

Analysis of embryonic motoneuron gene regulation: derepression of general activators function in concert with enhancer factors

Soo-Kyung Lee, Linda W. Jurata*, Junichi Funahashi†, Esmeralda C. Ruiz and Samuel L. Pfaff‡

Gene Expression Laboratory, The Salk Institute for Biological Studies, La Jolla, CA 92037, USA

*Present address: Psychiatric Genomics, 19 Firstfield Rd., Gaithersburg, MD 20878, USA

†Present address: Department of Molecular Neurobiology, Institute of Development, Aging and Cancer, Tohoku University, Seiryomachi 4-1, Aoba-ku, Sendai 980-8575, Japan

‡Author for correspondence (e-mail: pfaff@salk.edu)

Development 131, 3295-3306
Published by The Company of Biologists 2004
doi:10.1242/dev.01179

Accepted 22 March 2004

Summary

The underlying transcriptional mechanisms that establish the proper spatial and temporal pattern of gene expression required for specifying neuronal fate are poorly defined. We have characterized how the *Hb9* gene is expressed in developing motoneurons in order to understand how transcription is directed to specific cells within the developing CNS. We found that non-specific general-activator proteins such as E2F and Sp1 are capable of driving widespread low level transcription of *Hb9* in many cell types throughout the neural tube; however, their activity is modulated by specific repressor and activator complexes. The general-activators of *Hb9* are suppressed from triggering inappropriate transcription by repressor proteins *Irx3* and *Nkx2.2*. High level motoneuron expression is achieved by assembling an enhancesome on a

compact evolutionarily-conserved segment of *Hb9* located from –7096 to –6896. The ensemble of LIM-HD and bHLH proteins that interact with this enhancer change as motoneuron development progresses, facilitating both the activation and maintenance of *Hb9* expression in developing and mature motoneurons. These findings provide direct support for the derepression model of gene regulation and cell fate specification in the neural tube, as well as establishing a role for enhancers in targeting gene expression to a single neuronal subtype in the spinal cord.

Supplemental data available online

Key words: Motoneuron, Development, Hb9, Enhancer, Gene regulation, Derepression

Introduction

The circuits that control behavior comprise numerous subtypes of neurons that perform distinct functions based on their physiological properties and their pre- and postsynaptic connections. Therefore many developmental studies of the CNS have examined how the process of neuronal diversification occurs, with particular attention given to the identification of transcriptional networks that control gene expression (Goulding et al., 2002; Jessell, 2000; O'Leary and Nakagawa, 2002; Rubenstein and Rakic, 1999; Shirasaki and Pfaff, 2002). Nevertheless, the underlying regulatory mechanisms that direct gene expression to the proper neuronal subtypes at the correct time in development remain poorly characterized. To address this issue, we have examined how the *Hb9* gene is properly activated in newly formed spinal motoneurons while being excluded from neighboring cell populations in the developing spinal cord.

Motoneurons form synapses with muscles and directly mediate the control of locomotion, whereas a variety of different types of spinal cord interneurons coordinate and modulate motoneuron activity (Butt et al., 2002; Sharma and Peng, 2001). Motoneurons and individual locomotor-interneuron classes emerge from distinct progenitor cell domains along the dorsoventral axis of the neural tube. Progenitor cells in each domain are specified by graded sonic hedgehog (Shh) signaling (Ericson et al., 1997), leading to the

expression of unique combinations of homeodomain and basic helix-loop-helix (bHLH) transcription factors (Briscoe et al., 2000; Mizuguchi et al., 2001; Novitsch et al., 2001).

These findings have raised the question of how cells interpret small differences in Shh at progenitor cell boundaries where signaling is close to the threshold for two alternative differentiation pathways. One mechanism that contributes to the accurate assignment of cell fate is a process of cross-inhibitory transcriptional interactions between factors from different domains (Briscoe et al., 2000; Muhr et al., 2001). Many of the progenitor factors contain an eh1 motif homologous to the Engrailed transcription factor (Muhr et al., 2001), which serves as a docking site for Gro/TLE corepressors that recruit histone deacetylases associated with chromatin modifications that reduce transcription (Chen et al., 1999). Interactions with Gro/TLE corepressors not only mediate cross-repression, but are also necessary for the progenitor factors to specify which types of neurons are generated (Briscoe et al., 2000; Muhr et al., 2001). Taken together, these observations have suggested that gene regulation is controlled in specific areas of the developing neural tube by cell-type specific repressors acting to inhibit general activators of transcription (Muhr et al., 2001) (reviewed by Lee and Pfaff, 2001). Thus, the genes expressed by neuronal subtypes are predicted to be transcribed through a process of 'derepression', though mechanistic studies of

gene regulation in the neural tube have not directly tested this model.

The transcription factors known to be involved in the early stages of motoneuron differentiation include the homeodomain proteins Nkx6.1/6.2, Pax6 and Mnr2; and the bHLH proteins Olig1/2 and Ngn2 (Briscoe et al., 2000; Ericson et al., 1997; Lu et al., 2002; Marquardt and Pfaff, 2001; Mizuguchi et al., 2001; Novitch et al., 2001; Scardigli et al., 2001; Tanabe et al., 1998; Vallstedt et al., 2001; Zhou and Anderson, 2002). As the progenitor cells for motoneurons depart from the cell cycle and begin to differentiate, the LIM homeodomain proteins Lhx3/4 and Isl1 become expressed. This combination of LIM proteins forms a higher-order complex with the LIM co-factor nuclear LIM interactor (NLI) through cell-type specific protein-protein interactions that specifies the motoneuron fate (Thaler et al., 2002). The initiation of postmitotic motoneuron differentiation is accompanied by the downregulation of the progenitor cell factors and the selective expression of the Hb9 homeodomain protein in these neurons (Pfaff et al., 1996; Tanabe et al., 1998).

In humans, hereditary mutations of *HB9* (*HLXB9* – Human Gene Nomenclature Database) result in sacral agenesis due to haploinsufficiency (Ross et al., 1998). Null mutants of *Hb9* (*Hlxb9* – Mouse Genome Informatics) in mice reveal a crucial role for this transcription factor in motoneuron development (Arber et al., 1999; Thaler et al., 1999). Mice deficient in Hb9 generate defective motoneurons that inappropriately express V2 interneuron genes, such as *Chx10*, and fail to migrate and extend axons properly. The suppressive function of Hb9 on V2 interneuron genes is presumably required because motoneurons and V2 interneurons have a close developmental relationship and share several regulatory proteins such as Lhx3 and Lhx4, which contribute to the specification of both motoneurons and V2 interneurons (Sharma et al., 1998; Tanabe et al., 1998; Thaler et al., 2002). Consequently, when Hb9 is ectopically expressed in the adjacent population of V2 interneurons, it blocks the development of these cells (Tanabe et al., 1998). Thus, proper cell fate specification in the spinal cord is dependent upon the exclusion of Hb9 from the adjacent interneuron populations and its expression in motoneurons as the cells become postmitotic.

Studies in transgenic mice have identified a 9 kb upstream region of the *Hb9* promoter that is sufficient to target reporter gene expression to motoneurons (Arber et al., 1999; Thaler et al., 1999). We reasoned that this regulatory region of *Hb9* could be used as a substrate to characterize the molecular mechanisms responsible for directing the proper temporal and spatial pattern of gene expression to specific cell types in the developing nervous system. The combination of LIM factors Isl1 and Lhx3, together with the bHLH factors NeuroM and Ngn2 are found to bind to the *Hb9* promoter and activate its transcription (Lee and Pfaff, 2003). It remains unclear, however, whether the temporal and spatial pattern of *Hb9* is controlled entirely by motoneuron-specific positive regulators, or whether repressive mechanisms also contribute to its specific pattern of expression.

