RESEARCH ARTICLE

vHnf1 Regulates Specification of Caudal Rhombomere Identity in the Chick Hindbrain

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The homeobox-containing gene *variant hepatocyte nuclear factor-1 (vHnf1)* has recently been shown to be involved in zebrafish caudal hindbrain specification, notably in the activation of *MafB* and *Krox20* expression. We have explored this regulatory network in the chick by in ovo electroporation in the neural tube. We show that misexpression of *vHnf1* confers caudal identity to more anterior regions of the hindbrain. Ectopic expression of *mvHnf1* leads to ectopic activation of *MafB* and *Krox20*, and downregulation of *Hoxb1* in rhombomere 4. Unexpectedly, *mvhnf1* strongly upregulates *Fgf3* expression throughout the hindbrain, in both a cell-autonomous and a non-cell-autonomous manner. Blockade of FGF signaling correlates with a selective loss of *MafB* and *Krox20* expression, without affecting the expression of *vHnf1*, *Fgf3*, or *Hoxb1*. Based on these observations, we propose that in chick, as in zebrafish, *vHnf1* acts with FGF to promote caudal hindbrain identity by activating *MafB* and *Krox20* expression. However, our data suggest differences in the *vHnf1* downstream cascade in different vertebrates. *Developmental Dynamics 234:567–576, 2005.* © 2005 Wiley-Liss, Inc.

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INTRODUCTION

The Central Nervous System (CNS) arises from the neural plate, a homogenous sheet of epithelial cells that forms the dorsal surface of the embryo at the gastrula stage. The neural plate subsequently forms the neural tube, the anterior end of which partitions into a series of vesicles that will develop into the fore-, mid-, and hindbrain. The development of morphological features is accompanied by position-specific expression of regulatory genes that dictate the overall plan of the CNS and predict its regional specialization. Neural inducers and modifiers produced by adjacent tissues during gastrulation establish an initial crude anteroposterior (AP) pattern in the overlying neural plate. This pattern is progressively refined, resulting in a precise regional variation in cell identity along the AP and dorsoventral (DV) axes of the neural tube (Lumsden and Krumlauf, 1996).

In the hindbrain region of the vertebrate CNS, AP regionalization involves a segmentation process leading to the formation of 7–8 morphological bulges called rhombomeres (r) (for review see Lumsden and Krumlauf, 1996; Schneider-Maunoury et al., 1998). Pairs of rhombomeres cooperate to generate a metameric organization that underlies the repeating sequences of cranial branchiomotor nerves (Lumsden and Keynes, 1989). The rhombomeres also constitute cellular compartments since clonal analysis has revealed that rhombomere boundaries are partitions across which cell migrations are restricted (Fraser et al., 1990). This restriction of cell migration is thought to be required for each segment to maintain a specific pattern of gene expression and thus a distinct AP identity (Wilkinson, 1995). Rhombomeric organization is

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also critical for segmental specification and migration of neurogenic and branchial neural crest cells (Trainor and Krumlauf, 2001).

Several genes coding for transcription factors, such as *Krox20, MafB*, and *Hox* genes of paralogous groups 1 to 3, are involved in the formation of different rhombomeres or groups of rhombomeres and/or in the specification of their identity (Giudicelli et al., 2001, 2003; McClintock et al., 2001, 2002; Voiculescu et al., 2001; reviewed by Schneider-Maunoury et al., 1998; Lumsden, 2004). Three of these genes will be particularly referred to in this report: *Krox20, MafB*, and *Hoxb1*. *Krox20* is activated in the hindbrain in two transverse stripes that prefigure r3 and r5 (Irving et al., 1996; Wilkinson et al., 1989). This transcription factor has a dual function in segment formation and specification of odd- versus even-numbered rhombomere identity (Schneider-Maunoury et al., 1993, 1997; Giudicelli et al., 2001; Voiculescu et al., 2001). *MafB* is normally expressed in prospective r5 and r6, and is involved in the specification of these two rhombomeres and in *Krox20* activation in r5 (Eichmann et al., 1997; Cordes and Barsh, 1994; Moens et al., 1996, 1998; Prince et al., 1998; Manzanares et al., 1999; Giudicelli et al., 2003; Mechta et al., 2003). *Hoxb1* is expressed in the neural plate with an anterior limit at the prospective r3/r4 boundary. It is later upregulated in r4 and downregulated in r5 and r6. *Hoxb1* acts synergistically with its paralogue *Hoxa1* to specify r4 identity (Studer et al., 1996, 1998).

Recently, the homeodomain transcription factor, variant Hepatocyte Nuclear Factor 1 (vHNF1), was shown to be involved in caudal hindbrain patterning in zebrafish embryos. Zebrafish *vhnf1* is expressed from the end of gastrulation onward in a caudal domain with an anterior limit that coincides with the prospective r4/r5 boundary (Sun and Hopkins, 2001; Wiellette and Sive, 2003; Lecaudey et al., 2004). Four strong hypomorphic or null alleles of *vhnf1* have been isolated in zebrafish (Sun and Hopkins, 2001; Wiellette and Sive, 2003). Analysis of *vhnf1* mutant embryos reveals that *vhnf1* acts in synergy with FGF signals from prospective r4, to activate the expression of *valentino* (*val*, the zebrafish ortholog of *MafB*/*kreisler*) in r5 and r6, and of *krx20* in r5 (Maves et al., 2002; Walshe et al., 2002; Sun and Hopkins, 2001; Wiellette and Sive, 2003; Hernandez et al., 2004). In addition, *vhnf1* represses the expression of *hoxb1a*, the functional homologue of mouse *hoxb1*, independently of FGF signals, thereby limiting its expression to prospective r4 (Wiellette and Sive, 2003; Hernandez et al., 2004). Mouse *vHnf1* mutants have also been obtained. However, since *vHnf1*-deficient mouse embryos die shortly after implantation due to abnormal extraembryonic visceral endoderm formation (Barbacci et al, 1999; Coffinier et al., 1999), the *vHnf1* function in the mouse hindbrain has yet to be examined.

