Distinct domains within Mash1 and Math1 are required for function in neuronal differentiation versus neuronal cell-type specification

Yuji Nakada, Thomas L. Hunsaker, R. Michael Henke and Jane E. Johnson*

Center for Basic Neuroscience, UT Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390-9111, USA
*Author for correspondence (e-mail: jane.johnson@utsouthwestern.edu)

Summary

Many members of the basic helix-loop-helix (bHLH) family of transcription factors play pivotal roles in the development of a variety of tissues and organisms. We identify activities for the neural bHLH proteins Mash1 and Math1 in inducing neuronal differentiation, and in inducing the formation of distinct dorsal interneuron subtypes in the chick neural tube. Although both factors induce neuronal differentiation, each factor has a distinct activity in the type of dorsal interneuron that forms, with overexpression of Math1 increasing dI1 interneurons, and Mash1 increasing dI3 interneurons. Math1 and Mash1 function as transcriptional activators for both of these functions. Furthermore, we define discrete domains within the bHLH motif that are required for these different activities in neural development. Helix 1 of the Mash1 HLH domain is necessary for Mash1 to be able to promote neuronal differentiation, and is sufficient to confer this activity to the non-neural bHLH factor MyoD. In contrast, helix 2 of Math1, and both helix 1 and 2 of Mash1, are the domains required for the neuronal specification activities of these factors. The requirement for distinct domains within the HLH motif of Mash1 and Math1 for driving neuronal differentiation and cell-type specification probably reflects the importance of unique protein-protein interactions involved in these functions.

Key words: bHLH transcription factors, Spinal cord development, MyoD, Ngn1, Dorsal interneuron specification, Lim homeodomain factors, Neurogenesis

Introduction

Basic helix-loop-helix (bHLH) transcription factors are required for proper formation of the vertebrate and invertebrate nervous systems (Bertrand et al., 2002). The vertebrate neural bHLH transcription factors have been classified into subgroups based on their temporal pattern of expression (Lee, 1997) and on their homology to *Drosophila* proneural genes (Bertrand et al., 2002). The factors Mash1, a homolog of the *Drosophila Achaete-scute* genes (Johnson et al., 1990), and Math1, a homolog of the *Drosophila atonal* gene (Akazawa et al., 1995), both belong to the early expressed sub-group of bHLH factors because of their expression in progenitor cells of the developing neural tube, but they reside in distinct sub-classes based on sequence homology (Bertrand et al., 2002). Gene knockout studies have demonstrated essential roles for these factors in the formation of specific populations of neurons (Ben-Arie et al., 1997; Bermingham et al., 1999; Bermingham et al., 2001; Fode et al., 2000; Gowan et al., 2001; Guillemot et al., 1993; Parras et al., 2002). Gain-of-function studies in multiple systems provide evidence that these genes are capable of inducing neuronal differentiation (Cai et al., 2000; Cepko, 1999; Farah et al., 2000; Kim et al., 1997). Additional functions in neuronal specification have also been reported (Gowan et al., 2001; Parras et al., 2002; Perez et al., 1999). We have extended these studies to define common and distinct activities of Mash1 and Math1 in neural tube development, and

have identified the structural elements required for these activities.

The neural bHLH factors belong to a much larger family of transcription factors that are essential for diverse processes such as myogenesis (Davis et al., 1987), hematopoiesis (Bain et al., 1994) and pancreas development (Naya et al., 1997). The greater HLH transcription factor family has been grouped into classes based on tissue distribution, dimerization capabilities and DNA-binding specificities (Massari and Murre, 2000). Mash1 and Math1 belong to Class II, as do other tissue-specific bHLH factors such as the myogenic factor MyoD. Class II bHLH proteins preferentially form heterodimers with the broadly expressed Class I bHLH factors called E-proteins (E2a, HEB and E2-2) (Massari and Murre, 2000). These heterodimers characteristically bind E-box DNA consensus sites (CANNTG) and modulate transcription (Murre et al., 1989). Crystal structures of MyoD (Ma et al., 1994) and E47 (Ellenberger et al., 1994) show that the HLH domain is involved in dimer formation, and the basic region interacts with DNA. While it is probable that in vivo these neural bHLH factors have distinct DNA targets relative to each other and relative to other non-neural members of the family such as MyoD, in vitro these proteins can bind similar E-box sites (Johnson et al., 1992) (P. Ebert and J.E.J., unpublished data). The increase in specificity in the function of these proteins in

vivo is hypothesized to reflect additional context-dependent protein-protein interactions or post-translational modifications.

