained through the four-somite stage (Sun and Hopkins, 2001). *hoxb1a* expression begins at 90% epiboly, initially throughout the posterior neural plate (not shown) (Prince et al., 1998). *hoxb1a* and *vhnf1* expression overlap significantly at the end of gastrulation (Fig. 2E). However, during the tailbud stage, *hoxb1a* expression decreases in the posterior of its expression domain and, by the two-somite stage, it is limited to a stripe in presumptive r4 (Prince et al., 1998). At this stage, there is a gap between *hoxb1a* expression and *vhnf1* expression (Fig. 2F). In summary, *vhnf1* expression begins at midgastrula stages, and its anterior boundary is limited from an early stage to a location that lies within the r5 *krox20* expression domain (Fig. 2G).

Fgf signals are epistatic to *vhnf1* function

To understand better the molecular interactions that allow *vhnf1* to generate r5+r6 identity, we considered other defects

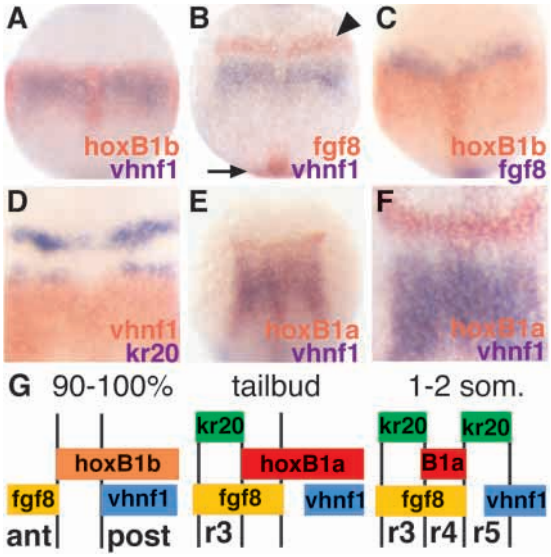

Fig. 2. Early expression of *vhnf1* in the posterior hindbrain. (A) Co-localization of *hoxb1b* (orange) and *vhnf1* (purple). At 100% epiboly, the anterior boundary of *vhnf1* expression is posterior to that of *hoxb1b*. (B) Co-localization of *fgf*8 (orange) and *vhnf1* (purple). At 100% epiboly, *fgf*8 expression is apparent in the presumptive anterior hindbrain (arrowhead) and in the germ ring (arrow), and there are cells between the posterior boundary of *fgf*8 and the anterior boundary of *vhnf1* that express neither gene. (C) Co-localization of *fgf*8 (purple) and *hoxb1b* (orange). At 100% epiboly, *fgf*8 and *hoxb1b* domains of expression are adjacent. (D) Co-localization of *vhnf1* (orange) and *krox20* (purple). At the one-somite stage, *vhnf1* expression overlaps the posterior half of r5 *krox20*. (E,F) Co-localization of *vhnf1* (purple) and *hoxb1a* (orange). At tailbud stage (E), the anterior boundary of *hoxb1a* expression lies anterior to that of *vhnf1* but *hoxb1a* expression overlaps that of *vhnf1* significantly throughout the posterior region. By the two-somite stage (F), *hoxb1a* expression has resolved to a single stripe and there is no overlap with *vhnf1*. (A-F) All embryos are dorsal view, anterior to the top. (G) Summary of expression data, anterior to the left.

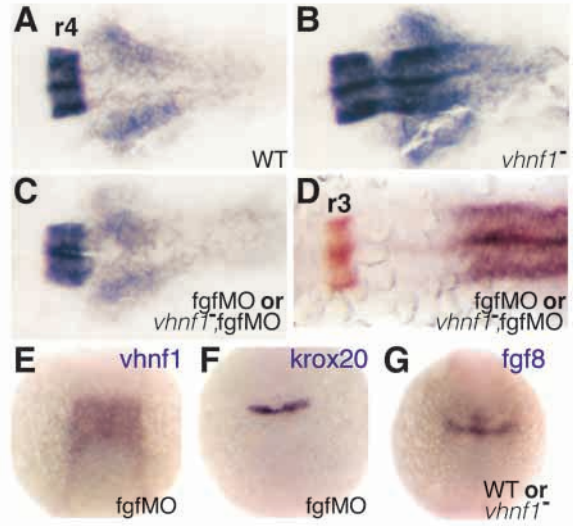

Fig. 3. Fgf function is epistatic to *vhnf1* function in the posterior hindbrain. (A-D) 18-somite stage embryos, dorsal view, anterior to the left. (A,B) *hoxb1a* expression is normally limited to the presumptive r4 domain (A) but is expanded to the posterior in *vhnf1* mutant embryos (12/47 embryos) (B). (C,D) Morpholino oligos (MOs) directed against both *fgf*3 and *fgf*8 injected into embryos derived from *vhnf1* heterozygous parents. Most (37/40) embryos show *hoxb1a* expression limited to the r4 domain (C). Expression of *hoxB4* (purple) and *krox20* (orange); all (56/56) embryos show no anterior expansion of *hoxB4* expression (D). (E-G) One-somite-stage embryos, dorsal view, anterior to the top. (E,F) Injection of MOs directed against *fgf*8+*fgf3* does not affect *vhnf1* expression (E), although is sufficient to inhibit r5 *krox20* expression in sibling embryos (F). (G) Clutches of embryos produced by *vhnf1* heterozyogous parents show consistent expression of *fgf*8 in all embryos.

that cause a similar phenotype. *fgf3* and *fgf8* are expressed in the presumptive anterior hindbrain (Fig. 2G), and their partially redundant functions can be ablated by injection of MOs (Raible and Brand, 2001). Loss of Fgf signaling in the hindbrain results in loss of r5+r6 identity (Maves et al., 2002; Walshe et al., 2002). However, although loss of *vhnf1* function results in expansion of r4 identity (Fig. 3A,B), loss of Fgf signals gives no expansion of *hoxb1a* (Fig. 3C) (Maves et al., 2002; Walshe et al., 2002). In this *fgf* loss-of-function background, *hoxB4* expression does not expand to the anterior, suggesting that there is no posteriorization of the tissue (Fig. 3D).