The mechanisms through which the caudal hindbrain is specified seem to differ slightly among vertebrate species. A striking example is in the expression and regulation of FGFs. In zebrafish, *fgf3* and *fgf8* are expressed in r4, and are involved in the patterning of both anterior and posterior hindbrain, as well as in otic vesicle formation (Phillips et al., 2001; Leger and Brand, 2002; Maroon et al., 2002; Walshe et al., 2002; Maves et al., 2002; Rhinn et al., 2003; Wiellette and Sive, 2004). Notably, *fgf3* expression in particular is repressed by *val* in r5 and r6 (Kwak et al., 2002). In contrast, in the mouse, *Fgf3* is expressed in r5 and r6 and its expression is positively controlled by *MafB* (McKay et al., 1994). The role of the MafB transcription factor is also controversial depending on the species. In zebrafish, *MafB*/*val* has been suggested to be responsible for the maturation and subdivision of a pro-rhombomeric territory into definitive r5 and r6 (Moens et al., 1996, 1998). In the mouse, *MafB* function has been analysed in the X-ray-induced *kreisler* mutant (McKay et al, 1994; Cordes and Barsh, 1994). In this species, *MafB* is only involved in the formation of r5, the generation of a definitive r6 territory being independent of *kreisler* (Manzanares et al., 1999).

Given the divergence of gene function and regulation in the caudal hindbrain between different vertebrate species, we sought to determine whether *vHnf1* function, initially elucidated in zebrafish, is conserved in other vertebrates. To this end, we first characterized the expression pattern of *vHnf1* in chick at early stages of neural tube development, and analyzed its role in caudal hindbrain patterning by gain-of-function experiments using in ovo electroporation. In addition, we analyzed its possible interaction with the FGF signaling pathway. We show that *vHnf1* promotes caudal hindbrain identity along with FGF signals, by activating *MafB* and *Krox20* expression in prospective r5/6 and r5, respectively, and downregulating *Hoxb1*. Blockade of FGF signals correlates with a selective loss of *MafB* and *Krox20* expression, without affecting the expression of *vHnf1, Fgf3*, or *Hoxb1* in the hindbrain. Surprisingly, *vHnf1* is also a very efficient activator of *Fgf3* expression. These data demonstrate an early requirement for *vHnf1* and FGF function in hindbrain patterning and suggest differences in the progressive specification of the caudal hindbrain in different vertebrate species.

RESULTS

vHnf1 Is Expressed in the Neural Plate in a Segment-Restricted Manner

vHnf1 is transiently expressed in the posterior hindbrain and neural tube in both mouse and zebrafish (Barbacci et al., 1999; Sun and Hopkins, 2001). We extended these observations by undertaking a detailed analysis of *vHnf1* expression during early chick neurulation. In situ hybridization (ISH) experiments revealed a dynamic expression pattern of *vHnf1*. *vHnf1* was first expressed at the end of gastrulation at the one somite stage (0–1 ss) in the posterior neural plate (Fig. 1a) with a sharp anterior border of expression. During early segmentation stages, *vHnf1* expression persisted in the caudal most part of the hindbrain until 10–11 ss (Fig. 1a–c,g, and data not shown). Then, it was expressed in the lateral plate mesoderm, and expression in the neuroepithelium ceased (results not shown). At early stages of embryonic development, *vHnf1* expression was restricted to the neuroepithelium and was absent in the notochord and floor

plate (see transverse sections in Fig. 1d–f). In zebrafish, *vHnf1* is expressed in the caudal neural plate at early somite stages, with a rostral limit that lies at the prospective r4/r5 boundary (Lecaudey et al., 2004). The anterior limit of *vHnf1* expression was also very sharp in the chick, as shown in Figure 1a–c, suggesting a segment-restricted expression profile.

To accurately determine the position of the rostral limit of *vHnf1* expression, we performed double ISH with different hindbrain markers. First, we used probes for *vHnf1* and *Wnt8c*, a gene that is conventionally used to identify pre-r4 (Hume and Dodd, 1993). To check whether *Wnt8c* and *vHnf1* expression territories were actually adjacent, we performed single ISH for *Wnt8c* (Fig. 1h,k) and one-color (Fig. 1j,m) and two-color (Fig. 1i,l) double ISH for *Wnt8c* and *vHnf1* using NBT/BCIP (blue) and INT/BCIP (red) staining, and followed the expression of the two genes. No gap was observed between *Wnt8c* and *vHnf1* expression domains before 3–4ss (Fig. 1i,j). However, by the 5–6ss, the anterior border of *vHnf1* expression was found to be posterior to *Wnt8c* (single- and double-colored ISHs with *Wnt8c* and *vHnf1* probes, Fig. 1l,m). Note the gap that emerged between *Wnt8c* and *vHnf1* expression domains in the flat mounted neural tube preparations from 5–6ss embryos when compared with those from 3–4ss embryos (Fig. 1j,m). Since *Wnt8c* is not expressed in r4 beyond 10ss, and *Hoxb1* is not yet singularized in r4 at that stage, it was not possible to follow the precise correspondence between *vHnf1* expression and the posterior boundary of r4 at later stages (data not shown).

Further insight into the spatial regulation of *vHnf1* in r5 was obtained by comparing *vHnf1* and *Krox20* (Fig. 1n). *Krox20* is expressed at around 4–5ss in pre-r3, and by 8ss a second more caudal band of sparse cells activate *Krox20* expression in r5 (Giudicelli et al., 2001). We performed two-color double ISH with *Krox20* and *vHnf1* genes. At 5–6ss, *Krox20* expression is only detectable in r3 (Giudicelli et al., 2001 and data not shown). As shown in Figure 1n, in 10ss embryos as *Krox20* expanded in r5, *vHnf1* expression decreased in that rhombomere, suggesting that *vHnf1* regressed from r5 as described in zebrafish (Lecaudey et al, 2004).

In summary, *vHnf1* was expressed in the prospective hindbrain at early neurula stages, with an anterior limit of expression lying at the prospective r4/r5 boundary. However, by the 9–10ss *vHnf1* expression regressed caudally and disappeared in r5 coincident with the onset of *Krox20* expression in r5, suggesting that the action of *vHnf1* on r5-cells may be transient and stage-specific.