We developed an in vivo method for characterizing gene regulation using electroporation of chick neural tube cells to further characterize the mechanisms that control gene expression in the developing spinal cord. In addition to an enhancer located at –7096 to –6896 for activating high level *Hb9* expression in motoneurons, general activator proteins E2F

and Sp1 appear to interact with the proximal segment of the gene from –550 to –1 and promote non-cell-type-specific transcription. The widespread activity of the general activators of *Hb9* are inhibited in non-motoneuron cells through the use of multiple repressor proteins that interact with sites located both within and outside the enhancer region. These studies provide direct support for the hypothesis of Muhr et al. (Muhr et al., 2001), which predicts that progenitor cell transcription factors in the developing spinal cord regulate cell fate through a derepression mechanism.

Materials and methods

DNA constructs

Mouse and human *Hb9* genomic sequences (26 kb) spanning the coding exons and flanking regions were aligned using BLAST 2 and limiting nucleotide gaps to 2% or less. The sequences are numbered by defining the start site of translation as 0. A 9.2 kb 5' flanking sequence of the mouse *Hb9* gene was isolated (Arber et al., 1999; Thaler et al., 1999) and mapped using various restriction enzymes. A series of deleted 2.5 kb distal elements were generated using PCR and subcloned into the following reporter vectors: Δ *NheI* proximal-GFP, Δ *NheI* proximal-*nlacZ*, synthetic TATA-GFP, d4GFP (Clontech) and TK-luciferase (gift of R. Evans). Mutations in the M250 enhancer were introduced using a PCR-based mutagenesis method (Stratagene, QuikChange), and verified by sequencing. Rat Isl1, Hb9 and Nkx6.1; mouse Lhx3, Ngn2, Nkx2.2, Pax6, Irx3, Hb9 and E47; chick NeuroM and NeuroD were cloned into pCS2 (Turner and Weintraub, 1994) and/or pcDNA3 (Invitrogen) containing a HA, flag or myc-epitope tag. The homeodomain of Hb9 (amino acids 226-308) linked to EnR repressor domain or VP16 activation domain was cloned into pCS2-myc.

In ovo electroporation and immunocytochemistry

Chick embryos (SPAFAS, McIntyre Farms) were incubated in a humidified chamber and staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951). DNA constructs were injected into the lumens of HH stage 12-13 chick embryonic spinal cords. Electroporation was performed using a square wave electroporator (BTX) as described previously (Nakamura et al., 2000). Incubated chicks were harvested and analyzed at HH stage 20-24, fixed in 4% paraformaldehyde and cryosectioned. Immunohistochemistry was performed as described previously (Thaler et al., 1999) using the following antibodies: mouse anti-MNR2/HB9 (5C10) (Tanabe et al., 1998), rabbit anti-Hb9 (Thaler et al., 1999), rabbit anti-Isl1 (Ericson et al., 1992), rabbit anti-*lacZ* (Sigma), mouse anti-Myc (9E10, DSHB) and rabbit anti-GFP (Molecular Probes).

Generation of transgenic mice

The wild-type *Hb9* enhancer (M250) and mutated derivatives were fused upstream of the Δ *NheI* proximal element-GFP or synthetic TATA-GFP. Restriction enzymes were used to cleave the promoter+reporter fragments from the plasmids, and the purified DNA was injected into mouse oocyte pronuclei. After microinjection, the fertilized embryos were transferred into pseudo-pregnant females and embryos were removed at E11.5 for analysis. Genotyping was performed by PCR with the GFP primers: 5'-AGAAACCATGG-ACCTTGACAGCTCGT and 5'-GGTCGCCACCATGGTGAGCAA.

Cell culture and transient transfection assays

293, CV1 and P19 cells were cultured in Dulbecco's modified Eagle medium or α -minimum essential medium supplemented with 10% bovine fetal serum. Cells were seeded into 48-well plates and transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. A CMV- β -galactosidase

plasmid was co-transfected for normalization of transfection efficiency, and empty vectors were used to equalize the total amount of DNA. Cells were harvested 36 hours after transfection. Cell extracts were assayed for luciferase activity and the values were corrected with β -galactosidase activity. Data are represented as means of triplicate values obtained from representative experiments. All transfections were repeated at least three times.

Electrophoretic mobility shift assays (EMSA)

EMSA was performed as previously described (Lee and Pfaff, 2003). The sequence of the sense strands of individual oligonucleotides is shown below. M3-oligo, CTCTCAGCCTTTCAGTGGAAATT; 3'CD-A, AATAGACCAATTAGCCAGGACTTCTGCCATTATCTGCTGTAGAC; 3'CD-B, TAGACTTGGGCATTAGTACGCCCTGATTCACCAATAATTTCAAGTCA; 3'CD-C, AAGTCAGAACACACTAAAAAATTCACCTACCGAGGCATTAATAATGT. The sequence of M50, M100A and M100C has been described previously (Lee and Pfaff, 2003).

Results

The *Hb9* promoter is active in chick embryo motoneurons

In order to understand how *Hb9* becomes selectively expressed by motoneurons, we focused on the regulatory elements in the upstream 9.2 kb non-coding region of the gene, as this DNA segment is sufficient to target reporter gene expression to postmitotic motoneurons in transgenic mice (Arber et al., 1999; Thaler et al., 1999). The expression of *Hb9* in motoneurons is evolutionarily conserved in many species ranging from humans to *Drosophila* (Arber et al., 1999; Broihier and Skeath, 2002; Ferrier et al., 2001; Ross et al., 1998; Saha et al., 1997; Thaler et al., 1999; Vult von Steyern et al., 1999). Therefore, we compared the nucleotide sequence of the *Hb9* promoter region from mouse and human to identify regions of conservation that might be involved in regulating gene expression. The alignment revealed several segments of >80% nucleotide homology interspersed with regions of lower homology (Fig. 1A).

In order to determine whether these evolutionarily conserved DNA segments represented cis-elements for gene regulation we sought a method for rapid in vivo characterization of reporter construct activity. We found that the upstream 9.2 kb segment of *Hb9* active in transgenic mice was sufficient to drive GFP expression in postmitotic (*Hb9*⁺) motoneurons from chick embryos (Fig. 1C). DNA was introduced into one side of the chick embryo neural tube using in ovo electroporation (Nakamura et al., 2000). Although GFP expression was detected in a few non-motoneurons per section, the overall fidelity of gene regulation was high and >95% of the GFP-labeled cells were *Hb9*⁺ motoneurons. Because this technique for monitoring gene expression in the developing spinal cord was rapid and highly reproducible, we used it as an assay to define the cis-element sequences required for the proper spatial and temporal pattern of *Hb9* expression.

Characterization of an enhancer active in motor neurons

The evolutionarily conserved regions within the *Hb9* promoter were scattered across the 9.2 kb fragment, therefore we began by making a series of deletions to localize the regulatory elements (Fig. 1A). Truncating the distal 8.5 kb segment of the

promoter ($\Delta NheI$, -1386 to -1) disrupted the normal activity of the promoter and resulted in low levels of GFP expression throughout the neural tube (Fig. 1D). Further mapping of the promoter found that a distal 2.5 kb segment from -8129 to -5575 with four areas of high nucleotide conservation was the main region responsible for directing motoneuron-specific expression of GFP when fused to the proximal segment of the *Hb9* promoter (Fig. 1A,E).

To better understand the positive regulation of *Hb9* in motoneurons, we next subdivided the 2.5 kb distal fragment into smaller segments using a series of 5' and 3' deletions around the evolutionarily conserved sequences (Fig. 1B). This mapping led to the identification of a 231 nucleotide sequence (M250, -7121 to -6890) containing two subregions of extremely high sequence conservation, M50 (-7096 to -7051) and M100 (-6997 to -6896). Although neither M50 nor M100 alone were sufficient to direct motoneuron expression, the combination was active in motoneurons (Fig. 1B,F) (Lee and Pfaff, 2003). As with the $\Delta NheI$ construct, however, low level GFP expression was also detected in non-motoneurons, suggesting that elements outside of the M250 region might contribute to the inhibition of *Hb9* expression in non-motoneurons (see below).