To test whether an epistatic relationship exists between *vhnf1* and Fgf signals, synthetic double mutants were made. Injection of *fgf3* + *fgf8* MOs into embryos derived from *vhnf1*^{wi408} carrier parents produced embryos all of the same phenotype, with *hoxb1a* expression limited to the r4 domain and *hoxB4* expression limited to the r6-r7 boundary (Fig. 3C,D). Because the loss of the combination of *vhnf1* function and Fgf signals gives the same phenotype as loss of Fgf signals alone, Fgf signaling appears to be epistatic to *vhnf1* function in hindbrain pattern formation.

To determine whether the epistasis between *vhnf1* and Fgfs results from regulation of transcript levels, expression of each gene was tested in the other mutant background. Knockdown of Fgf signaling by injection of a mix of *fgf3* + *fgf8* MOs results in no change in the anterior-posterior pattern of *vhnf1* expression (Fig. 3E). The effectiveness of the MOs to knock down Fgf function in the posterior hindbrain was monitored by loss of r5 *krox20* expression in sibling embryos (Fig. 3F). Embryos generated from *vhnf1*^{wi408} heterozygous parents all show the same pattern of *fgf8* and *fgf3* transcript expression at the one-somite stage, and so *vhnf1* function is not required for normal *fgf8* or *fgf3* expression at this stage (Fig. 3G; not shown). Thus, Fgf signals function epistatically to *vhnf1* to generate rhombomere identity in the posterior hindbrain, and this epistatic relationship is not based on regulation of RNA levels during gastrulation (Fig. 3H).

vhnf1 function and Fgf signals synergize to regulate caudal hindbrain genes

Although both Fgf signals and *vhnf1* function are required to specify r5+r6, neither factor alone is sufficient. Ectopic *vhnf1* induces r5 identity only in the r4 domain, and ectopic Fgf has a limited ability to induce r5+r6 identity at late somitogenesis and primarily within the caudal hindbrain (Maves et al., 2002; Sun and Hopkins, 2001). Therefore, the combination of these two factors was tested for an enhanced ability to induce r5+r6 identity.

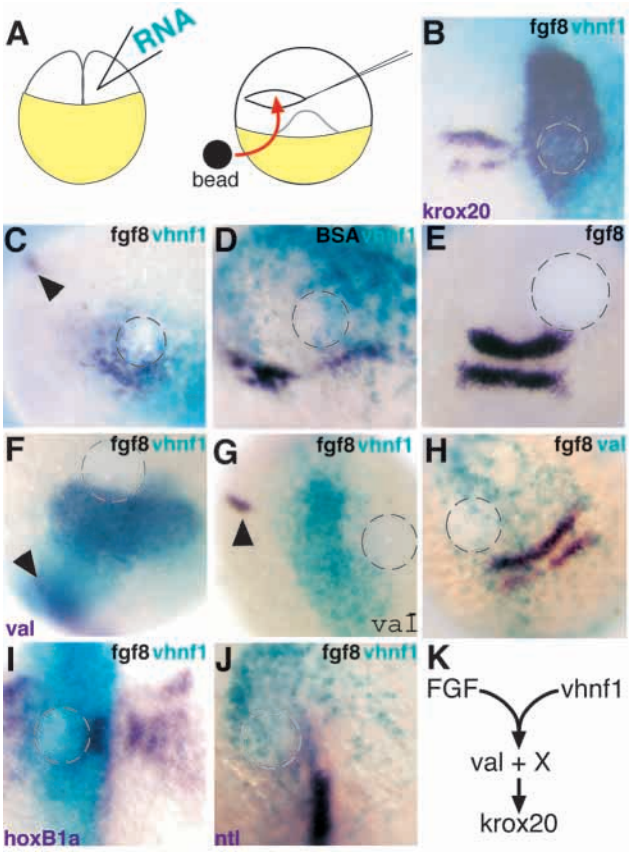

Fig. 4. The combination of *vhnf1* expression and Fgf signal is sufficient to activate posterior hindbrain gene expression. (A) The experimental method consisted of injection of the indicated RNA into one cell at the two-cell stage, then implantation of a protein-coated bead after the shield stage. Embryos were aged to about the three-somite stage and fixed for analysis. (B,C) Injection of *vhnf1* RNA and implantation of a Fgf8-coated bead resulted in significant induction of *krox20* expression both within the neural plate (B) and in lateral ectoderm (C). Arrowhead indicates the location of endogenous *krox20* expression. (D) Injection of *vhnf1* RNA and implantation of a BSA-coated bead does not induce *krox20* expression. (E) The Fgf8-coated bead alone is not sufficient to induce ectopic *krox20* expression. (F) Injection of *vhnf1* RNA and implantation of a Fgf8-coated bead induces *valentino* (*val*) expression. Arrowhead indicates the location of endogenous *val* expression. (G) Injection of *vhnf1* RNA into a *val* mutant embryo (no endogenous r5 *krox20* expression) and implantation of an Fgf8-coated bead does not induce *krox20* expression. This dorsal view of the neural plate shows that r3 *krox20* is repressed by *vhnf1*. Arrowhead indicates endogenous r3 *krox20* on the uninjected side. (H) Injection of *val* RNA and implantation of a Fgf8-coated bead is not sufficient for induction of *krox20* expression. (I) Injection of *vhnf1* RNA and implantation of a Fgf8-coated bead results in localized repression of *hoxb1a* expression. (J) Injection of *vhnf1* RNA and implantation of a Fgf8-coated bead does not induce expression of the axial mesoderm marker *no tail* (*ntl*). (K) Summary of data. The combination of Fgf8+*vhnf1* is sufficient to induce *val* and *krox20* expression, and *val* function is required along with other Fgf+*vhnf1*-inducible factor(s) (X) for *krox20* induction.