Ectopic Expression of *vHnf1* in the Hindbrain Neuroepithelium Confers Caudal Identity to More Anterior Hindbrain Regions

In order to perform gain-of-function studies of *vHnf1*, we constructed a series of expression vectors that allowed the expression of a bicistronic mRNA under the control of the β-actin promoter. In the vHnf1 expression vector *vHnf1-GFP*, the first cistron encoded the mouse *vHnf1* cDNA and the second the Green Fluorescent Protein (GFP); the two cistrons were separated by an internal ribosome entry site (IRES) (see Experimental Procedures section). Controls were performed using the pIRES-GFP vector or a vector containing a form of *vHnf1* with a Q136E substitution in the POU-specific domain that completely abolishes DNA-binding (see Experimental Procedures section; Barbacci et al., 2004). Embryos were electroporated just before the formation of the rhombomeres at 3–4ss (HH8) and analyzed after 16–22 hr, at approximately 16ss (HH12). Before analysis, electroporated embryos were screened for GFP expression under a fluorescence microscope.

Since *vHnf1* is expressed in the caudal hindbrain up to the prospective r4/r5 boundary, we assessed the consequences of *vHnf1* misexpression in more anterior rhombomeres. Electroporation of the *vHnf1* expression construct induced the appearance of *Krox20*-positive cell patches in r4 (Fig. 2d–f; n = 15/23). Neither the vector alone nor the mutated *vHnf1* construct had any effect on *Krox20* expression (Fig. 2a–c and data not shown; n = 10/10).

Somewhat surprisingly, *vHnf1*-electroporated embryos exhibited cell patches that did not express *Krox20* in r3 and r5 (Fig. 2d,e), where it is normally expressed. We performed double ISH with *mvHnf1* and *Krox20* to elucidate whether the *Krox20*-negative patches were indeed expressing *mvHnf1*. As shown in Figure 2f, many *mvHnf1*-positive cells in r3 and r5 (Fig. 2f in red) did not express *Krox20* (Fig. 2f in blue) (n = 6/6, see arrow). On the other hand, some of the ectopic *Krox20*-positive cell patches in r4 were negative for *vHnf1* (Fig. 2f, see arrowhead). Thus, misexpression of *vHnf1* resulted in: (1) an ectopic cell- and non-cell autonomous *Krox20* expression in r4, and (2) cell-autonomous downregulation of *Krox20* expression in r3 and r5. However, it is possible that low levels of expression of one of the genes could not be detected due to high expression levels of the other, or that high levels of vHNF1 protein exerted non-specific effects.

MafB is normally expressed in the caudal hindbrain from 5–6 ss, in prospective r5 and r6, and it is known to be involved in hindbrain segmentation and in specification of AP regional identity (Eichmann et al., 1997; Cordes and Barsh, 1994; Giudicelli et al., 2003; Mechta et al., 2003). Misexpression of *vHnf1* caused a rostral expansion of *MafB* expression (Fig. 2g–I). Distinct *MafB*-positive patches were always observed within r3 and r4 (Fig. 2g–i, n = 16/20), and it is possible that other ectopic patches with low levels of *MafB* expression were masked by the high levels of electroporated *mvHnf1* (Fig. 2h). Ectopic *MafB* expression levels were always equivalent to those of the endogenous gene.

To further explore the disruption of rhombomere identity caused by *mvHnf1* misexpression, we analyzed the expression of *Hoxb1*, which is a major determinant of r4 identity (Studer et al., 1994). At the stages under study (16ss), *Hoxb1* is evenly expressed at high levels in r4, as well as at lower levels in r7/r8 and the spinal cord (Sundin and Eichele, 1990). *vHnf1* electroporation resulted in downregulation of *Hoxb1* in r4, as revealed by the appearance of patches of cells in r4 that did not express *Hoxb1*

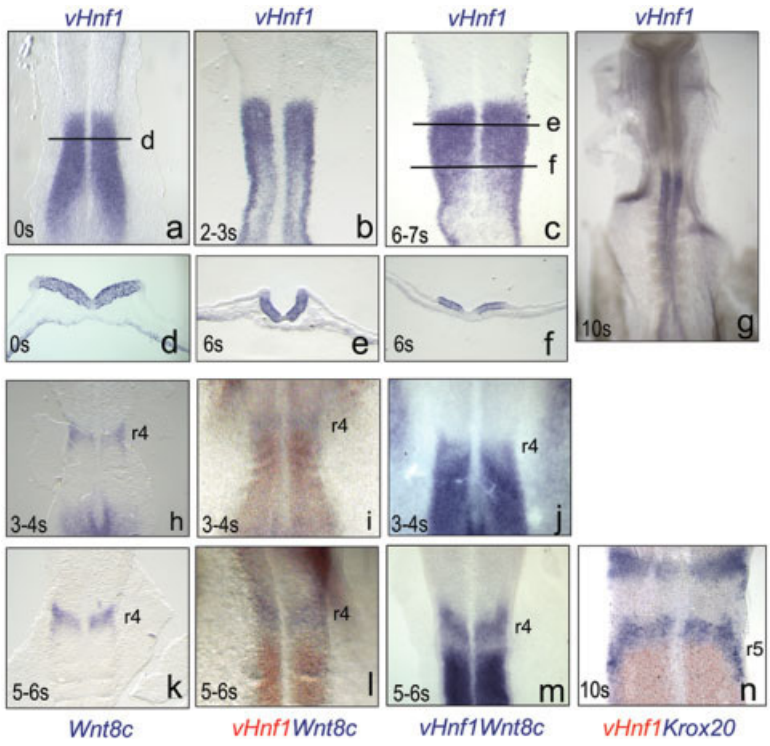

Fig. 1.

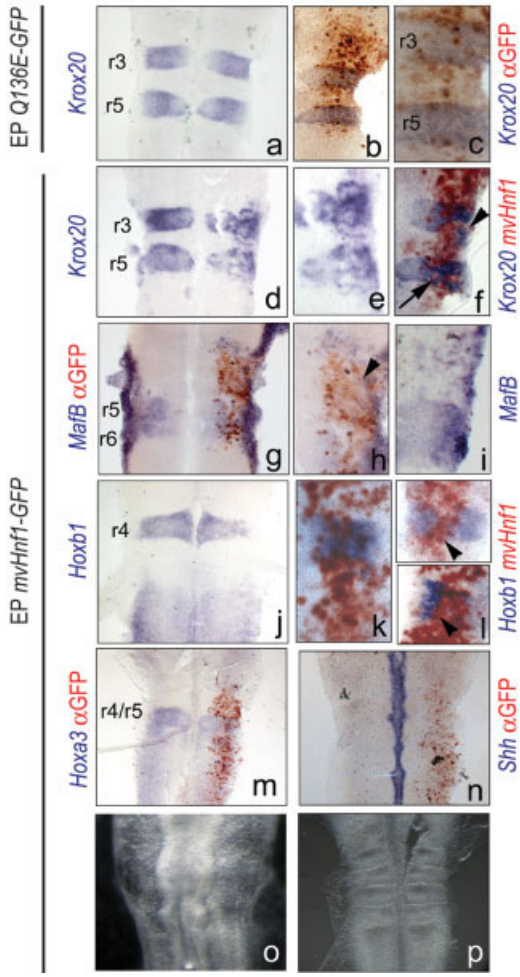

Fig. 2.