As predicted from the crystal structures, much of the phenotypic specificity of the bHLH transcription factors resides within the bHLH domain itself. Studies with the *Drosophila* proneural genes *atonal* and *scute* demonstrated that the ability to promote ectopic chordotonal organ and external sensory organ formation, respectively, resides within the bHLH domain, with specificity arising from the basic region and the HLH serving a required but more general function (Chien et al., 1996). Importantly, the residues in the basic region that interact with DNA are common between Atonal and Scute, and amino acids that differ face away from the DNA. Thus, it was proposed by Chien et al., that additional proteins probably interact with the basic region to provide the specificity of activity (Chien et al., 1996). In addition, studies with the muscle-specific bHLH factor MyoD, indicated that specific residues in the basic region are important in promoting non-muscle cells to differentiate into muscle cells (Weintraub et al., 1991). In contrast, in *Xenopus* the domain responsible for differences in the ability of Xash1 and X-ngnr1 (*Xenopus* homologs of Mash1 and Ngn) to induce specific targets in neural development mapped to helix 1 of the HLH domain (Talikka et al., 2002). Together these data demonstrate the importance of the bHLH domain to the function of Class II bHLH transcription factors but suggest that different domains within the bHLH are important in different cellular contexts.

For the studies reported here, we have exploited an over-expression paradigm, in ovo chick electroporation, to define distinct activities of the neural bHLH factors Mash1 and Math1. We show that each of these factors induces neuronal differentiation as defined by cell cycle exit, translocation of the cells laterally out of the ventricular zone, and expression of neuronal markers. However, overexpression of each neural bHLH factor directs distinct changes to the composition of the dorsal interneuron sub-types formed. The domains responsible for the different activities map not to the DNA interaction domains but to the HLH protein-protein interaction domains. The importance of the HLH domain for the specificity of function of each bHLH factor, rather than the DNA interaction domain, suggests that context-dependent protein-protein interactions are important for the distinct function of each neural bHLH factor.

Materials and methods

In ovo chick electroporation

Fertilized White Leghorn eggs were obtained from the Texas A&M Poultry Department (College Station, TX) and incubated at 37°C for 3 days. Solutions of supercoiled plasmid DNA (2 mg/ml) in PBS/0.02% Trypan Blue were injected into the lumen of the closed neural tube and electroporated into epithelial cells on one side of the neural tube by placing electrodes on either side of the embryo (Timmer et al., 2001). Square-wave current (three 50-msecond pulses of 25 mV) was generated using a BTX (San Diego, CA) T820 electroporator connected to 4-mm gold electrodes. A GFP expression vector (CMV-EGFP; Clontech) was co-injected as a control to monitor efficiency and extent of electroporation. Injection and electroporation were performed at Hamburger and Hamilton stages 14-16 (HH14-16) (Hamburger and Hamilton, 1951). 24 hours later (HH23-24), embryos were harvested, fixed in 4% formaldehyde for 2 hours at 4°C, rinsed in PBS, sunk in 30% sucrose, embedded in OCT

(Tissue Tek), and cryosectioned at 30 µm. All sections shown were taken between the upper and lower limb regions. Embryos were processed for immunofluorescence as described below. For each experiment, multiple sections from at least three electroporated embryos were analyzed.

Immunofluorescence

Immunofluorescence was performed by overnight incubation with the appropriate dilution of primary antibody in PBS/1% goat serum/0.1% Triton X-100 at 4°C, followed by hybridization with appropriate secondary goat-anti-rabbit or goat-anti-mouse IgG, conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, Inc.). Primary antibodies used for this study include: mouse anti-Mash1 (Lo et al., 1991), rabbit anti-LH2A/B (Lhx2/9) (Liem et al., 1995), mouse anti-BOSS1 (Krämer et al., 1991), mouse anti-BrdU (Becton-Dickinson, Inc), mouse anti-c-Myc (9E10 Santa Cruz Biotechnology), rabbit anti-Pax2 (Zymed), Tuj1 (Lee et al., 1990), and from the Developmental Studies Hybridoma Bank, 39.4D5 mouse anti-Islet1 and 4F2 mouse anti-Lim1/2 (Lhx1/5). In experiments where anti-BrdU was used, HH22-23 chick embryos were injected with 50 µl of 100 mM BrdU solution 1 hour prior to sacrifice. Sections from BrdU-injected embryos were treated with 2 N HCL for 15 minutes to denature the DNA, followed by a 15-minute neutralization step with 0.1 M sodium borate pH 8.5, prior to the addition of primary antibody. Imaging of the immunofluorescence was performed by confocal analysis on a Bio-Rad MR-1024 confocal microscope. GFP signal was imaged using the standard FITC filter.