vhnf1 RNA was injected into one cell at the two-cell stage. Injected embryos were grown to shield stage, at which point an Fgf8-coated bead was inserted. Embryos were then grown to approximately the three-somite stage and fixed (Fig. 4A). Injection of *vhnf1* RNA and implantation of an Fgf8-coated bead results in strong induction of *krox20* expression limited to the cells expressing *vhnf1* and lying close to the Fgf8 bead (18/20 embryos). This effect is particularly strong throughout the neural plate (Fig. 4B), and is also robust in non-neural ectoderm (Fig. 4C). Embryos in which a BSA-coated bead was implanted near cells overexpressing *vhnf1* show no induction of *krox20* expression (6/6 embryos) (Fig. 4D). The Fgf8 bead alone does not induce any *krox20* expression (10/10 embryos) (Fig. 4E), unless the bead lies adjacent to the normal domain of *krox20* expression (not shown). To determine whether induced *krox20* expression has r5-specific identity, induction of *val* expression was also examined and the combination of *vhnf1* expression with the Fgf8 bead was found to result in induction of *val* expression (5/5 embryos) (Fig. 4F). It appears that the combination of *vhnf1* and Fgf8 is sufficient for induction of early r5+r6 gene expression.

To determine whether induction of ectopic *krox20* in response to *vhnf1*+Fgf8 is mediated by *val* function, the experiment was carried out in embryos produced by *val* heterozygous parents. Embryos were characterized as *val* mutants by their lack of r5 *krox20* expression in the uninjected half of the embryo. *val* mutant embryos show no induction of *krox20* expression in cells expressing *vhnf1* and located near the implanted Fgf8 bead (Fig. 4G) (7/7 embryos), whereas their wild-type siblings show strong induction of *krox20* (not shown). Thus, ectopic induction of *krox20* expression in the presence of *vhnf1* and Fgf8 is dependent on *val* function.

To determine whether Fgf8 and *val* can synergize in a manner similar to Fgf8 and *vhnf1*, *val* RNA was injected and Fgf8-coated beads were added to the embryos. In this case, no induction of *krox20* expression is observed (15/19 embryos) (Fig. 4H), although weak *krox20* induction is observed in a few cases in which the bead is located close to the endogenous *krox20* domain (4/19 embryos). Thus, Fgf8+*val* is insufficient to activate ectopic *krox20* expression, suggesting that *vhnf1* is specifically required to make hindbrain cells competent to respond to Fgf8 signals. In addition, this result shows that *val* RNA alone is insufficient to induce ectopic *krox20* expression (Fig. 4H). Therefore, it is likely that additional factors induced by Fgf+*vhnf1* are required in collaboration with *val* to activate *krox20* expression.

To test whether *vhnf1*+Fgf8 can induce caudal hindbrain identity other than r5+r6, the expression of *hoxb1a*, a presumptive r4 marker, was analysed. *hoxb1a* expression is partially repressed in the region where *vhnf1* expression overlaps endogenous *hoxb1a* expression (10/10 embryos) and is not ectopically induced in regions where the Fgf8 bead lies close to the *vhnf1* expression (4/4 embryos) (Fig. 4I). Fgf+*vhnf1* is therefore insufficient to induce ectopic r4 identity.

Fgf8 can induce mesoderm identity (Griffin et al., 1998; Rodaway et al., 1999) and mesoderm is a source of neural posteriorizing signals including Fgfs (Koshida et al., 1998; Kudoh et al., 2002; Woo and Fraser, 1997). The expression of *no tail* (*ntl*) was examined to determine whether mesoderm was induced by Fgf8 under these experimental conditions and could mediate the synergy between *vhnf1* and Fgf8. However, no ectopic *ntl* expression is observed in most embryos (13/15)

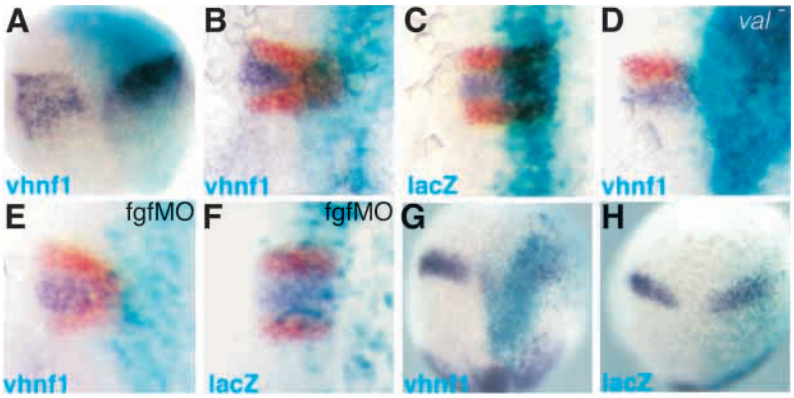

Fig. 5. *vhnf1* functions as a repressor of anterior identity independently of Fgf and *val* functions.
(A-H) Overexpression of *vhnf1* marked by co-injected *lacZ* (light blue stain). Panels labeled *lacZ* have only *lacZ* RNA injected. Dorsal view, anterior to the top.
(A-F) *hoxb1a* expression (purple) and *krox20* expression (orange) at six-somite stage. (A-C) *vhnf1* does not affect *hoxb1a* expression at 100% epiboly (A) but represses *hoxb1a* expression (B). *krox20* expression marks future r3 and r5 domains (orange). (C) When *lacZ* alone is injected, no repression of *hoxb1a* is observed. (D) Injection of *vhnf1* into *valentino* (*val*) mutant embryos results in repression of *hoxb1a* expression at the six-somite stage and repression of the anterior (r3) *krox20* expression. (E) Co-injection of *fgf3* and *fgf8*-targeted morpholino oligos with *vhnf1* RNA results in repression of *hoxb1a* expression. (F) Coinjection of *fgf3* and *fgf8* MOs with *lacZ* RNA has no effect on *hoxb1a* expression. (G,H) Expression of *fgf8* RNA at tailbud stage. Injection of *vhnf1* represses the anterior hindbrain expression of *fgf*8 (G), whereas injection of *lacZ* alone has no effect on *fgf*8 expression (H).

