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ScienceDirect
Developmental Biology 309 (2007) 344–357
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

Genomes & Developmental Control

Direct regulation of *vHnf1* by retinoic acid signaling and MAF-related factors in the neural tube

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Received for publication 19 April 2007; revised 21 June 2007; accepted 4 July 2007
Available online 10 July 2007

Abstract

The homeodomain transcription factor vHNF1 plays an essential role in the patterning of the caudal segmented hindbrain, where it participates in the definition of the boundary between rhombomeres (r) 4 and 5 and in the specification of the identity of r5 and r6. Understanding the molecular basis of *vHnf1* own expression therefore constitutes an important issue to decipher the regulatory network governing hindbrain patterning. We have identified a highly conserved 800-bp enhancer element located in the fourth intron of *vHnf1* and whose activity recapitulates *vHnf1* neural expression in transgenic mice. Functional analysis of this enhancer revealed that it contains two types of essential motifs, a retinoic acid response element and two half T-MARE sites, indicating that it integrates direct inputs from the retinoic acid signaling cascade and MAF-related factors. Our data suggest that MAFB, which is itself regulated by vHNF1, acts as a positive modulator of *vHnf1* in r5 and r6, whereas another MAF-related factor is absolutely required for the expression of *vHnf1* in both the hindbrain and the spinal cord. We propose a model accounting for the initiation and maintenance phases of *vHnf1* expression and for the establishment of the r4/r5 boundary, based on cooperative contributions of Maf factors and retinoic acid signaling.

Keywords: Hindbrain segmentation; Pattern formation; Transcriptional enhancers; MAFB

Introduction

The vertebrate hindbrain is subjected to a transient segmentation process along the anteroposterior (AP) axis that results in the establishment of a series of 7/8 metameric transversal territories called rhombomeres (r) (Lumsden and Keynes, 1989; Lumsden and Krumlauf, 1996). This subdivision presages the stereotyped pattern of neuronal differentiation in the hindbrain (Clarke et al., 1998; Lumsden and Keynes, 1989). It also underlies the pathways of neural crest cell migration into the branchial arches and participates in their patterning (Ghislain et al., 2003; Lumsden et al., 1991; Serbedzija et al., 1992; Trainor and Krumlauf, 2000; Trainor et al., 2002 and references therein), thus playing an essential role in craniofacial morphogenesis.

Numerous genes, including a large proportion of transcription factor genes, have been shown to present evolutionarily conserved, restricted patterns of expression along the AP axis, with limits corresponding to prospective or established rhombomere boundaries (reviewed in Lumsden and Krumlauf, 1996), and many of them have been implicated at different levels of the segmentation process (see Chomette et al., 2006 for references). Understanding the principles governing hindbrain segmentation will clearly require to decipher the details of the regulation of these segmentally restricted genes themselves. Although data are still limited on this matter, they suggest that the initial establishment and later evolution of their expression patterns result from the combination of at least three types of inputs: morphogenetic gradients of diffusible signaling molecules such as retinoic acid (RA), Fgf and Wnt (Dupe and Lumsden, 2001; Gavalas and Krumlauf, 2000; Kudoh et al., 2002; Marin and Charnay, 2000; Nordstrom et al., 2006; Walshe et al., 2002), cross-regulations between the segmentally

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0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.07.003

expressed genes (Maconochie et al., 1997; Manzanares et al., 1997; Sham et al., 1993) and autoregulatory mechanisms (Chomette et al., 2006; Manzanares et al., 2001; Popperl et al., 1995).

vHnf1 (variant hepatocyte nuclear factor 1, also known as *Hnf1β* or *Tcf2*) is one of these evolutionarily conserved, segmentally restricted genes, playing an essential role in hindbrain development. It encodes a transcription factor with an atypical homeodomain, closely related to HNF1α (De Simone et al., 1991; Rey-Campos et al., 1991). In the mouse neural tube *vHnf1* expression is initiated at around embryonic day (E) 7.8 in the caudal hindbrain and spinal cord, and at E8 its anterior limit was shown to coincide with prospective r4/r5 boundary (Barbacci et al., 1999; Coffinier et al., 1999; Sirbu et al., 2005), as in other vertebrate species (Aragon et al., 2005; Lecaudey et al., 2004; Sun and Hopkins, 2001). Later this anterior limit progressively retracts posteriorly in the hindbrain and the spinal cord expression becomes restricted to the roof plate and a ventral region (Aragon et al., 2005; Barbacci et al., 1999; Coffinier et al., 1999; Lecaudey et al., 2004). Knock-out of the mouse gene was shown to be lethal around E6 due to the absence of extraembryonic visceral endoderm where *vHnf1* is also expressed (Barbacci et al., 1999; Coffinier et al., 1999). Recent work in the zebrafish has shown that impairment of *vHnf1* function leads to mis-specification of r5 and r6, which acquire r4-like identity (Hernandez et al., 2004; Sun and Hopkins, 2001; Wiellette and Sive, 2003). More precisely, vHNF1 was shown to repress an r4 fate posterior to the r4/r5 boundary, to cooperate with Fgf signals from r4 in activating *MafB* expression in r5 and r6 and to cooperate with MAFB in establishing r5 and r6 identities, including the direct transcriptional activation of *Krox20* in r5 (Chomette et al., 2006; Hernandez et al., 2004).