To determine whether the proximal -1386 to -1 region of *Hb9* was necessary for M250 function, we replaced this segment with a minimal 70 nucleotide synthetic TATA element (Colgan and Manley, 1992). The M250+TATA construct was expressed at high levels in motoneurons, whereas the TATA element alone had little or no activity (Fig. 1G, data not shown). GFP expression from the M250+TATA construct was more restricted to motoneurons than the M250+ $\Delta NheI$ construct (compare Fig. 1F with G). This difference is probably due to the presence of an element/s for a general activator/s located within the proximal -1386 to -1 area of the *Hb9* promoter that is not present in constructs with a synthetic TATA element (see below).

The identification of a compact regulatory region from -7121 to -6890 (M250) in *Hb9* that was sufficient to promote reporter gene expression in chick motoneurons led us to test whether the cis-elements in the M250 DNA segment were also active in mouse motoneurons. The linearized M250+ $\Delta NheI$:GFP construct was injected into mouse oocyte pronuclei, and embryos were examined at E11.5 shortly after motoneurons are postmitotically generated (Fig. 1H). GFP⁺ motoneurons were detected in four out of five independent transgenic embryos (Table 1). Double labeling with motoneuron and interneuron markers revealed that the highest levels of GFP were detected in *Hb9*⁺, *Isl1/2*⁺ motoneurons, although lower levels of ectopic expression was detected in cells near to but not overlapping with the V2 interneuron population marked by *Lhx3* and *Chx10*, and dorsal root ganglion cells flanking the neural tube (see Fig. S1 at <http://dev.biologists.org/supplemental>). Taken together, our findings indicate that M250 is an evolutionarily conserved regulatory region, which is further supported by the finding that M250 is active in transgenic mouse motoneurons when linked to a synthetic TATA element (M250+TATA, data not shown). Because the M250 region of *Hb9* from -7121 to -6890 is a module that contributes to cell-type-specific gene expression in a distance and orientation-independent manner (data not shown), we conclude that M250 is a motoneuron enhancer

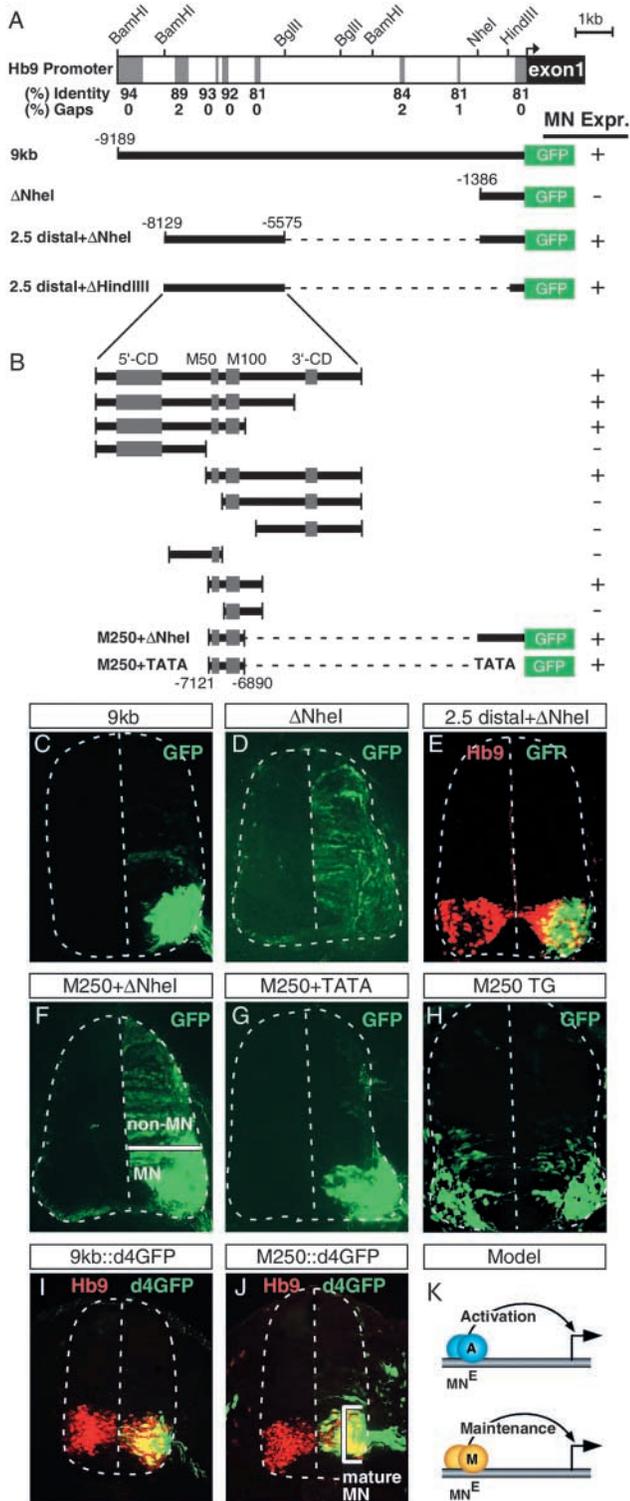


Fig. 1. Characterization of a motoneuron enhancer. (A) Schematic representation of mouse genomic sequences of *Hb9*. Gray boxes represent areas of high homology between mouse and human. Percent nucleotide conservation is shown and percent gapping in the alignment is listed for each area of homology. Mouse promoter sequences (black line) were fused to the reporter GFP gene to determine the activity of each DNA fragment. The presence (+) or absence (-) of high level GFP expression in motoneurons from HH stage 24 chicks electroporated with each reporter construct is listed on the right (MN Expr.). Each result is representative of ~20 embryos. (B) Fragments of the 2.5 kb distal fragment -8129 to -5575 were subcloned into a Δ NheI:GFP or TATA:GFP vector as indicated, electroporated into chick embryos and GFP expression was analyzed at HH stage 24. Gray rectangles correspond to evolutionarily conserved sequences 5'-CD (5' conserved domain), M50 (~50 nucleotide segment from enhancer), M100 (~100 nucleotide segment from enhancer) and 3'-CD (3' conserved domain). A 231 nucleotide segment (M250, -7121 to -6890) contains an enhancer active in embryonic motoneurons. (C-G) GFP expression in HH stage 24 chick embryos following electroporation of *Hb9*:GFP reporter constructs listed in A and B. (C) The upstream 9.2 kb region of *Hb9* directs motoneuron-specific expression of GFP. (D) The proximal Δ NheI fragment of *Hb9* drives weak GFP expression along the entire dorsoventral axis of the neural tube. (E) The 2.5 kb distal element produces a strong GFP signal (green) in *Hb9*⁺ motoneurons (red). Medial red cells lacking GFP are probably Mnr2⁺ motoneuron progenitors that label with the anti-Hb9 antibody. (F) A 231 nucleotide fragment (M250), drove high levels of GFP in motoneurons (MN), but some ectopic expression of the reporter is also detected in the dorsal neural tube (non-MN). The M250+ Δ NheI construct corresponds to MN^E::GFP in Lee and Pfaff (Lee and Pfaff, 2003). (G) M250 linked to synthetic TATA box is active in motoneurons without labeling ectopic cells. (H) E11.5 transgenic mouse embryos with M250+ Δ NheI:GFP reporter stained with anti-GFP antibody. (I, J) *Hb9*::d4GFP (destabilized GFP) reporter activity in HH stage 20 chick embryos. The 9.2 kb promoter of *Hb9* and M250 enhancer (-7121 to -6890) are both active in 'mature' (laterally-located) motoneurons (MN, bracket). (K) The enhancer of *Hb9* (MN^E) mediates activation (blue factors) and maintenance (yellow factors) of transcription in developing motoneurons.