(Fig. 4J), although, when the bead lies deep under the epiblast and close to the prechordal plate, ectopic *ntl* is observed close to the beads in deep tissue regions (2/15 embryos). Therefore, the co-operativity of Fgf signals and *vhnf1* function appears to occur within the ectoderm. In summary, Fgf signals synergize with *vhnf1* to activate expression of r5+r6-specific genes, including the activation of *val* and *krox20* expression (Fig. 4K).

vhnf1 represses *hoxb1a* independent of Fgf signals and *val* function

The expansion of *hoxb1a* in *vhnf1*^{wi408} mutants (Fig. 1B), paired with the suppression of *hoxb1a* by injection of *vhnf1* RNA (Fig. 4I), suggests that one of the ways in which *vhnf1* functions is to repress r4-specific *hoxb1a* expression. Therefore, we further examined *hoxb1a* expression in response to ectopic *vhnf1*. Expression of *hoxb1a* at 90-100% epiboly is normal when *vhnf1* is overexpressed (Fig. 5A). However, by the six-somite stage, expression of *hoxb1a* is completely repressed by overexpression of *vhnf1*, and *krox20* expression appears in a single broad band (32/32 embryos) (Fig. 5B). Overexpression of *lacZ* alone has no effect on *hoxb1a* or *krox20* expression (17/17 embryos) (Fig. 5C). Thus, ectopic *vhnf1* is able to repress *hoxb1a* expression in presumptive r4.

To test whether the repression of *hoxb1a* by *vhnf1* occurs through *val* function, the ability of *vhnf1* to repress *hoxb1a* in a *val* mutant background was tested. Ectopic *vhnf1* is able to repress *hoxb1a* expression in this background (10/10 embryos) (Fig. 5D). *val* mutant embryos injected with *lacZ* do not show repression of *hoxb1a* (7/7 embryos) (data not shown). No *krox20* expression remains after *vhnf1* overexpression in *val* mutants, indicating that ectopic *vhnf1* represses r3-specific *krox20* as well as r4-specific *hoxb1a*. Therefore, it appears that ectopic *vhnf1* transforms both presumptive r3 and r4 towards the r5 identity autonomously of *val* function.

To test whether the repression of *hoxb1a* by *vhnf1* requires the co-function of Fgf signals, the ability of ectopic *vhnf1* to repress *hoxb1a* in a *fgf*-compromised background was tested. Embryos were injected with MOs directed against *fgf*3 and *fgf*8 along with the *vhnf1* RNA. Ectopic *vhnf1* represses *hoxb1a* expression in the absence of Fgf signals (22/22 embryos) (Fig. 5E) but co-injection of *lacZ* with the *fgf* MOs does not reduce the extent of *hoxb1a* expression (59/59 embryos) (Fig. 5F). These data imply that *vhnf1* functions independently of Fgf signals to repress *hoxb1a* expression. Because knockdown of *fgf*s based on the MOs used in this experiment does not lead to complete repression of r5 *krox20* expression, it is interesting that there is no remaining *krox20* expression in the tissue injected with *vhnf1* RNA and anti-*fgf* MOs. This might reflect a synergistic effect that results from repression of *fgf8* expression by *vhnf1* (Fig. 5G,H), which might give a stronger loss of *fgf* functions and therefore a complete repression of r5 *krox20* expression. *fgf*8 is expressed throughout presumptive anterior hindbrain tissue at tailbud stage (Reifers et al., 1998), and *vhnf1* appears to be sufficient to repress anterior hindbrain identity during gastrulation (Fig. 5G).

val can partially rescue loss of *vhnf1* function

The expression of multiple genes is dependent on *vhnf1* function, including *val*, *krox20* and *hoxb3*. Of these, *val* is the earliest expressed, and loss of *val* function also results in loss of *krox20* in presumptive r5 and loss of *hoxb3* expression (Moens et al., 1996; Prince et al., 1998). To test whether *vhnf1* is independently required for regulation of *krox20* and *hoxb3*, *val* RNA was injected into *vhnf1*^{wi408} mutant embryos and expression of target genes was analyzed. Expression of *krox20* is unaffected in wild-type embryos by overexpression of *val* (Fig. 6A). However, injection of *val* RNA is sufficient to rescue r5 *krox20* expression in *vhnf1*^{wi408} mutant embryos in the injected side of the embryo (7/8 embryos) and it is striking that *krox20* expression induced by *val* RNA is limited to the presumptive r5 domain (Fig. 6B). *lacZ* RNA alone has no ability to rescue *krox20* expression (Fig. 6C).

In contrast to the strong rescue of *krox20* expression by *val* RNA, expression of *hoxb3* is infrequently rescued, although *hoxb3* expression is occasionally observed after *val* injection into *vhnf1*^{wi408} embryos (Fig. 6D-F). However, the frequency of rescue is lower (5/14 embryos) than observed for *krox20* rescue, suggesting that the *val* RNA injected is not sufficient to rescue to *hoxb3* expression. This result could be due to a requirement for *vhnf1* functions not mediated by *val* function or to a requirement for a higher concentration of *val* RNA for *hoxb3* expression.