Plasmid description

All electroporations utilized the expression vector pMiwIII, which drives expression through a chick β-actin promoter (Muramatsu et al., 1997). The coding regions of rat *Mash1* and mouse *MyoD* were cloned into the pMiwIII expression vector using convenient restriction sites. With the exception of rat *Mash1*, all genes inserted into the pMiwIII vector were myc-tagged (5 copies) at the N terminus. All amino acid swaps between Mash1/MyoD or Mash1/Math1 were made by recombinant PCR (Erlich, 1989; Higuchi et al., 1988). Mash1 DNA binding mutant, Mash1NR-AQ, changed two amino acids (NR to AQ) in the basic region similar to that previously described for Ngn1AQ (Gowan et al., 2001; Sun et al., 2001). Two additional Mash1 DNA binding mutants (Mash1R-G and Mash1E-G) were generated that mutated residues predicted to contact DNA based on modeling Mash1 to the MyoD crystal structure (Ma et al., 1994). PCR products were digested with *Nco*I and *Xba*I, purified, and ligated to *Nco*I- and *Xba*I-digested pMiwIII-(myc)5 expression vector. The bHLH domain of Math1 or Mash1 was fused to the myc-tagged VP16 activation cassette (Triezenberg et al., 1988) or the myc-tagged EnR repressor domain (Smith and Jaynes, 1996). Note, Mash1 bHLH fusions only functioned when Mash1 sequences were N-terminal to the VP16 or EnR cassettes. In addition, five amino acid residues N-terminal to the Mash1 bHLH domain shown in Fig. 4 (PAAVA) were required for Mash1bHLH-VP16 or -EnR fusions to function. All constructs generated by PCR were sequence verified before use, and protein expression was verified by immunofluorescence with antibodies to the epitope tag myc or to Mash1. The precise sequences of the bHLH regions used for the chimeric proteins and mutants are shown.

Modeling Mash1 structure

The Mash1 primary sequence was submitted to the 3D-pssm web server (http://www.sbg.bio.ic.ac.uk/~3dpssm/) where it was matched to known secondary structures (Fischer et al., 1999; Kelley et al., 1999; Kelley et al., 2000). The 3D-pssm program predicted that the best overall structural match was to the MyoD homodimer (Ma et al., 1994). A feature of the program allowed a MyoD-based modeling of the Mash1 secondary structure. This structure was positioned in 3D-Mol Viewer (Vector NTI Suite 7.0), and then transferred to Adobe Illustrator for labeling.

Results

Neural bHLH factors induce neuronal differentiation in the neural tube

In the developing neural tube, proliferating precursor cells reside in the ventricular zone. As these cells differentiate they move laterally out of the ventricular zone, exit the cell cycle, and begin to express neuronal-specific markers. Using these three criteria for neuronal differentiation, we demonstrate that overexpression of neural bHLH factors by in ovo electroporation of DNA into chick neural tube cells induced the cells to undergo neuronal differentiation within 24 hours. Electroporation of a control expression vector at stage HH14-16, followed by harvest 24 hours later, revealed electroporated cells throughout the ventricular zone as well as lateral to this zone on the electroporated side of the neural tube (Fig. 1A, red). In contrast, electroporation of expression vectors encoding the neural bHLH factors Mash1 or Math1 resulted in the electroporated cells being preferentially located lateral to the ventricular zone in the mantle layer where differentiating neurons reside (Fig. 1C,E, red). Furthermore, these electroporated cells had begun to express a marker of neuronal differentiation, Tuj1 (Fig. 1C,E,Q), and have exited the cell cycle as determined by the lack of BrdU incorporation (Fig. 1D,F,R). Although Mash1 appears to function more efficiently in this assay than Math1, by all three criteria, cells with excess expression of each of these neural bHLH factors are induced to undergo neuronal differentiation. This neuronal differentiation activity of Mash1 and Math1 is also mimicked by Ngn1 (data not shown) and Ngn2 (Dubreuil et al., 2002). The bHLH factors require DNA binding for their neurogenic activity as demonstrated by the loss of this activity in DNA binding mutants for Mash1 (Fig. 1G-J,Q,R). Furthermore, not all class II bHLH factors have this activity since MyoD, a myogenic bHLH factor, did not induce neuronal differentiation (Fig. 1K,L,Q). MyoD did, however, moderately reduce proliferation in the ventricular zone as seen in the reduction in the percentage of BrdU-expressing electroporated cells relative to the control (Fig. 1R), consistent with previous reports that MyoD induces cell-cycle exit (Lassar et al., 1994).