(MN^E). However, this enhancer in isolation is not sufficient to restrict gene expression to only motoneurons (Fig. 1F,H), necessitating the involvement of other regulatory inputs to control *Hb9* expression (see below).

Isl1, Lhx3 and NeuroM (and/or Ngn2) cooperate to specify motoneuron identity and synergize to trigger *Hb9* transcription

via elements located within M250 (Lee and Pfaff, 2003). Nevertheless, this array of LIM and bHLH proteins exists for only a brief period in motoneuron development as the cells emerge from the ventricular zone. Thus, it was unclear whether the enhancer at -7121 to -6890 mediated only the initial activation of *Hb9*, or whether it could also contribute to its maintenance in 'mature' motoneurons that have migrated away from the ventricular zone. GFP expression was detected in both young and mature motoneurons with M250-based constructs (Fig. 1F,H), but we were uncertain whether the labeling of mature cells was simply due to the stability of the GFP protein after its initial expression in newly generated motoneurons. To determine whether the enhancer of *Hb9* contributed to the maintenance of gene expression in motoneurons following their initial generation we used a destabilized form of GFP fused to the ornithine decarboxylase gene encoding a PEST sequence to accelerate protein degradation of the reporter (d4GFP, $t_{1/2}$ =4 hours) (Li et al., 1998). We found that mature motoneurons located laterally in the neural tube were labeled by the destabilized form of GFP when expressed with either the 9.2 kb (9 kb::d4GFP) or the M250-based (M250::d4GFP)

Table 1. Expression of GFP in independent lines of E11.5 transgenic mouse embryos containing wild-type and mutant derivatives of M250 linked to the Δ NheI:GFP construct

| | Total number of transgenic embryos | Number of embryos with GFP ⁺ motoneurons (%) |
|-----------|------------------------------------|---|
| Wild type | 5 | 4 (80.0) |
| E box mt | 12 | 2 (16.7) |
| A/T mt | 6 | 1* (16.7) |

*A small number of weak GFP⁺ motoneurons were detected per section. This embryo was selected for the image in Fig. 4L.

construct containing the isolated enhancer from -7121 to -6890 (Fig. 1I,J). These findings suggest that cis-elements within M250 contribute to the activation and maintenance of *Hb9* in developing motoneurons (Fig. 1K).

E box elements are necessary for *Hb9* expression in motor neurons

Examination of the M250 sequence (-7121 to -6890) revealed several candidate binding sites for transcription factors, including two consensus E box elements for bHLH protein binding (CANNTG) characterized in a previous study by Lee and Pfaff (Lee and Pfaff, 2003) (Fig. 2A). To test the function of these E box elements in vivo, point mutations were used to alter the sequences (Fig. 2B). Mutations in the M50 E box had a minimal effect on M250 activity, whereas mutation of the M100 E box reduced motoneuron expression more

dramatically (data not shown). The combination of M50 and M100 mutations disrupted the normal activity of M250 in chick motoneurons, as previously reported (Fig. 2C,D) (Lee and Pfaff, 2003). Mutation of the E boxes had two effects on transgene expression: GFP labeling in motoneurons was markedly reduced, whereas GFP labeling in non-motoneurons became more apparent (Fig. 2D). These findings suggest that the E box elements might function to enhance expression in motoneurons and suppress *Hb9* in non-motoneurons. Next, we tested whether the E box elements were necessary for *Hb9* expression in mice. Transgenic mouse embryos with both E boxes mutated exhibited a dramatic reduction in the level and frequency of reporter gene expression in motoneurons compared with the wild-type enhancer element (Fig. 2E,F; Table 1).

Developing motoneurons sequentially express several bHLH proteins, including *Ngn2* in the progenitor cells followed by *NeuroM* in the early postmitotic motoneurons and *NeuroD* in the more mature cells (Roztocil et al., 1997; Sommer et al., 1996). Our previous studies had determined that *Ngn2* and *NeuroM* contribute to the activation of *Hb9* during the initial stages of motoneuron development (see Fig. S2A,B at <http://dev.biologists.org/supplemental>) (Lee and Pfaff, 2003), but it remained unclear whether *NeuroD* in the mature cells could also stimulate *Hb9* expression. To compare the activity of these transcription factors, P19 cells were transfected with expression constructs encoding bHLH proteins together with a luciferase reporter containing seven E box elements (Nakashima et al., 2001). Under these conditions *Ngn2* activated the reporter much more than either *NeuroM* or *NeuroD* (Fig. 3A). Despite this inherent difference in trans-activation, *Ngn2*, *NeuroM*, and *NeuroD* each synergized in a similar way with the LIM factors *Isl1* and *Lhx3* to trigger *Hb9* expression (Fig. 3B) (Lee and Pfaff, 2003). Likewise, each bHLH factor dimerized with E47 and bound to the M50 and M100 E box elements in a sequence-specific manner (Fig. 3C,D; see Fig. S2C), and exhibited a similar ability to promote motoneuron differentiation from transfected P19 embryonic carcinoma cells when expressed with *Isl1* and *Lhx3* (Fig. 3E-H) (Lee and Pfaff, 2003). Taken together, these findings suggest that the initial activation of *Hb9* expression is dependent on *Ngn2* and *NeuroM* as motoneurons become

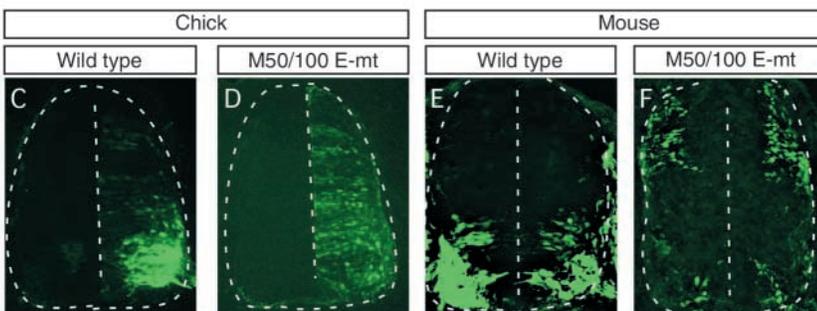
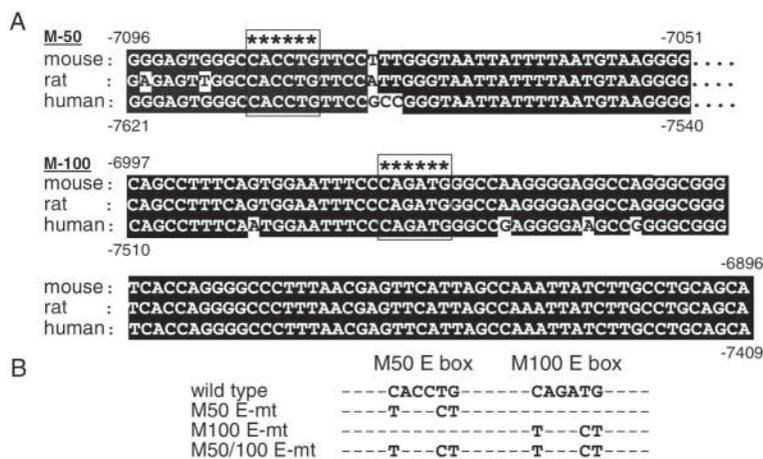
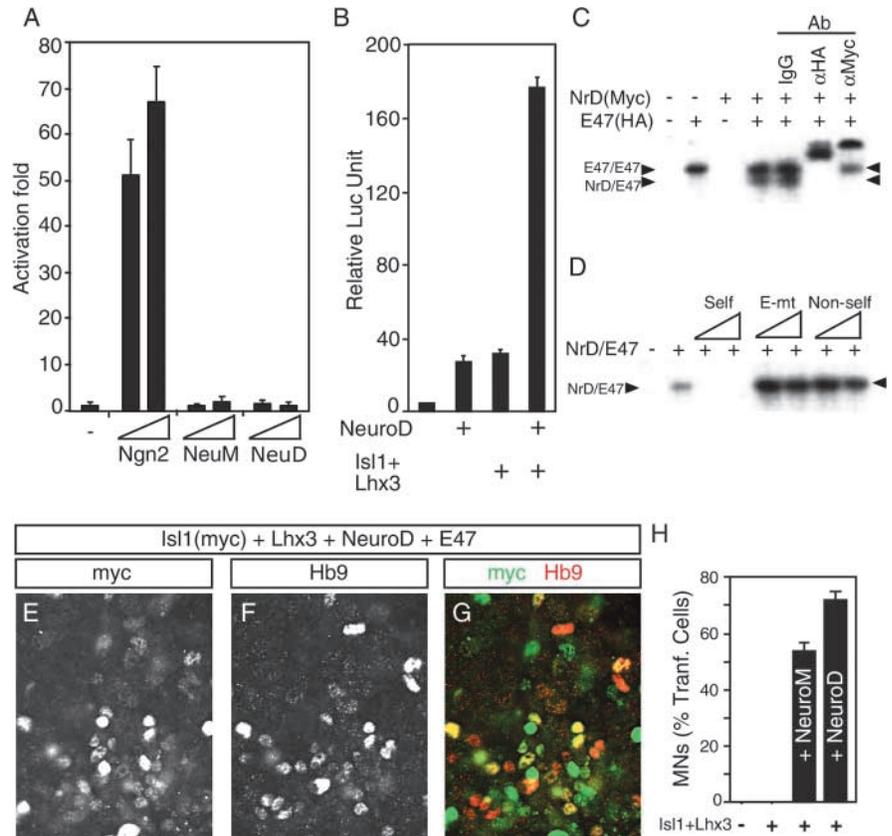


