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Identification of a conserved 125 base-pair *Hb9* enhancer that specifies gene expression to spinal motor neurons

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Abstract

The homeobox gene *Hb9* is expressed selectively by motor neurons (MNs) in the developing CNS. Previous studies have identified a 9-kb 5′ fragment of the mouse *Hb9* gene that is sufficient to direct gene expression to spinal MNs in vivo. Here, we sought to identify more discrete MN-specifying elements, using homology searches between genomic sequences of evolutionarily distant species. Based on homology screening of the mouse and human *Hb9* promoters, we identified a 3.6-kb *Hb9* enhancer that proved sufficient to drive MN-specific *lacZ* expression. We then compared mouse, human, and pufferfish (*Fugu rubripes*) genomic sequences, and identified a conserved 438-bp sequence, consisting of noncontiguous 313-bp and 125-bp fragments, residing within the 3.6-kb *Hb9* enhancer. The zebrafish (*Danio rerio*) *Hb9* genomic region was then found to have two identical copies of the 125-bp sequence, but no counterpart for the 313-bp sequence. Transgenic analysis showed that the 125-bp alone was both necessary and sufficient to direct spinal MN-specific *lacZ* expression, whereas the 313-bp sequence had no such enhancer activity. Moreover, the 125-bp *Hb9* enhancer was found to harbor two *Hox*/*Pbx* consensus-binding sequences, mutations of which completely disrupted thoracolumbar *Hb9* expression. These data suggest that *Hox*/*Pbx* plays a critical role in the segmental specification of spinal MNs. Together, these results indicate that the molecular pathways regulating *Hb9* expression are evolutionarily conserved, and that MN-specific gene expression may be directed and achieved using a small 125-bp 5′ enhancer.

Keywords: Motor neurons; Promoters; Spinal muscular atrophy; Amyotrophic lateral sclerosis

Introduction

In the vertebrate CNS, the specification of neural identity is initiated by humoral inductive factors, that impose a specific profile of transcription factor expression on neural progenitor cells, thereby restricting their phenotypic differentiation (Goridis and Brunet, 1999; Jessell and Melton, 1992). In the developing spinal neuroepithelium, motor neuron (MN) progenitors arise in part in response to the ventralizing action of Sonic hedgehog (Shh) (Briscoe and Ericson, 2001). The specification of MN progenitors by Shh is mediated through the patterned expression of homeodomain (HD) and basic helix–loop–helix (bHLH) transcription factors; these function primarily as transcriptional repressors (Muhr et al., 2001), whose cross-regulatory interactions establish distinct progenitor domains (Briscoe et al., 1999, 2000; Jessell, 2000; Vallstedt et al., 2001). Through this general scheme, MN progenitors are restricted to a narrow region of the ventral neural tube termed the pMN domain (Briscoe et al., 2000; Jessell, 2000; Pierani et al., 2001). Within this domain, two HD proteins, *Nkx6.1* and *Pax6*, and a bHLH protein, *Olig2*, characterize MN

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progenitors. Together, these proteins serve to initiate the expression of distinct MN transcription factors, which include the HD protein HB9.

HB9 is expressed selectively by post-mitotic spinal MNs in the developing vertebrate CNS, and serves as a marker for the MN phenotype (Arber et al., 1999; Tanabe et al., 1998). Genetic studies in mice have suggested its importance in the consolidation and maintenance of MN identity (Arber et al., 1999; Thaler et al., 1999). A 5′ 9-kb *Hb9* promoter has been shown to drive MN-specific expression in vivo (Arber et al., 1999; Wichterle et al., 2002). Nonetheless, the regulatory control of *Hb9* gene expression is only poorly understood. In this study, we sought to identify *cis*-acting regulatory elements of the *Hb9* gene specifically active in MNs, so as to predict factors that might regulate MN induction. Using cross-species homology analysis with enhancer screening by transgenesis, we identified a highly conserved 125-bp *cis*-acting regulatory sequence, which appears to direct gene expression to spinal MNs. Moreover, by site-directed mutagenesis, we found that disrupting *Hox*/*Pbx* binding sites within this 125-bp *Hb9* enhancer completely abolished *β-gal* expression in the thoracic and lumber spinal cord, without affecting the reporter gene expression in the cervical levels of the cord. Thus, the 5′ 9-kb *Hb9* promoter harbors a highly conserved 125-bp element that directs vertebrate MN gene expression, and *Hox*/*Pbx* binding sites within this element appear necessary for thoracolumbar MN specification.