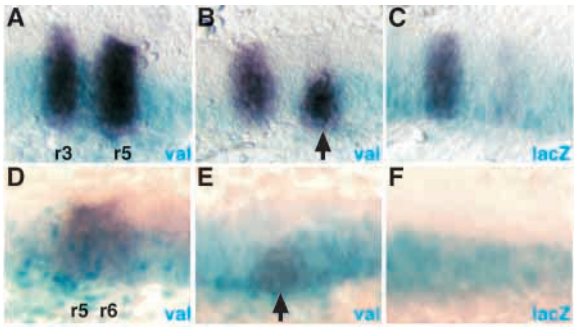

Fig. 6. *valentino* (*val*) RNA is sufficient to recover some posterior hindbrain identity in *vhnf1* mutants. (A-F) Injection of *val+lacZ* RNA or *lacZ* alone. Embryos are 12-somite stage. (A) Injection of *val* RNA into wild-type embryos has no effect on endogenous *krox20* expression. (B) Injection of *val* RNA into *vhnf1* embryos recovers r5 *krox20* expression (arrow) within the injected cells in 7/8 mutant embryos. (C) Injection of *lacZ* RNA alone is not sufficient to recover *krox20* expression. (D) Injection of *val* RNA into wild-type embryos at the 12-somite stage does not affect *hoxb3* expression (arrow). (E) Injection of *val* RNA into *vhnf1* mutant embryos recovers some *hoxb3* expression in 5/14 embryos. (F) Injection of *lacZ* RNA alone does not rescue *hoxb3* expression.

DISCUSSION

We have shown that *vhnf1* functions in the zebrafish hindbrain to generate individual rhombomere identity in the caudal hindbrain. *vhnf1* functions through two mechanisms to specify rhombomeres 5 and 6, first by synergizing with Fgf signals to activate r5+r6-specific genes and second by limiting *hoxb1a* expression to presumptive r4 independently of *fgf3* and *fgf8* function.

Subdivision of the caudal hindbrain domain

The data presented here define the mechanism by which the initially broad expression domains of the future hindbrain are subdivided (Fig. 7). Soon after *hoxb1b* expression appears with an anterior limit near the future r3-r4 boundary, *vhnf1* expression begins, with a more posterior limit of expression, probably within the future r5 region. It is not clear how *vhnf1* expression is initiated or what limits its anterior boundary. Expression of *vhnf1* is sensitive to retinoic acid (RA) (E. Wiellette and H. Sive, unpublished) and RA receptor binding sites have been identified in the mouse *vhnf1* promoter (Power and Cereghini, 1996), so it is possible that RA directly regulates *vhnf1* transcription. However, it is also possible that the observed RA sensitivity is mediated by *hoxb1b* function; when Hox gene function is removed from zebrafish by making embryos devoid of *pbx* gene functions, *vhnf1* transcription is not initiated (Waskiewicz et al., 2002).

In late gastrula stages, *fgf*3 and *fgf*8 expression begins in the anterior hindbrain (Maves et al., 2002; Reifers et al., 1998; Walshe et al., 2002), and the combination of Fgf signals with *vhnf1* is sufficient to initiate expression of *val* and *krox20*. It seems likely, based on the demonstrated significance of the presumptive r4 domain in caudal hindbrain pattern formation (Maves et al., 2002; Walshe et al., 2002), that the significant Fgf signals received by *vhnf1*-expressing cells are secreted from the anterior hindbrain domain. Consistent with this, the cellular response as measured by *val* expression does not begin until after the anterior hindbrain expression of *fgf*3 and *fgf*8 begins.

vhnf1 function is required for activation of *val* and *krox20* expression. However, the anterior boundary of *vhnf1* expression lies posterior to the anterior boundary of *krox20* r5 expression, which is comparable to that of *val* (Moens et al., 1998). Because *vhnf1* encodes a putative transcription factor, it seems likely that Vhnf1 protein is not acting directly to regulate the transcription of *val* or *krox20* but might rather be regulating transcription of an extracellular signal, which works at a distance of one or two cell diameters to control the expression of *val* and *krox20*.

Also, at the end of gastrulation, *hoxb1a* expression appears in a broad domain of the posterior neural plate, with an anterior boundary similar to that of *hoxb1b*. Restriction of *hoxb1a* expression to presumptive r4 during somitogenesis is conserved in mouse (Murphy and Hill, 1991), and downregulation of *hoxb1a* might be necessary to allow normal development of r5-r7. Although *hoxb1a* expression is activated throughout the posterior hindbrain in a *vhnf1*-insensitive manner, *hoxb1a* can be repressed by ectopic *vhnf1* starting at tailbud stage, the same stage at which *hoxb1a* transcripts are first downregulated outside of r4. *hoxb1a* repression in

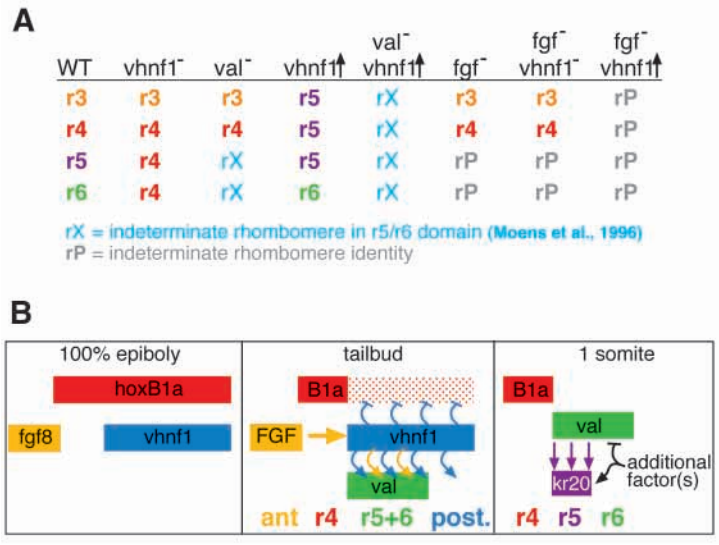

Fig. 7. Model for the method by which *vhnf1* functions to generate rhombomere-specific identity. (A) Summary of the data. rX represents the combined r5+r6 domain, a partially differentiated rhombomere that develops in *val* mutants. rX identity is distinct from rP, the unspecified tissue that remains when Fgf signals are reduced. (B) Model of the role of *vhnf1* in generation of rhombomere identity. After *hoxb1a*, *fgf8* and *vhnf1* gene expression is established, *vhnf1* functions to repress *hoxb1a* throughout the domain in which *vhnf1* is expressed. In addition, Fgf signals from the anterior are received by posterior hindbrain cells, and the combination of Fgf signal transduction and *vhnf1* function result in activation of *val* expression within the domain in which the overlap occurs. *val* function is then sufficient to activate *krox20* expression, although this activation is limited to the presumptive r5 domain, suggesting that other factors limit this activation outside of r5 or promote it within r5.