(Fig. 2j, n = 6/7), or more severe and homogeneous downregulation of *Hoxb1* expression (Fig. 2k,l, see arrowheads). Since the repression of *Hoxb1* occurred within the *mvHnf1*-electroporated region Fig. 2k,l), it is unlikely to be a direct repression by *vHnf1* as it was proposed in zebrafish (Wielette and Sive, 2003; Hernandez et al., 2004).

Hoxa3 is normally expressed in the caudal hindbrain up to the r4/r5 boundary (Grappin-Botton et al., 1995). As shown in Figure 2m, *Hoxa3* expression was not significantly altered following misexpression of *vHnf1* (n = 8/8). *Hoxa3* has been shown in mice to be a direct transcriptional target of *MafB* in r5 and r6 (Manzanares et al., 2001). However, misexpression of *MafB* by electroporation in chick leads to a weak ectopic activation of *Hoxa3* in r3 (Giudicelli et al., 2003). In our experiments, ectopic expression of *vHnf1* in r3 and r4 did

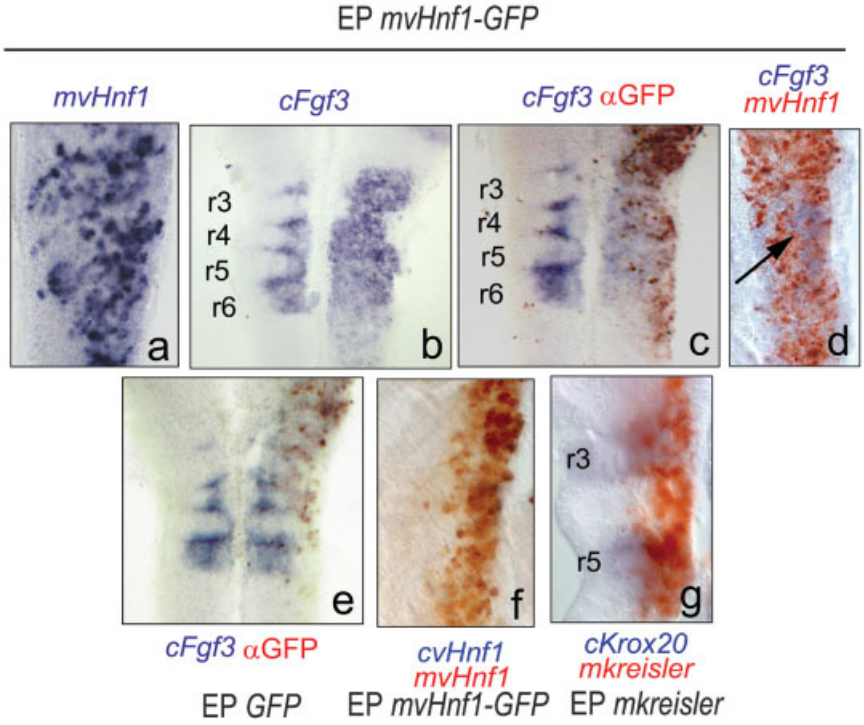

Fig. 3.

not lead to the ectopic activation of *Hoxa3* in r3 (Fig. 2m). This may be because the level of ectopic *MafB* in r3 following *vHnf1* misexpression is insufficient to activate *Hoxa3* transcription, or that *vHnf1* alone is not sufficient to change the complete identity of r4.

To assess whether the above changes in rhombomeric molecular markers were followed by disruption of the morphological segmentation process, we analyzed a marker that gave us some morphological rhombomeric landmarks. In situ hybridization for *Shh* labels the floor plate and allows visualization of the rhombomeric swellings in the ventral part of the neural tube. As shown in Figure 2n, no gross morphological effects were observed in response to *vHnf1* misexpression. Nomarski analysis of whole mount and flat-mounted hindbrains electroprated with *vHnf1* showed well-defined morphological rhombomeric boundaries (Fig. 2o,p).

In summary, ectopic expression of *vHnf1* led to changes in rhombomere identity manifested by the ectopic activation of *MafB* and *Krox20* in more anterior regions, and to the repression of *Hoxb1* in r4.

vHnf1 Upregulates *Fgf3* Expression Throughout the Hindbrain

Previous reports have addressed the importance of *fgf3* and *fgf8* in organizing the hindbrain in zebrafish (Maves et al., 2002; Walshe et al., 2002; Wiellette and Sive, 2004). In mice, *Fgf3* is expressed in r5 and r6 under the control of *MafB* (McKay et al., 1994), suggesting that the role of FGFs may differ among vertebrate species. This prompted us to examine whether FGF signaling could mediate the ectopic activation of *MafB* and *Krox20* in chick. Thus, we analyzed the effect of misexpressing *vHnf1* on *Fgf3* expression. In the chick, *Fgf3* is normally expressed in r4 and r5 from late streak stage and expands to r6 during neurulation (from 15ss), later becoming restricted to rhombomere boundaries (Wilkinson et al., 1988; Mahmood et al., 1995; data not shown). Misexpression of *vHnf1* anterior to the r4/r5 boundary led to a widespread upregulation of *Fgf3* anterior to r4, up to the r1/r2 boundary (Fig. 3b–d). *Fgf3* expression became homogeneous within the electroporated rhombomeres (Fig. 3b–d, n = 18/21), its characteristic boundary-restricted pattern at that stage being masked by the high levels of ectopic *Fgf3* expression. Electroporation of the vector alone or the mutated form of vHNF1 did not have any effect on *Fgf3* expression (Fig. 3e).