Substantial evidence has accumulated to implicate the RA signaling pathway in the activation of *vHnf1* expression and fixation of its anterior boundary. Depletion of RA during early embryogenesis, using mutations in the major RA synthesizing enzyme gene, *Raldh2* (Hernandez et al., 2004; Sirbu et al., 2005), or application of a specific RALDH2 molecular antagonist (Hernandez et al., 2004; Maves and Kimmel, 2005), led to almost complete abolition of *vHnf1* expression. Conversely, *vHnf1* was ectopically induced in the anterior neural tube following elevation of the endogenous RA level obtained by treatment with RA (Maves and Kimmel, 2005; Sirbu et al., 2005) or by preventing RA degradation (Hernandez et al., 2007). In addition, the fixation of the rostral limit of *vHnf1* expression and therefore of the r4/r5 boundary has also been shown to involve a mechanism of mutual repression between vHNF1 and the Iro7 transcription factor (Lecaudey et al., 2004). However it is not known whether *vHnf1* constitutes a direct target of RA signaling and/or Iro7, or whether their actions are relayed by other segmentally expressed genes. Indeed perturbation in the expression of *Hox* paralogous group 1 genes and of their associated factors genes, *Pbx* and *Meis*, have been shown to dramatically affect *vHnf1* expression in zebrafish (Choe and Sagerstrom, 2004; Waskiewicz et al., 2002) and some of these genes have been shown to be under RA control (Gould et al., 1998; Langston and Gudas, 1992; Marshall et al., 1994). Understanding the details of *vHnf1* regulation therefore requires the identification of its direct upstream regulators. In the present work, to initiate such an analysis, we have searched for the *cis*-acting regulatory elements responsible for *vHnf1* expression in the hindbrain and spinal cord. We have screened a 193-kb genomic region surrounding the mouse gene and identified a highly conserved 800-bp transcriptional enhancer element located in the fourth intron of *vHnf1* and whose activity recapitulates *vHnf1* neural expression in transgenic mice. Analysis of this enhancer revealed that it contains a conserved RA response element (RARE) essential for its activity, establishing that *vHnf1* constitutes a direct target of RA signaling. The enhancer also contains two half T-MARE motifs and their mutation abolished enhancer activity. T-MARE and half T-MARE constitute binding sites for members of the MAF family of proteins that are basic-leucine zipper (bZIP) transcription factors belonging to the AP1 superfamily (for a review see Motohashi et al., 1997, 2002). MAF proteins can homo- and heterodimerize with each other as well as with other members of the AP1 family such as Jun and Fos (Kataoka et al., 1994; Matsushima-Hibiya et al., 1998). Our data indicate that MAFB, which is itself regulated by vHNF1, acts as a positive modulator of *vHnf1* in r5 and r6 and suggest the absolute requirement of (an)other MAF-related factor(s) for the regulation of *vHnf1* in both the hindbrain and the spinal cord.

Materials and methods

DNA constructions and mutagenesis

BAC clone RPCI23-304H7 was obtained from a mouse genomic library at the Children's Hospital Oakland Research Institute (BACPAC Resources). Mouse fragments #2 to 5 were cloned by PCR from the BAC clone using primers indicated in Supplementary Table S1. Chicken fragments #8 to 12 were cloned by PCR from chicken BAC clone CH261-68C12 (BACPAC resources) using primers indicated in Supplementary Table S1. Mutagenesis of the RARE (Studer et al., 1994) in fragment #5 was performed by PCR using primers indicated in Supplementary Table S1. Mutagenesis of the MAF-binding sites (Manzanares et al., 2002) in fragment #5 was performed using the Quickchange Multi Site Directed Mutagenesis Kit (Stratagene) with primers indicated in Supplementary Table S1. Fragments #2 to 12 were cloned into pBGZ40 (Yee and Rigby, 1993) upstream of the minimal *β-globin* promoter/*lacZ* reporter gene.

Generation of transgenic mice, genotyping and in ovo electroporation

Purification of fragments #2 to 7, 10 and 11 and microinjection of fertilized mouse eggs were performed as described previously (Ghislain et al., 2002; Sham et al., 1993). Transgenic embryos were identified by PCR with primers indicated in Supplementary Table S1. BAC RPCI23-304H7 DNA was isolated using alkaline lysis and cesium chloride gradient ultracentrifugation. It was co-injected as a supercoiled plasmid in fertilized mouse eggs together with a 1.5-kb *vHnf1* minimal promoter-*lacZ* reporter fragment in equimolar amounts as described (Chomette et al., 2006). Transgenic embryos were identified by PCR using the BAC vector and minimal promoter-*lacZ* reporter-specific primers indicated in Supplementary Table S1. The *kreisler* allele (Frohman et al., 1993) was maintained in S129 background and construct #3 transgene in a mixed C57Bl6/DBA2 background. In ovo electroporation into the chick neural tube was performed as previously described (Giudicelli et al., 2001) at stages HH8–9 and embryos were collected at HH11–13 for X-gal staining. Co-electroporation