future r5-r7 is probably brought about at least in part by the function of *vhnf1*, independent of Fgf and *val* functions. The separate roles of *vhnf1*, as an activator in conjunction with Fgf signals and as a repressor independent of Fgf signals, might reflect distinct molecular interactions, either with cofactor proteins or with DNA. Two different forms of Vhnf1 protein, which result from alternative splicing, have been characterized as having different DNA binding affinities and transactivation strengths (Ringeisen et al., 1993). It is possible that these isoforms provide the different functional specificities in the caudal hindbrain.

Nonequivalence of *vhnf1* and *val* functions

Although one of the central functions of *vhnf1* in hindbrain pattern formation is activation of *val* expression, loss of *vhnf1* has a more severe phenotype in the forming hindbrain than loss of *val* function. Loss of *val* results in the production of a narrowed 'rX' domain in place of r5 and r6 (Moens et al., 1996). Like the mis-specified r5 and r6 domain in *vhnf1* mutant embryos, the *val* mutant rX domain does not express *hoxb3*, and the posterior boundary of *hoxb1a* and the anterior boundary of *hoxB4* expression are similarly indistinct (Prince et al., 1998). However, the rX domain of *val* mutants is significantly narrower than the combination of r5 and r6 domains, whereas *vhnf1* mutants show no apparent reduction of tissue. In addition, the reticulospinal neurons in *val* mutants are correctly specified (Moens et al., 1996).

The genetic distinctions between *vhnf1* and *val* are paralleled by differences in molecular capacities. The combination of *val*+Fgf is not sufficient to induce r5 identity outside the r5 domain, whereas the embryo is broadly sensitive to *vhnf1*+Fgf function. Conversely, Fgf+*vhnf1* cannot induce ectopic *krox20* in a *val* mutant background, suggesting that each transcription factor has unique and necessary functions in hindbrain pattern formation. Although overexpression of *val* in the *vhnf1*^{wi408} mutant background results in recovery of *krox20* expression, this appears only in the r5 domain, with no ectopic *krox20* expression detected. This suggests that only limited domains are competent to respond to *val* function, potentially based on the presence of a cofactor.

Potential conservation of *vhnf1* function in hindbrain pattern formation

Knockout of the murine *vhnf1* gene results in early death as a result of failure to form visceral endoderm (Barbacci et al., 1999; Coffinier et al., 1999). As yet, no studies of later loss-of-function of *vhnf1* have been published. However, *vhnf1* is expressed in the mouse hindbrain in a broad domain that lies close to the otic placode, a position that is similar to the domain of expression in zebrafish (Barbacci et al., 1999; Coffinier et al., 1999). In addition, *vhnf1* expression is detected before the onset of *kreisler* expression (Cordes and Barsh, 1994), suggesting that *vhnf1* could have a conserved role in regulation of *kreisler* expression in the developing mouse hindbrain. Furthermore, expression of mouse *hoxb1*, the most likely functional homolog of zebrafish *hoxb1a*, is downregulated in the most posterior hindbrain at a time soon after initiation of *vhnf1* transcription initiation (Murphy and Hill, 1991). Finally, although *fgf*8 transcript expression is restricted to r1 and the midbrain-hindbrain boundary (MHB) in mice, *fgf*3 expression is observed in r4 at the same time that *kreisler* expression is induced in presumptive r5+r6, and before the upregulation of *fgf*3 in the r5+r6 domain (Cordes and Barsh, 1994; Joyner et al., 2000; Mahmood et al., 1996). Thus, it is likely that the restriction of rhombomere-specific identities in the caudal hindbrain of the mouse follows a molecular mechanism similar to the one we have described for zebrafish.

Competence to respond to Fgf signals in the MHB and posterior hindbrain

Fgf signals are reused throughout development and yet the cellular response to the signal varies based on time and location. In the developing zebrafish brain, *fgf8* function is required not only for pattern formation in the caudal hindbrain but also for formation of the MHB (Reifers et al., 1998). In the posterior hindbrain, Fgf signaling results in activation of *val* expression, whereas, at the MHB, Fgf signaling results in activation of *gbx2*, *fkd3* and *spry4* (Reim and Brand, 2002). During late gastrulation stages, the anterior hindbrain expresses *fgf3* and *fgf8* in a domain that lies between the forming MHB and the posterior hindbrain. Thus, it appears that the anterior hindbrain domain provides a source of Fgf signals for both the MHB and posterior hindbrain. However, the molecular and morphological results of this signaling are different.

Various features of Fgf signal transduction might provide distinct cellular responses in the MHB and posterior hindbrain, including different Fgf receptor interactions and negative feedback regulation of Fgf signal transduction. One potential distinction in Fgf signaling outcome is the presence of intracellular cofactors. We have shown here that the expression of *vhnf1* in or near cells receiving Fgf signals is sufficient to promote activation of posterior hindbrain gene expression. Similarly, it has been shown that the presence of *pou2* in cells receiving Fgf signals is required for activation of MHB target genes including *gbx2*, *fkd3* and *spry4* (Reim and Brand, 2002). Thus, it is possible that one of the ways in which cells generate a differential response to Fgf signals is through the presence of a transcriptional cofactor such as *pou2* or *vhnf1*, which provides promoter selection specificity. Further work to characterize the unique cellular responses to Fgf signals in the presence of *pou2* or *vhnf1* will help determine the role of transcription factors as mediators of signaling specificity.