Materials and methods

Homology screening

A 9-kb 5′ non-coding fragment of the mouse *Hb9* gene (a kind gift from Dr. Thomas Jessell) was sequenced. The corresponding human genome sequence was obtained from Ensembl genome browser (www.ensemble.org). Blast 2 sequences program (www.ncbi.nih.gov/blast/bl2seq/bl2.html) was used to identify conserved non-coding regions of the *Hb9* gene between mouse and human. Ensembl genome browser was also used to identify evolutionarily conserved non-coding sequences of the *Hb9* genes among mouse, rat, human, pufferfish, and zebrafish, and then multiple sequence alignments were conducted using MacVector (Accelrys).

Transgenic constructs

Construct #1 (*3.6-kb Hb9 enhancer-β-globin-lacZ*) was generated by inserting a 3.6 kb *Not*I/*Sse*8387I fragment derived from a 9-kb 5′ non-coding fragment of the mouse *Hb9* gene into the *Not*I/*Pst*I sites of the reporter construct BGZA, which contains the *β-globin* minimal promoter, *lacZ* gene, and *SV40* polyadenylation cassette (a gift of Dr. Jane Johnson). Construct #2 (*5.4-kb Hb9 promoter-lacZ*) contains a 5.4-kb *Sse*8387I/*Pme*I fragment of the 9-kb *Hb9* promoter region into the *Pst*I/*Sma*I sites of *placZpA*. Construct #3 (*438-bp Hb9 enhancer-β-globin-lacZ*) was obtained by cloning 313-bp and 125-bp fragment derived from Constructs #4 and #5, respectively, to BGZA.

Construct #4 (*313-bp Hb9 enhancer-β-globin-lacZ*): A 313-bp fragment was PCR amplified with the primers:

5′-ATAGCATAGCGGCCGCTGAATAAATTTAAGCAGGCT-3′, 5′-GCTCTAGAAGCCCCATCCCCCTTCAAT-3′,

and cloned into BGZA.

Construct #5 (*125-bp Hb9 enhancer-β-globin-lacZ*): A 125-bp fragment was PCR amplified with the primers:

5′-GACTAGTAGAGTGGTTAGCTGATGAAT-3′, 5′-TCACCCGGGTCTAATCAGCCTGCCTAGCT-3′,

and cloned into BGZA. Constructs #4 and #5 contain three copies of the 313-bp and 125-bp fragments, respectively, in order to drive the reporter gene expression more efficiently.

Construct #6: The site-directed mutagenesis construct was generated according to Ho's method (Ho et al., 1989) using the primers:

5′-ATAGCATAGCGGCCGCTGAATAATTTAAGCAGGCT-3′,
5′-T**CG**TT**C**GTTTTTGTCAA**CG**CA**CG**AGCTAACCACTCTGGCTGGA-3′,
5′-T**CG**TG**CG**-TTGACAAAAAC**G**AA**CG**AGCTTCGAGCTTTATTGGGAAACAGGT-3′,
5′-TCACCCGGGTCTAATCAGCCTGCCTAGCT-3′.

All PCR-amplified fragments were verified by sequencing.

Production and genotyping of transgenic mice

Transgenes for injection were separated from vector sequences using 1% Seakim LE agarose (BMA), purified on QIAquick gel extraction kit (Qiagen), precipitated in injection buffer. Transgenic mice were generated by standard procedures (Hogan et al., 1994) using fertilized eggs from B6D2F1 9C57BL/6 × DBA) crosses. Transgenics were identified by PCR with the *lacZ* primers 5′-CGAGTGTGATCATCTGGTCG-3′ and 5′-TTACCTTGTGGAGCGACATC-3′ using genomic DNA extracted from yolk sacs or tails.

lacZ detection

β-gal staining of whole-mount embryos

Staged embryos were dissected from the uterus in cold PBS and fixed for 45 min at 4°C in fixing solution (1%