Fig. 2. Enhancer function is dependent on bHLH factors. (A) Two evolutionarily conserved blocks, M50 and M100, are found in the M250 enhancer. Mouse, rat and human sequences of M50 and M100 are aligned, and nucleotide coordinates of the mouse and human sequences are indicated. Two canonical E box sites are marked with asterisks (*). (B) Mutations were introduced into the E boxes as shown, and functional activity was tested in the context of the M250+ Δ NheI:GFP construct shown in Fig. 1B,F. (C,D) GFP expression in HH stage 24 chick embryos following electroporation of each reporter construct. (E,F) GFP expression in E11.5 transgenic mouse embryos containing wild-type or E box mutant constructs.

Fig. 3. NeuroD contributes to the activation of *Hb9*. (A) P19 cells were transfected with a luciferase reporter containing reiterated E boxes and increasing amounts of expression constructs encoding bHLH factors. Ngn2 strongly activated transcription, whereas NeuroM (NeuM) and NeuroD (NeuD) exhibited markedly less inherent trans-activating function. (B) NeuroD activates M250 enhancer-mediated transcription in a synergistic manner with Isl1 and Lhx3 in P19 cells. (C) NeuroD/E47 dimer binding to the M250 enhancer was examined using gel retardation assays. Myc-tagged NeuroD [NrD(Myc)] and HA-tagged E47 were translated in vitro separately and incubated with the M50 oligonucleotide in the presence or absence of antibodies directed against each epitope. This translation condition favored the formation of E47:E47 complexes. Similar results were seen with oligos containing the M100 E box (not shown). (D) The binding of co-translated NeuroD/E47 on M50 was challenged by 20- or 100-fold molar excess of wild type (self), E-box mutated (E-mt) or unrelated (non-self) DNA. Co-translation of proteins favored the formation of NeuroD:E47 heterodimers, which bind with high specificity to the E box sites within the enhancer. (E-H) The co-expression of NeuroD with Isl1 and Lhx3 triggers the differentiation of Hb9⁺ motoneurons in >70% of the transfected P19 cells. This activity is comparable with that seen with NeuroM (Lee and Pfaff, 2003).



postmitotic (Lee and Pfaff, 2003), and that NeuroD contributes to the maintenance of *Hb9* expression in mature motoneurons.

Multiple positive-acting elements are located in the enhancer

To determine whether additional regulatory sequences in M250 were necessary for enhancer activity, we generated mutations at nine locations within the -7096 to -6896 segment (M1-M9) (Fig. 4A). The M4, M5, M6 and M7 mutations did not significantly alter reporter gene expression compared to the wild-type enhancer (Fig. 4B,F-I). This was in contrast to the effect of the M1, M2, and M3 mutations which markedly reduced GFP expression in motoneurons (Fig. 4C-E). The M8 and M9 mutations did not entirely block enhancer activity, but the intensity of reporter gene labeling was consistently reduced compared to controls (Fig. 4J,K). Previous studies of the LIM-HD factors Isl1 and Lhx3 have shown that these proteins dimerize and bind to two separate sites at the positions of the M1-M2 and M8-M9 mutations (Jurata et al., 1998; Lee and Pfaff, 2003; Thaler et al., 2002). To determine whether these elements for LIM-HD binding have a conserved function in mice, transgenic animals were generated in which both the M1 and M9 mutations were incorporated into the enhancer. The mutation of these elements reduced the level and frequency of GFP expression in mouse embryo motoneurons, indicating these sequences are essential for proper expression of *Hb9* (Fig. 4L; Table 1).

Mutations at the M3 site reduce transcription in motoneurons as well as increased expression in non-motoneurons, suggesting this could represent a bi-functional

regulatory element (Fig. 4A,E). The protein/s that bind to this element were not known, prompting us to test whether the M3 element represented another site for Isl1/Lhx3 binding. We found, however, that the M3 sequence was unable to compete for Isl1/Lhx3 DNA binding, suggesting that an unidentified protein/s binds to this element (Fig. 4A,M,N). These findings in combination with those from previous studies (Lee and Pfaff, 2003) demonstrate that the enhancer from -7121 to -6890 contains binding sites for Isl1/Lhx3, Ngn2/E47, NeuroM/E47 and NeuroD/E47, and probably other proteins that function to activate transcription in developing and mature motoneurons.

Proximal elements are necessary for enhancer function

Deletions of the *Hb9* promoter had two effects on transcription: they disrupted high-level motoneuron expression, and they led to low level but widespread ectopic GFP labeling (Fig. 1D). This ectopic labeling appeared to be dependent on sequences within the proximal -1386 to -1 segment of *Hb9*, as replacement of this region with a synthetic TATA element preserved the motoneuron labeling but eliminated the ectopic expression (compare Fig. 1F with 1G). To examine the 'general' activators of *Hb9*, we optimized the reporter-labeling to more easily monitor transcription. We found that using immunocytochemistry to detect *nlacZ* was more sensitive than GFP fluorescence (compare Fig. 1D with Fig. 5D).