We thank members of the Sive lab for helpful comments. In addition, we would like to thank L. Maves and C. Moens for helpful discussions, and members of the Moens lab for sending plasmids, protocols and fish. Finally, we thank N. Hopkins and Z. Sun for the *vhnf1* cDNA and for communicating results prior to publication. We are indebted to B. Pratt for excellent fish husbandry. Supported by NIH HD08704 to E.L.W. and NIH MH059942 to H.S.

REFERENCES

Alexandre, D., Clarke, J. D., Oxtoby, E., Yan, Y. L., Jowett, T. and Holder, N. (1996). Ectopic expression of *Hoxa-1* in the zebrafish alters the fate of the mandibular arch neural crest and phenocopies a retinoic acid-induced phenotype. *Development* **122**, 735-746.

Barbacci, E., Reber, M., Ott, M. O., Breillat, C., Huetz, F. and Cereghini, S. (1999). Variant hepatocyte nuclear factor 1 is required for visceral endoderm specification. *Development* **126**, 4795-4805.

Bell, E., Wingate, R. J. and Lumsden, A. (1999). Homeotic transformation of rhombomere identity after localized *Hoxb1* misexpression. *Science* **284**, 2168-2171.

Coffinier, C., Thepot, D., Babinet, C., Yaniv, M. and Barra, J. (1999). Essential role for the homeoprotein vHNF1/HNF1β in visceral endoderm differentiation. *Development* **126**, 4785-4794.

Cordes, S. P. and Barsh, G. S. (1994). The mouse segmentation gene *kr* encodes a novel basic domain-leucine zipper transcription factor. *Cell* **79**, 1025-1034.

Dupe, V. and Lumsden, A. (2001). Hindbrain patterning involves graded responses to retinoic acid signalling. *Development* **128**, 2199-2208.

Frohman, M. A., Martin, G. R., Cordes, S. P., Halamek, L. P. and Barsh, G. S. (1993). Altered rhombomere-specific gene expression and hyoid bone differentiation in the mouse segmentation mutant, *kreisler* (*kr*). *Development* **117**, 925-936.

Gavalas, A. and Krumlauf, R. (2000). Retinoid signalling and hindbrain patterning. *Curr. Opin. Genet. Dev*. **10**, 380-386.

Gavalas, A., Studer, M., Lumsden, A., Rijli, F. M., Krumlauf, R. and Chambon, P. (1998). Hoxa1 and Hoxb1 synergize in patterning the hindbrain, cranial nerves and second pharyngeal arch. *Development* **125**, 1123-1136.

Giudicelli, F., Gilardi-Hebenstreit, P., Mechta-Grigoriou, F., Poquet, C. and Charnay, P. (2003). Novel activities of Mafb underlie its dual role in hindbrain segmentation and regional specification. *Dev. Biol*. **253**, 150-162.

Griffin, K. J., Amacher, S. L., Kimmel, C. B. and Kimelman, D. (1998). Molecular identification of *spadetail*: regulation of zebrafish trunk and tail mesoderm formation by T-box genes. *Development* **125**, 3379-3388.

Joyner, A. L., Liu, A. and Millet, S. (2000). Otx2, Gbx2 and Fgf8 interact to position and maintain a mid-hindbrain organizer. *Curr. Opin. Cell Biol.* **12**, 736-741.

Kolm, P. J. and Sive, H. L. (1995). Regulation of the *Xenopus* labial homeodomain genes, *HoxA1* and *HoxD1*: activation by retinoids and peptide growth factors. *Dev. Biol*. **167**, 34-49.

Koshida, S., Shinya, M., Mizuno, T., Kuroiwa, A. and Takeda, H. (1998). Initial anteroposterior pattern of the zebrafish central nervous system is determined by differential competence of the epiblast. *Development* **125**, 1957-1966.

Kudoh, T., Wilson, S. W. and Dawid, I. B. (2002). Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm. *Development* **129**, 4335-4346.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1115.

Mahmood, R., Mason, I. J. and Morriss-Kay, G. M. (1996). Expression of Fgf-3 in relation to hindbrain segmentation, otic pit position and pharyngeal arch morphology in normal and retinoic acid-exposed mouse embryos. *Anat. Embryol.* **194**, 13-22.

Manley, N. R. and Capecchi, M. R. (1997). Hox group 3 paralogous genes act synergistically in the formation of somitic and neural crest-derived structures. *Dev. Biol*. **192**, 274-288.

Manzanares, M., Trainor, P. A., Nonchev, S., Ariza-McNaughton, L., Brodie, J., Gould, A., Marshall, H., Morrison, A., Kwan, C. T., Sham, M. H. et al. (1999). The role of *kreisler* in segmentation during hindbrain development. *Dev. Biol*. **211**, 220-237.

Marin, F. and Charnay, P. (2000). Hindbrain patterning: FGFs regulate *Krox20* and *mafB*/*kr* expression in the otic/preotic region. *Development* **127**, 4925-4935.

Maves, L., Jackman, W. and Kimmel, C. B. (2002). FGF3 and FGF8 mediate a rhombomere 4 signaling activity in the zebrafish hindbrain. *Development* **129**, 3825-3837.

McClintock, J. M., Kheirbek, M. A. and Prince, V. E. (2002). Knockdown of duplicated zebrafish *hoxb1* genes reveals distinct roles in hindbrain patterning and a novel mechanism of duplicate gene retention. *Development* **129**, 2339-2354.

Moens, C. B. and Prince, V. E. (2002). Constructing the hindbrain: insights from the zebrafish. *Dev. Dyn*. **224**, 1-17.

Moens, C. B., Yan, Y. L., Appel, B., Force, A. G. and Kimmel, C. B. (1996). *valentino*: a zebrafish gene required for normal hindbrain segmentation. *Development* **122**, 3981-3990.

Moens, C. B., Cordes, S. P., Giorgianni, M. W., Barsh, G. S. and Kimmel, C. B. (1998). Equivalence in the genetic control of hindbrain segmentation in fish and mouse. *Development* **125**, 381-391.