Thus, misexpression of *vHnf1* resulted in an expansion of *Fgf3* along the hindbrain, with concomitant loss of its boundary restricted expression pattern.

It is worth noting that the effects described were observed when electroporation was performed between 3–4ss and 7ss (stages HH8 and HH9, respectively). When electroporation was performed after 10ss, no alteration in the expression of hindbrain segmentation genes was observed (results not shown). This suggested a precise temporal window for *vHnf1* function, a possibility consistent with its fleeting expression in r5.

Cell-Autonomous and Non-Cell-Autonomous Effects of *vHnf1* Exogenous Expression

A common characteristic of the changes in gene expression (either activation or repression) following ectopic expression of *vHnf1* was their occurrence in patches of cells (Fig. 3a). Because the electroporation is expected to hit isolated cells, the existence of such patches may be explained by clonal expansion of a single electroporated cell, by non-cell-autonomous modifications of gene expression around the transfected cells, or by cell movements.

To address that more directly, we performed double-labeling experiments to detect both exogenous *vHnf1* and possible target genes. mvHNF-GFP was detected with an anti-GFP antibody developed with INT-BCIP (red) and *cFgf3* was detected with a riboprobe developed with NBT/BCIP (blue). Double-labeling experiments in *vHnf1*-electroporated embryos indicated that the GFP (and thus, likely the mouse vHNF1 protein) was not present in all cells expressing *cFgf3*. mvHNF1 was always expressed in cells that were within or bordering *Fgf3*-positive patches (Fig. 3c,d see arrow, n = 9/9). We also analyzed expression of *MafB* and *vHnf1* by combination of ISH for *MafB* and immunodetection of GFP. As with *Fgf3*, exogenous vHNF1 was always detected in cells that were either within or/and surrounding the *MafB*-positive domains (Fig. 2g,h, see arrow-

Fig. 1. Expression profile of *vHnf1* during early chick embryogenesis. In situ hybridization with *cvHnf1* shown in flat-mounts (**a–c**) or whole mount (**g**); panels show dorsal views. Transverse sections of embryos shown in a and c are shown in **d** and **e, f**, respectively. Note that *vHnf1* is expressed in the neuroectoderm. Flat-mounted single (**h,k**) and double (**i,j, l–n**) whole-mount in situ hybridization showing dorsal views. *Wnt8c* is shown in blue and *vHnf1* in red in two-color double in situ hybridization (i) and (l), or both in blue in one-color double in situ hybridization (j) and (m). *Krox20* is shown in blue and *vHnf1* in red in two-color double in situ hybridization (n). Anterior is to the top. Somite stages are indicated at the bottom left.

Fig. 2. Effects of misexpression of *vHnf1* on hindbrain patterning. Microinjection and electroporation of cDNA constructs was performed at 4–7ss and embryos were incubated overnight until 16–18ss. Efficiency of electroporation was assessed by visualization of GFP expression under the fluorescence microscope (not shown). Embryos of 16 ss after electroporation with the mutated form of *mvHnf1* named *Q136E-GFP* (**a–c**), or with *mvHnf1-GFP* (**d–p**), were assayed for in situ hybridization for *cKrox20* (a–f), *cMafB* (g–i), *cHoxb1* (j–l), *cHoxa3* (m), and *cShh* (n). Whole-mount in situ hybridization with the genes of interest was revealed in blue and immunostaining with anti-GFP in red (b, c, g, h, m, n), or double in situ hybridization with *cKrox20* (f) or *cHoxb1* (k, l) in blue and *mvHnf1* in red. Nomarski analysis of whole-mount (o) or flat-mounted (p) embryos at 16 and 25ss, respectively, shows that rhombomeric boundaries are not affected. In all cases, the electroporated side is the right one, and anterior is to the top. Images show flat-mounted hindbrains at 20× (a, b, d, f, g, j, m, n), 40× (e, h, i), or 63× (c, k, l, o).

Fig. 3. *cFgf3* is upregulated in response to misexpression of *vHnf1*. Microinjection and electroporation of cDNA constructs was performed at 4–7ss and embryos were incubated overnight until they reached 14ss. Whole-mount in situ hybridization with *cFgf3* was revealed in blue (**a–e**) and immunostaining with anti-GFP in red in c, e. Embryos are shown at 14ss following electroporation with *mvHnf1-GFP* (a–d, **f**), *GFP* (e), or *mkreisler* (**g**). Flat-mounted two-color ISH with *cvHnf1* (blue) in embryos electroporated with *mvHnf1* (red) is shown in f, or with *cKrox20* (blue) in embryos electroporated with *mkreisler* (red) in g. Anterior is to the top. The electroporated side of the embryos is to the right.