We began by systematically creating 5' deletions across the 9.2 kb upstream region (Fig. 5A). Deletion of the distal 5.6 kb segment of *Hb9* (Δ BamHI), which contained the majority of

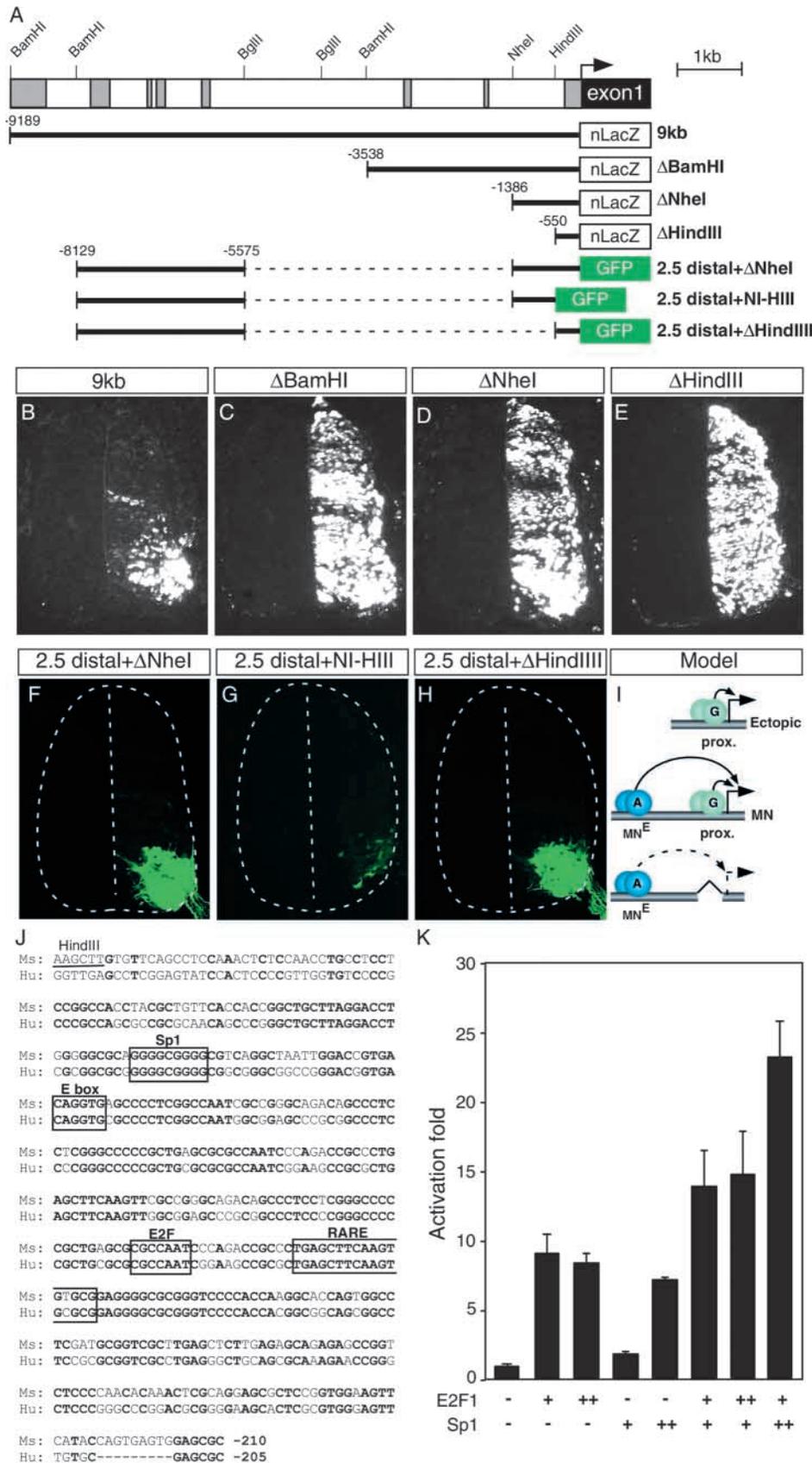
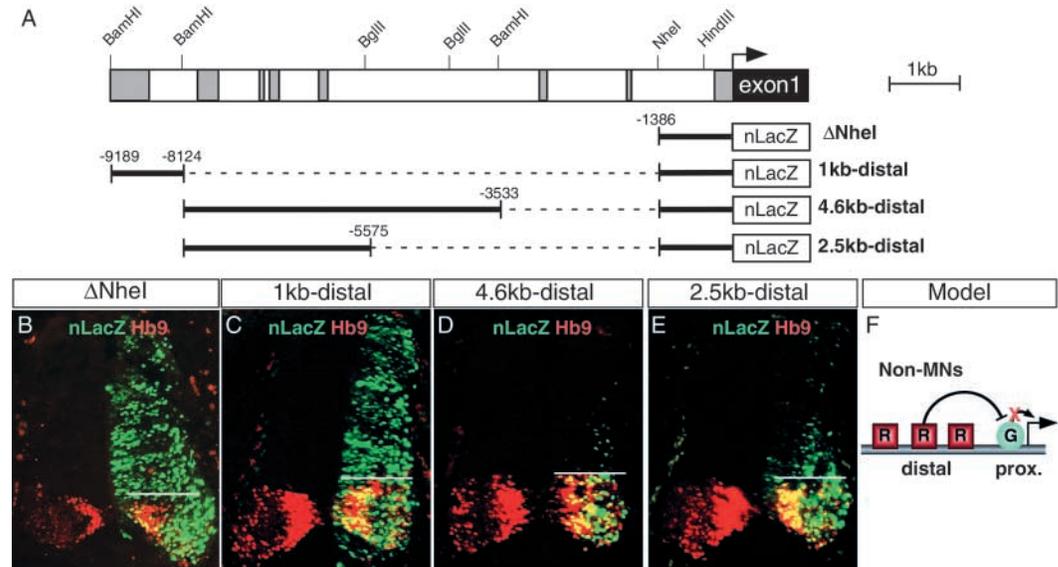


Fig. 5. General-activators of *Hb9* in non-motoneurons. (A) Fragments of the mouse *Hb9* promoter were linked to the reporter nuclear *lacZ* or GFP. (B-H) DNA constructs were electroporated into chick embryos and reporter expression was detected using immunocytochemistry at HH stage 24. (B) The 9.2 kb promoter of *Hb9* drives motoneuron-specific gene expression. (C) Deletion of the distal 5.6 kb *Bam*HI fragment impairs the motoneuron-specific activity leading to widespread ectopic reporter expression. (D,E) Likewise, large 5' deletions to the *Nhe*I and *Hind*III sites leaving only 1386 and 550 nucleotides of *Hb9* sequences, respectively, continued to promote widespread ectopic reporter expression. (F-H) The 2.5 kb distal element (-8129 to -5575) was linked to three different proximal fragments of *Hb9* as indicated in A. 2.5 distal + Δ *Nhe*I or Δ *Hind*III drove high levels of GFP expression in motoneurons, but 2.5 distal + NI-HIII was less active, suggesting the -550 proximal segment of *Hb9* is necessary for enhancer function. (I) General activators are predicted to interact with the proximal (prox.) region of *Hb9* and promote widespread transcription of the gene. The enhancer of *Hb9* (*MN^E*) in combination with general-activators drive motoneuron-specific expression. Deletion of the proximal segment of *Hb9* disrupts enhancer function. (J) The nucleotide sequence of the proximal 550 nucleotide region of *Hb9* from mouse and human are aligned. The conserved sequences in both species are marked in bold, and potential binding sites for activators are boxed and labeled. This sequence lacks an obvious TATA box motif. The longest cDNA extends to -195 in mouse *Hb9* (-181 in human). Because the precise start of transcription has not been mapped, we use the start of translation as the reference point for the coordinates shown with the sequences (see Materials and methods). (K) CV1 cells were transfected with increasing concentrations of plasmids encoding E2F1 and/or Sp1 together with a luciferase reporter linked to *Hb9* (2.5 distal+ Δ *Nhe*I).

Fig. 6. Repressors interacting with dispersed elements suppress ectopic *Hb9* expression. (A) Distal fragments were linked to the proximal -1386 segment ($\Delta NheI$) of *Hb9* to map elements involved in repressing ectopic gene expression. (B-E) DNA clones were electroporated into chick embryos, and immunocytochemistry was used to detect *lacZ* (green) and *Hb9* (red) at HH stage 24. White bar indicates dorsal limit of *Hb9*⁺ motoneuron population. (B) The proximal segment of *Hb9* drives widespread reporter expression. (C) The 1 kb distal fragment does not restrict the ectopic expression of *Hb9*. (D) The 4.6 kb distal fragment limited reporter expression to motoneurons. (E) The 2.5 kb distal fragment expressed *nLacZ* in motoneurons and a few cells in the V2 interneuron region. (F) The proximal 550 nucleotide segment of *Hb9* activates transcription throughout the spinal cord. In non-motoneurons (non-MNs), this activation is suppressed by factors that require elements in the 4.6 kb distal region of the promoter.



that progenitor cell factors such as *Nkx2.2* and *Irx3* expressed by non-motoneuron cells suppress the expression of *Hb9*.