Murphy, P. and Hill, R. E. (1991). Expression of the mouse labial-like homeobox-containing genes, *Hox 2.9* and *Hox 1.6*, during segmentation of the hindbrain. *Development* **111**, 61-74.

Oxtoby, E. and Jowett, T. (1993). Cloning of the zebrafish *krox-20* gene (*krx-20*) and its expression during hindbrain development. *Nucleic Acids Res.* **21**, 1087-1095.

Power, S. C. and Cereghini, S. (1996). Positive regulation of the *vHNF1* promoter by the orphan receptors COUP-TF1/Ear3 and COUP-TFII/Arp1. *Mol. Cell. Biol*. **16**, 778-791.

Prince, V. E., Moens, C. B., Kimmel, C. B. and Ho, R. K. (1998). Zebrafish Hox genes: expression in the hindbrain region of wild-type and mutants of the segmentation gene, *valentino*. *Development* **125**, 393-406.

Raible, F. and Brand, M. (2001). Tight transcriptional control of the ETS domain factors Erm and Pea3 by Fgf signaling during early zebrafish development. *Mech. Dev*. **107**, 105-117.

Reifers, F., Bohli, H., Walsh, E. C., Crossley, P. H., Stainier, D. Y. and Brand, M. (1998). Fgf8 is mutated in zebrafish *acerebellar* (*ace*) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. *Development* **125**, 2381-2395.

Reim, G. and Brand, M. (2002). *Spiel-ohne-grenzen/pou2* mediates regional competence to respond to Fgf8 during zebrafish early neural development. *Development* **129**, 917-933.

Ringeisen, F., Rey-Campos, J. and Yaniv, M. (1993). The transactivation potential of variant hepatocyte nuclear factor 1 is modified by alternative splicing. *J. Biol. Chem*. **268**, 25706-25711.

Rodaway, A., Takeda, H., Koshida, S., Broadbent, J., Price, B., Smith, J. C., Patient, R. and Holder, N. (1999). Induction of the mesendoderm in the zebrafish germ ring by yolk cell-derived TGF-beta family signals and discrimination of mesoderm and endoderm by FGF. *Development* **126**, 3067-3078.

Rossel, M. and Capecchi, M. R. (1999). Mice mutant for both *Hoxa1* and *Hoxb1* show extensive remodeling of the hindbrain and defects in craniofacial development. *Development* **126**, 5027-5040.

Sagerstrom, C. G., Grinbalt, Y. and Sive, H. (1996). Anteroposterior patterning in the zebrafish, *Danio rerio*: an explant assay reveals inductive and suppressive cell interactions. *Development* **122**, 1873-1883.

Sagerstrom, C. G., Kao, B. A., Lane, M. E. and Sive, H. (2001). Isolation and characterization of posteriorly restricted genes in the zebrafish gastrula. *Dev. Dyn*. **220**, 402-408.

Salzberg, A., Elias, S., Nachaliel, N., Bonstein, L., Henig, C. and Frank, D. (1999). A Meis family protein caudalizes neural cell fates in *Xenopus*. *Mech. Dev*. **80**, 3-13.

Schulte-Merker, S., Ho, R. K., Herrmann, B. G. and Nusslein-Volhard, C. (1992). The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* **116**, 1021-1032.

Seitanidou, T., Schneider-Maunoury, S., Desmarquet, C., Wilkinson, D. G. and Charnay, P. (1997). Krox-20 is a key regulator of rhombomere-specific gene expression in the developing hindbrain. *Mech. Dev*. **65**, 31-42.

Studer, M., Gavalas, A., Marshall, H., Ariza-McNaughton, L., Rijli, F. M., Chambon, P. and Krumlauf, R. (1998). Genetic interactions between *Hoxa1* and *Hoxb1* reveal new roles in regulation of early hindbrain patterning. *Development* **125**, 1025-1036.

Sun, Z. and Hopkins, N. (2001). *vhnf1*, the MODY5 and familial GCKD-associated gene, regulates regional specification of the zebrafish gut, pronephros, and hindbrain. *Genes Dev.* **15**, 3217-3229.

Trainor, P. A. and Krumlauf, R. (2001). Hox genes, neural crest cells and branchial arch patterning. *Curr. Opin. Cell Biol.* **13**, 698-705.

Vlachakis, N., Choe, S. K. and Sagerstrom, C. G. (2001). Meis3 synergizes with Pbx4 and Hoxb1b in promoting hindbrain fates in the zebrafish. *Development* **128**, 1299-1312.

Walshe, J., Maroon, H., McGonnell, I. M., Dickson, C. and Mason, I. (2002). Establishment of hindbrain segmental identity requires signaling by FGF3 and FGF8. *Curr. Biol*. **12**, 1117-1123.

Waskiewicz, A. J., Rikhof, H. A., Hernandez, R. E. and Moens, C. B. (2001). Zebrafish Meis functions to stabilize Pbx proteins and regulate hindbrain patterning. *Development* **128**, 4139-4151.

Waskiewicz, A. J., Rikhof, H. A. and Moens, C. B. (2002). Eliminating zebrafish Pbx proteins reveals a hindbrain ground state. *Dev. Cell* **3**, 723-733.

Weinberg, E. S., Allende, M. L., Kelly, C. S., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, O. G., Grunwald, D. J. and Riggleman, B. (1996). Developmental regulation of zebrafish MyoD in wild-type, *no tail* and *spadetail* embryos. *Development* **122**, 271-280.

Westerfield, M. (1995). *The Zebrafish Book*. Eugene, Oregon: University of Oregon Press.

Woo, K. and Fraser, S. E. (1997). Specification of the zebrafish nervous system by nonaxial signals. *Science* **277**, 254-257.

Woo, K. and Fraser, S. E. (1998). Specification of the hindbrain fate in the zebrafish. *Dev. Biol*. **197**, 283-296.