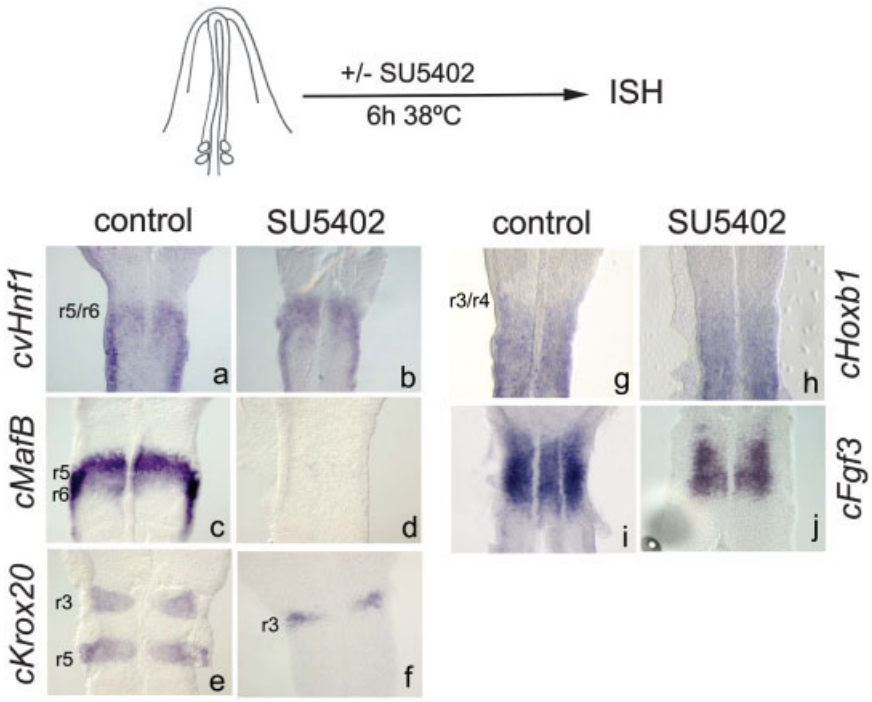

Fig. 4 FGFs signals are involved in hindbrain segmentation. Embryos were placed in culture at 2ss (**a,b**) or 3–4ss (**c–j**), according to the scheme shown in the top panel, and assayed for expression of *cvHnf1* (a,b), *cMafB* (c,d), *cKrox20* (e,f), *cHoxb1* (g,h), and *cFgf3* (l,j) by in situ hybridization. Embryos were cultured in DMEM containing 1%FCS in the absence (a, c, e, g, i) or presence (b, d, f, h, j) of SU5402. All panels show flat-mounted hindbrains from explanted embryos. Anterior is to the top.

head). Experiments using double ISH (for *Fgf3* or *MafB*, and *mvHnf1*) yielded similar results to the ones obtained with anti-GFP (data not shown). This reinforces the notion that ectopically expressed *vHnf1* could induce gene expression in both a cell- and non-cell-autonomous manner.

Another possible explanation for these results is that these cell patches expressed the endogenous *vHnf1* gene at stages when they should not, due to an autoregulatory *vHnf1* loop. To address this question, we performed double in situ hybridization experiments with the chicken *vHnf1* probe in blue and the mouse *vHnf1* probe in red, in embryos that had been electroporated at 2–3ss when *vHnf1* is expressed in the entire caudal hindbrain (Fig. 1b). As observed in Figure 3f, cells expressing the exogenous *mvHnf1* gene in the hindbrain did not activate the endogenous gene in that region (n = 5/5), the intermediate mesoderm that normally expresses *vHnf1* being a positive control for endogenous expression (not shown). Further studies will be required to unravel the mechanisms that control *vHnf1* expression in more detail.

We assessed whether the ectopic expression of *Krox20* after *vHnf1* misexpression was under the control of ectopic *MafB* by electroporation of *mkreisler/MafB* followed by analysis of *Krox20* expression. Double ISH experiments showed that ectopic expression of *mkreisler* (shown in red in Fig. 3g) did not activate *Krox20* expression (shown in blue in Fig. 3g; n = 9/9) as had been previously reported (Giudicelli et al., 2003). This suggests that *Krox20* expression in the chick is controlled by *vHnf1* independently of *kreisler/MafB*. Similarly, misexpression of *mKrox20* does not cause ectopic expression of *MafB* (Giudicelli et al., 2001 and results not shown). These experiments raised the question of the nature of the cell-to-cell signals that mediated the effects of *vHnf1*, the FGF pathway being the most attractive (see below and Marín and Charnay, 2000).

To further assess whether the FGF signaling pathway was involved in ectopic *Krox20* and *MafB* activation upon *vHnf1* misexpression, we performed a series of experiments of organotypic explants cultures using inhibitors of the FGF signaling pathway. To date, only *Fgf3* and *Fgf4* among the whole FGF family are expressed in the neural tube at those early stages (Shamim and Mason, 1999; and results not shown).

To determine whether *vHnf1, Krox20, MafB*, and *Hoxb1* were regulated by FGF signals, we explanted 1–2ss embryos and cultured them in

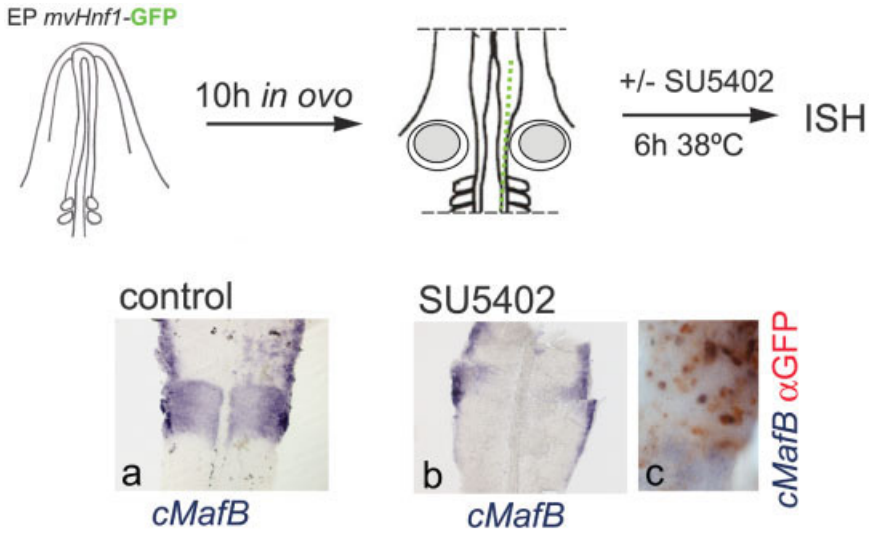

Fig. 5. Ectopic *MafB* after *mvHnf1*-electroporation requires the integrity of FGF signals. Embryos were electroporated with an expression construct for *mvHnf1*, explanted after 10 hr grown in ovo, and allowed to grow in culture in the presence (**b,c**) or absence (**a**) of 50 μM SU5402 for 6 hr, as depicted in the top panel. Embryos were assayed for in situ hybridization with *cMafB* (a–c) and immunostaining with anti-GFP (c). Electroporated side is the right one and anterior is to the top. All panels show flat-mounted hindbrains from explanted embryos.

the presence or absence of SU5402, a blocker of FGF receptor signaling (Marín and Charnay, 2000). No effects on *vHnf1* expression were detected in the presence of SU5402 as shown in Figure 4a,b (n = 7/7). These results indicate that expression of *vHnf1* does not rely on the FGF pathway. However, a clear inhibition of *MafB* was observed in SU5402-treated explants (Fig. 4c,d) (n = 9/9). Similar results were obtained for *Krox20* expression, which was dramatically reduced in response to inhibition of FGF pathway (Fig. 4e,f) (n = 9/9). In contrast, *Hoxb1* expression was not affected by FGF receptor blockade (Fig. 4g,h) (n = 3/3). Inhibition of FGF signaling did not have an observable effect in *Fgf3* expression (Fig. 4i,j, n = 10/10) suggesting that FGF3 regulation does not require an FGF loop. Next, we investigated whether ectopic *MafB* expression induced by *vHnf1*-misexpression was dependent on FGF signals. Embryos were electroporated with an *mvHnf1* expression construct, grown in ovo for 10 hr, explanted and incubated for another 6 hr with control medium or medium containing SU5402 (Fig. 5, see scheme in the top panel). Ectopic expression of *MafB* was inhibited in embryo explants incubated with SU5402 (compare Fig. 5a with b,c), indicating that the effects of *vHnf1* required an intact FGF signaling pathway.