Genetic studies have shown that *Hb9* feeds back negatively to modulate its own expression (Arber et al., 1999). We tested whether *Hb9* could suppress the activity of its enhancer when LIM and bHLH factors synergize to activate transcription. We found that the native *Hb9* protein and the EnR-*Hb9* repressor both inhibited transcription under these conditions, whereas the *Hb9*-HD and a fusion of *Hb9* to the VP16 activation domain (VP16-*Hb9*) lacked this activity (Fig. 7B). Thus, in developing motoneurons where *Hb9* transcription is synergistically activated, co-repressors such as those recruited by the engrailed fusion (EnR) appear to be involved in negative feedback regulation. Consistent with these findings, *Hb9* protein bound in a sequence-specific manner to the ATTA motifs in the enhancer (Fig. 7C,D).

Discussion

Studies of the developing spinal cord have identified a transcription factor pathway involved in the specification of motoneurons (Fig. 8A) (Briscoe and Ericson, 2001; Jessell, 2000; Marquardt and Pfaff, 2001). In this report, we have examined how the transcription of the *Hb9* gene is selectively targeted to motoneurons, in order to gain insight into the mechanistic relationship between gene regulation and neuronal specification. Our studies found evidence for three strategies that contribute to the proper spatial and temporal regulation of *Hb9* in postmitotic motoneurons. First, general activator proteins located throughout the neural tube interact with the proximal 550 nucleotides of the promoter and non-selectively stimulate *Hb9* transcription. Second, the non-specific function of these general activators is constrained by multiple repressor proteins, including *Nkx2.2* and *Irx3* in non-motoneuron cells. And third, high level *Hb9* expression in postmitotic

motoneurons is activated and maintained by a compact enhancer region from -7121 to -6890 that contains multiple cis-elements for positive-acting factors including *Ngn2*, *NeuroD*, *NeuroM*, *Isl1* and *Lhx3* (Lee and Pfaff, 2003).

Evidence for a derepression model of gene regulation in the neural tube

Shh signaling specifies distinct progenitor cell populations within the ventral neural tube that represent the precursors of individual neuronal subtypes involved in locomotion (Briscoe et al., 2000; Ericson et al., 1997). The progenitor cells within each of these domains express unique combinations of transcription factors that regulate cell fate within the neural tube (Fig. 8A) (Briscoe and Ericson, 2001; Briscoe et al., 2000). Because these progenitor cell factors repress transcription, it has been predicted that neuronal fate in the spinal cord is largely controlled indirectly via a poorly defined derepression mechanism for gene expression (Muhr et al., 2001). A prediction of this model is that 'general' activators play a key role in the activation of gene expression. Our analyses provide direct support for the derepression model of Muhr et al. (Muhr et al., 2001) by demonstrating that non-cell-type-specific activators such as the broadly expressed proteins *E2F* (Zhao et al., 1995) and *Sp1* (Saffer et al., 1991) promote the expression of *Hb9* via proximally located cis-elements. As predicted by the derepression model, the inappropriate expression of *Hb9* in non-motoneurons is inhibited by repressor proteins. Thus, the activation of *Hb9* in postmitotic motoneurons is dependent on the absence or inactivation of these repressors – leading to derepression of the promoter.

The general activation of *Hb9* via the proximal promoter occurs in dividing cells, postmitotic neurons, and neural crest derivatives such as the dorsal root ganglia (data not shown). The precise role of general transcription factors such as *E2F*, *Sp1* and their related family members needs to be further

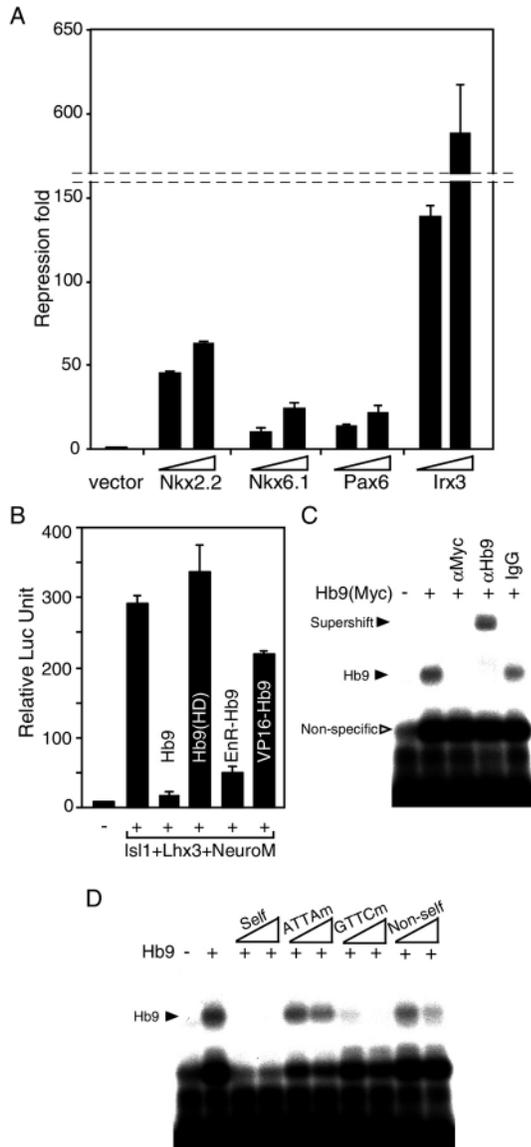


Fig. 7. Repression of *Hb9* by homeodomain transcription factors. (A) Plasmids encoding progenitor factors, Nkx2.2, Nkx6.1, Pax6 and Irx3, were co-transfected with the 2.5 kb distal+TK:luciferase reporter into 293 cells and luciferase activity was used to monitor the fold repression relative to vector-only transfections. (B) Hb9 and EnR-Hb9 (Hb9 homeodomain linked to eh1 engrailed repressor domain) repressed the synergistic activation of M250 by Isl1/Lhx3/NeuroM in transfected P19 cells. By contrast, Hb9-HD and Hb9-HD fused VP16 activation domain (VP16-Hb9) did not. (C,D) Gel retardation analysis reveals that Hb9 binds to the M50 and M100 (not shown) portions of its enhancer. Antibodies against Hb9 supershifted or disrupted the DNA:protein complex, whereas IgG control serum had no effect on DNA binding. Mutation of the ATTA sequences within M50 (ATTAm) disrupted Hb9 binding. The protein complex formed by Isl1/Lhx3 relies on the same ATTA elements for binding (Lee and Pfaff, 2003), suggesting Hb9 may compete with the LIM factors for access to the enhancer.

examined; however, it is interesting that these two proteins interact to promote transcription (Karlseider et al., 1996). Our findings raise the question of why a motoneuron-specific gene such as *Hb9* receives transcriptional inputs from non-specific