These data suggest that the ectopic expression of *MafB* and *Krox20* in response to *vHnf1* misexpression could occur through a neural tube mediator that depends on a FGF signal.

DISCUSSION

In this study, we have investigated the function of *vHnf1* in hindbrain patterning in the chick embryo, along with the possible involvement of FGF signals in this process. We show that *vHnf1* is expressed in the neural tube from very early stages of neural development. Gain-of-function experiments show that *vHnf1* is able to activate *Krox20* and *MafB* in more anterior hindbrain regions, and to repress *Hoxb1* in r4. Surprisingly, ectopic *vHnf1* induces an expansion of the *Fgf3* expression domain within the hindbrain. Blockade of FGF signaling correlates with a selective loss of *MafB* and *Krox20* expression without affecting the expression of *vHnf1, Fgf3*, and *Hoxb1* in the hindbrain. Based on these observations, we propose that *vHnf1* promotes caudal hindbrain identity along with FGFs signals. These data demonstrate an early requirement for *vHnf1* and FGF in chick hindbrain patterning and underlie the differences between distinct vertebrates in the regulatory hierarchy leading to caudal hindbrain patterning.

vHnf1 and the Specification of AP Regional Identity in the Hindbrain

We show that *vHnf1* is expressed in the neural tube of the chick in a segment-restricted manner, up to the r4/r5 boundary, at early stages of neural embryonic development. Coincident with the onset of *Krox20* expression in r5 cells, *vHnf1* expression regresses posteriorly, suggesting that the action of *vHnf1* in r5-cells is transient and stage-specific. These data are in agreement with previous observations in zebrafish that show that the anterior border of *vHnf1* lies at the r4/r5 boundary (Sun and Hopkins, 2001; Wiellette and Sive, 2002; Lecaudey et al., 2004).

Using a gain-of-function approach, we demonstrate that ectopic expression of *vHnf1* in the hindbrain disrupts the molecular properties of rhombomeres rostral to the *vHnf1* expression domain (anterior to r5), forcing them to acquire some, but not all, of the molecular characteristics of r5 or r6. The expression of *Krox20* was ectopically activated in r4, and that of *MafB* was ectopically activated in r2, r3, and r4. In addition, vHNF1 seemed to have a dual action on *Krox20*: in the normally non-expressing *Krox20* domains such as r4, where *Krox20* expression is induced by *vHnf1*, and in the normotopic *Krox20*-expression domains r3 and r5 where it is downregulated. Concerning this latter effect, we cannot exclude that a high level of *vHnf1* transcripts leads to an unspecific suppression of *Krox20* expression. Ectopic expression of *vHnf1* also leads to a repression of *Hoxb1* in r4. This repression is consistent with the zebrafish data. However, it occurs within a more restricted domain than the area in which *mvHnf1* is misexpressed; consequently, unlike the situation in zebrafish, downregulation of *Hoxb1* does not seem to be due to a direct repression by *vHnf1* (Wielette and Sive, 2003; Hernandez et al., 2004). A possibility is that inhibition of *Hoxb1* expression in r4 is the result of abnormal activation of *Krox20* in this rhombomere. Indeed, *Krox20* electroporation experiments in the chick neural tube showed that *Krox20* is able to repress *Hoxb1* expression (Giudicelli et al., 2001).

Taken together, these data show that in chick, as in zebrafish, *vHnf1* is involved in the acquisition of caudal hindbrain (r5–r6) identity.

FGF Is Involved Downstream of *vHnf1* in Caudal Hindbrain Specification

Unexpectedly, our ectopic expression results show a striking positive regulation of *Fgf3* expression, resulting from cell-autonomous and non-cell-autonomous effects. Data from zebrafish show that *fgf3* and *fgf8* are required in r4 for the expression of *krx20* and *val* in r5 and r5–r6, respectively (Maves et al., 2002; Walshe et al., 2002). Furthermore, *vHnf1* promotes r5 and r6 identity in synergy with FGF3/8 signals (Wiellete and Sive, 2003; Hernandez et al., 2004). In chick, *Krox20* and *MafB* can be ectopically induced by FGF beads at later stages of hindbrain development (Marín and Charnay, 2000), suggesting that Fgfs are involved in *MafB* activation by *vHnf1*. Our results from experiments using organotypic explants support this hypothesis. When FGF signaling is blocked by a chemical agent such as SU5402, *MafB* and *Krox20* expression is almost completely abolished, with the remaining expression of *Krox20* in r3 most probably due to the fact that transcription of the gene had already been initiated in r3 when the embryos were explanted. Moreover, incubation with SU5402 of *vHnf1*-electroporated embryos, prevents the appearance of ectopic *MafB* patches, supporting the hypothesis that in *vHnf1*-electroporated embryos, the *MafB*-positive patches occur as a result of ectopic *Fgf3* expression. The partial non-cell autonomy of *MafB* and *Krox20* ectopic activation in response to *vHnf1*

may, therefore, be explained by the involvement of an FGF signal in this process.

The only FGFs known to be expressed in the chick neural tube at those stages are *Fgf3* (Mahmood et al., 1995; and results not shown) and, at a significantly lower level, *Fgf4* (Shamim and Mason, 1999; and results not shown). In addition, in this organism there is a short time lapse in which *Fgf3* and *MafB*/*kreisler* overlap in the presumptive r5, *Fgf3* expression appearing prior to *MafB* signal (data not shown). Although we cannot exclude the possibility that other FGFs are involved, the absence of *Fgf4* upregulation in response to misexpression of *vHnf1* (data not shown) makes FGF3 the most likely candidate to be involved in the regulation of *MafB* and *Krox20* expression.