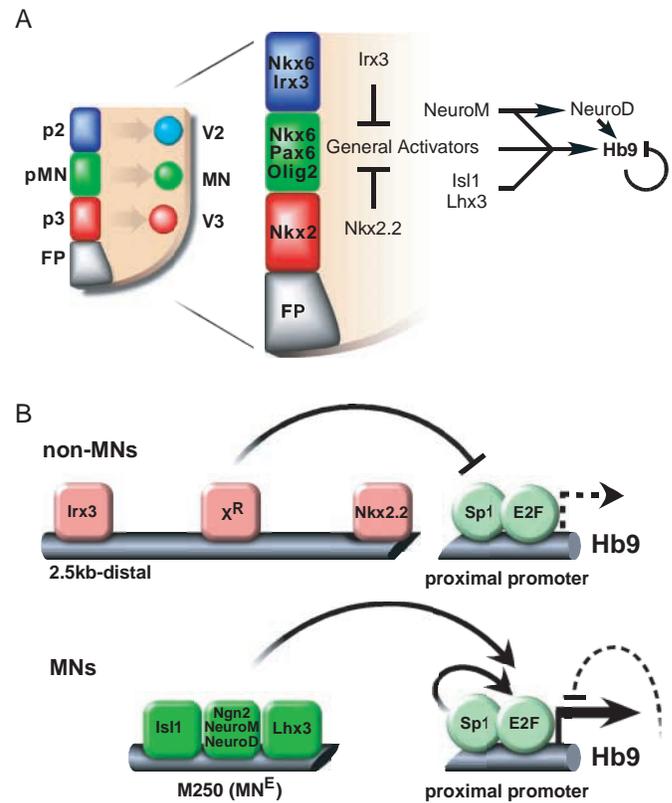


Fig. 8. Model of transcriptional regulation for motoneuron-specific gene expression. (A) Progenitor cell domains (blue, green, red) in the ventral neural tube express specific combinations of transcription factors involved in cell fate specification (Briscoe et al., 2000). The floor plate (gray, FP) is shown. Progenitor cell factors from the p2 and p3 domains repress the general-activators of *Hb9*, providing direct evidence for the derepression model of gene regulation in the neural tube (Muhr et al., 2001) (reviewed by Lee and Pfaff, 2001). To achieve high level expression of *Hb9* in motoneurons, however, enhancer factors Isl1, Lhx3 and NeuroM cooperate with general activators. (B) The spatial pattern of *Hb9* expression in motoneurons is regulated by repressors (red) in non-motoneuron cells binding to dispersed sites. This report demonstrates that Irx3 and Nkx2.2 repress *Hb9*, but it remains unclear whether they function directly or indirectly. The postmitotic repression of *Hb9* in non-motoneurons may be mediated by additional repressors (X^R). In differentiating motoneurons activators (green) bind to the enhancer (M250) and proximal 550 nucleotide segment of the promoter. Together this ensemble of specific and general proteins triggers high level transcription in motoneurons, which is modulated through negative feedback regulation by Hb9 protein.

factors? A general feature of cell specific gene regulation is that many activators appear to be insufficient to promote maximal levels of transcription, known as 'activator insufficiency' (Barolo and Posakony, 2002). In support of this, *Hb9* enhancer function was facilitated by the proximal -550 to -1 segment of the gene which mediates interactions with general activators.

The coordinated use of general activators with enhancer-factors to promote high level gene expression, however, raises the problem of how to prevent inappropriate expression of the gene in non-motoneurons. The strategy used to block the general activators of *Hb9* from promoting leaky expression in

non-motoneurons is based on transcriptional repression. The repressors we identified, *Nkx2.2* and *Irx3*, represent transcription factors implicated in the development of progenitor cells (Briscoe et al., 2000; Briscoe et al., 1999). The binding sites for repressors appear to be more widely dispersed, and fall both within and outside of the enhancer region (Fig. 8B). This organization may facilitate the function of both short and long range types of transcriptional repression that are mediated by different types of co-repressors such as Gro/TLE and CtBP (Zhang and Levine, 1999). Ectopic expression of *Nkx2.2* and *Irx3* suppress motoneuron differentiation in vivo (Briscoe et al., 2000; Briscoe et al., 1999; Muhr et al., 2001). Our finding that *Nkx2.2* and *Irx3* inhibit *Hb9* transcription in 293 cells is consistent with the possibility that these transcription factors bind directly to the *Hb9* promoter, as indirect gene regulation is likely to be less permissive in these non-neuronal cells, though further analyses are required to establish this definitively. If *Nkx2.2* and *Irx3* act directly to suppress *Hb9*, a remaining question is whether the co-repressors that these transcription factors recruit function by modulating the activity of the positive-acting factors in the enhancer and proximal promoter and/or by modifying histones to suppress transcription (Kuo and Allis, 1998).

Although *Irx3* is found to inhibit *Hb9* expression, this may occur in a context-dependent fashion because *Irx3* is also detected in some postmitotic motoneurons (Cohen et al., 2000). One possibility is that the function of *Irx3* is developmentally regulated as progenitor cells differentiate. In this scenario, *Irx3* would repress *Hb9* in dividing cells but not postmitotic motoneurons. Alternatively, *Irx3* may function constitutively as a repressor, but the activity of the motoneuron-specific factors acting via the enhancer may be sufficient to overcome the repressive activity of *Irx3*. As *Hb9* levels vary in different motor column subtypes (Arber et al., 1999; Tanabe et al., 1998; Thaler et al., 1999), it is possible that *Irx3* levels contribute to this finer level of *Hb9* regulation among motor subtypes.

Although the repressor factors identified in this study are known to be expressed in the progenitor cells of the neural tube, it is less clear what prevents *Hb9* expression in postmitotic interneurons. One possibility is that the repressors in the progenitor cells for interneurons imprint the gene with stable modifications that persist postmitotically. Alternatively, a new set of repressor proteins might function in interneurons (X^R , Fig. 8B), such as the postmitotic interneuron factors *Chx10*, *En1* and *Evx1/2* which interact with the Gro/TLE class of co-repressors (Han and Manley, 1993; Jaynes and O'Farrell, 1991; Liu et al., 1994). Although the underlying basis for repressing *Hb9* in postmitotic interneurons is poorly defined, the suppression of the general activators for *Hb9* present in both progenitor cells and interneurons suggests that transcriptional derepression is a mechanism that can operate within progenitor cells and postmitotic neurons.

A neuronal subtype enhancer

Although transcriptional repression is necessary to prevent *Hb9* from becoming transcribed in non-motoneurons, high level expression in motoneurons is facilitated by a 231 nucleotide enhancer region (MN^E) located from -7121 to -6890. Thus, derepression is not the only mechanism that

operates to control *Hb9* expression. The core sequences within the enhancer are conserved between mouse, rat and human, and function in both mouse and chick embryos (Lee and Pfaff, 2003). Results shown here, in combination with those from a previous study (Lee and Pfaff, 2003), demonstrate that point mutations within two E box elements for bHLH factors and four ATTA elements that bind homeodomain factors disrupt normal *Hb9* expression. The sequential expression of *Ngn2*, *NeuroM* and *NeuroD* in developing motoneurons suggests that the composition of the enhancer complex involved in activating and maintaining *Hb9* expression changes as these cells develop.

In this report, we have defined an in vivo method for characterizing gene regulation that should be applicable for the analysis of other promoters in the embryonic spinal cord (Timmer et al., 2001). High level *Hb9* expression in motoneurons appears to be facilitated by two types of activators – those that establish a neuron-specific enhancer-complex and general activators that function in a non-cell-type-specific manner. Our findings are consistent with the possibility that the two types of activators function cooperatively via a mechanism termed activator insufficiency (Barolo and Posakony, 2002). This regulatory strategy, however, necessitates the use of repressors to prevent the inappropriate activation of *Hb9*. Thus, in motoneurons, derepression of the activators is necessary to permit *Hb9* expression.

We are grateful to H. Juguilon in the R. Evans laboratory for assistance with transfections, and R. Tjian for Sp1. We thank K. Sharma for helping to initiate the early stages of this project; and G. Gill, M. Montminy and G. Lemke for comments on the manuscript. S.-K.L. was supported by a postdoctoral fellowship from the Human Frontiers Science Program, L.W.J. was supported by an NIH postdoctoral fellowship, and S.L.P. by the Mather's and PEW Foundations. This research was supported by NINDS grant RO1NS37116.

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