So far, we do not know whether *vHnf1* and *Fgf3* work in synergy to activate *MafB* and *Krox20*, as it was proposed in zebrafish. However, our preliminary data are not in favor of this hypothesis. Indeed, we were unable to see significant differences in ectopic *MafB* expression between embryos electroporated with *mvHnf1* alone and co-electroporated with *mFgf3* and *mvHnf1* (results not shown). Further experiments will be required to determine whether FGF3 can synergize with vHNF1 in the chick hindbrain.

Differences in the Molecular Mechanisms of Caudal Hindbrain Patterning Among Different Vertebrates

While previous reports highlighted the importance of *fgf3* and *fgf8* in organizing the hindbrain in zebrafish (Maves et al., 2002; Walshe et al., 2002; Wiellette and Sive, 2004), mouse embryos carrying mutations in *Fgf3* and *Fgf10*, the two FGFs known to be expressed in the mouse hindbrain, show no defects in hindbrain segmentation (Wright and Mansour, 2003). Results on the relation between *kreisler*/*MafB*/*val* and *Fgf3* in zebrafish and mice are somewhat complex and contradictory. In *val* zebrafish mutants, *fgf3* expression is expanded caudally in the hindbrain, suggesting that *val* (*MafB* ortholog) normally represses *fgf3* in r5 and r6 (Kwak et al., 2002). In contrast, experiments in the mouse suggest a positive regulation of *Fgf3* by the mouse ortholog *kreisler* (McKay et al., 1996). Here we show another difference between the chick and the zebrafish in caudal hindbrain patterning: in chick, *Fgf3* is a downstream target of *vHnf1* and is strictly required for the activation of *MafB* and *Krox20* in response to *vHnf1*. Altogether, these results show that although conservation of general mechanisms exists, conservation of particular gene networks for hindbrain patterning is not strict among vertebrates.

EXPERIMENTAL PROCEDURES

Fertilized hens' eggs (Granja Gibert, Tarragona, Spain) were incubated at 38°C for the designated times and embryos were staged according to Hamburger and Hamilton (1951). Embryos were dissected from the yolk and fixed by immersion in 4% paraformaldehyde in phospate-buffered saline (4%PFA/PBS) at 4°C.

Expression Constructs

A vHNF1-GFP expression construct, *vHnf1-GFP*, was prepared by inserting the 1.7-kb cDNA of the mouse vHNF1-A isoform (Cereghini et al., 1992) into the *Bam*HI site of the β-actin-pIRES2-EGFP vector. As a control in the electroporation experiments, we used either empty β-actin-pIRES2-EGFP or a Q136E mutation of vHNF1. This mutation, a Q136 to E substitution in the POU-specific domain found in a Mody5 patient, completely abolishes DNA binding (Barbacci et al., 2004). pAdRSVkreisler was as described in Giudicelli et al. (2003). pCSFGF3 was used for coelectroporation with *vhnf1-GFP*.

In Ovo Electroporation

Commercial fertilized hens' eggs were typically incubated for 26–29 hr, up to stage HH7–8, before DNA injection. Ectopic expression of *vHnf1, kreisler* or *Fgf3* within the hindbrain was generated by electroporation (Itasaki et al., 1999). DNA was resuspended at a concentration of 1 mg/ml in water and Fast Green (Sigma, St. Louis, MO) was added at a concentration of 1 mg/ml. The DNA solution was microinjected anteriorwards into the neural tube using a pulled glass capillary. A drop of M199 medium (Invitrogen, La Jolla, CA) was placed on the egg membrane and electroporation was performed using an INTRACELL electroporator with CUY611 platinum-coated electrodes (Nepagene), using the following parameters: four 50-ms pulses of 25 V at a frequency of 1 Hz. Following electroporation, M199 medium was added to the embryos and the eggs were sealed. Embryos destined for culture (see below) were incubated for a further 10 hr at 38°C and then explanted. All other embryos were incubated overnight at 38°C. Embryos were collected and fixed in 4% PFA/PBS at 4°C prior to analysis.

Whole-Mount In Situ Hybridization

In situ hybridization was performed using digoxigenin-labeled riboprobes, essentially as described previously (Wilkinson and Nieto, 1995). Digoxigenin was detected with NBT/BCIP (Roche), which generates a purple stain. For double in situ hybridization, fluorescein-labeled probes were used. INT-BCIP was used for the detection of fluorescein, generating an orange stain. The riboprobes were as follows: *mMafB*/*kreisler* (Cordes and Barsh, 1994); *cWnt8c* (Hume and Dodd, 1993); *cShh* (Levin et al., 1997); *cKrox20* (Giudicelli et al., 2001); *cMafB* (Giudicelli et al., 2003); *cHoxb1* (Guthrie et al., 1992); *cFgf3* (dEST Data Bank); and *cHoxa3* (Grapin-Botton et al., 1995). The *cvHnf1* riboprobe encompasses the entire C-terminal transactivation domain of chick *vHnf1* (796 bp) and was generated by RT-PCR using 4-somite stage chick embryo RNA and the following degenerated primers located, in the third helix of the homeodomain and in the termination codon of *vHnf1*, respectively: 5′ gag gts cgw gtc tac aac tgg tt 3′, 5′ tca cca ggc rtg rag wgg aca ctg tt 3′.

Whole-Mount Immunohistochemistry

Immunohistochemical detection of green fluorescent protein was performed in electroporated embryos using an anti-GFP polyclonal antibody at a 1/700 dilution (Molecular Probes, Eugene, OR). As a secondary anti-

body, horseradish peroxidase-conjugated sheep anti-rabbit was used at a 1/200 dilution (Dako, Carpinteria, CA). Horseradish peroxidase activity was detected using the AEC system (LabVision), which generates an orange/red stain.

Organotypic explanted embryos were prepared as described by Giraldez (1998). Briefly, 2–3 somite stage (ss) chick embryos were aseptically dissected and isolated. Explants were cultured for 6 hr in DMEM (Invitrogen) in the presence of 1% Fetal Calf Serum (Bio Whittaker) at 37°C in a humidified atmosphere containing 5% CO_2. Explants were grown either with medium alone or in the presence of 50 μM SU5402 (Calbiochem, San Diego, CA).

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