Differences in phenotype were noted with the different DNA binding mutants. The mutant Mash1NR-AQ was modeled after NgnAQ (Sun et al., 2001) and mutates two amino acid residues in the basic region just at the junction with helix 1. This mutation in Mash1 resulted in a loss of activity, since it was indistinguishable from the control (Fig. 1A,B,G,H,Q,R). The other two DNA binding mutants (Mash1R-G and Mash1E-G), each with a single amino acid change in a residue predicted to contact DNA (Ellenberger et al., 1994; Ma et al., 1994), also were unable to induce neuronal differentiation (Fig. 1I,Q and Fig. 4), however, in these cases there appeared to be a dominant negative phenotype. Rather than the electroporated cells being uniformly distributed throughout the neural tube as in controls, they were biased to the ventricular zone at the expense of the lateral regions containing the neurons (Fig. 1I,Q, and data not shown). This phenotype for a DNA binding mutant is consistent with Mash1 normally forming heterodimers with another protein, such as one of the E-proteins, in vivo. The lack of this effect with Mash1AQ suggests that this latter mutant blocks interaction of Mash1 with an in vivo partner.

Mash1 and Math1 induce formation of different neuronal populations

In a previous study, we demonstrated that Math1 and Ngn1 have distinct roles in the specification of interneurons in the dorsal neural tube (Gowan et al., 2001; Helms and Johnson, 2003). This suggests that neural bHLH factors share a role in inducing neuronal differentiation but have distinct roles in neuronal subtype specification. To determine whether Mash1, like Math1 and Ngn1, has a specific role in inducing a distinct interneuron population, we used double immunofluorescence with antibodies to LIM homeodomain (HD) factors that have been used to identify interneuron subtypes based on position in the dorsoventral axis, their initial migratory patterns, and their expression of specific LIM HD transcription factors (Fig. 2M) (Gross et al., 2002; Helms and Johnson, 2003; Müller et al., 2002). We found that overexpression of Mash1 increased dI3 neurons and decreased dI1, dI2 and dI4/6 neurons. This was detected as a 3-fold increase in Islet1 (dI3 marker) on the electroporated side (Fig. 2A,K, green), and a decrease in the number of Lhx2/9-expressing cells (dI1) (Fig. 2A,I, red), Lhx1/5+;Pax2– cells (dI2) (Fig. 2B,J, red) and Lhx1/5+;Pax2+ cells (dI4/6) (Fig. 2B,L, yellow). The increase in Islet1 was due to an increase in the dI3 population since MNR, a marker co-expressed with Islet1 in motor neuron populations was not increased (data not shown). Mash1-expressing progenitor cells are adjacent to dI3, dI4 and dI5 interneurons in the dorsal spinal cord (Gross et al., 2002; Müller et al., 2002). Markers for dI5 neurons were not included in this study, and the dI4 and dI6 populations are not easily distinguishable with the markers used. As a control, the inactive Mash1 mutant (Mash1NR-AQ) had no effect on the composition of these interneuron populations (Fig. 2C,D,I-L). The DNA binding mutant Mash1R-G that appeared to inhibit neuronal differentiation (see above), decreased dI1 and dI3 but not the Lhx1/5 containing interneuron populations relative to the control side (Fig. 2E,F,I-L). It is not clear why the Lhx1/5 (dI2) population was unaffected. One possibility is that Ngn1, which is known to be required for the dI2 population (Gowan et al., 2001), efficiently binds E-box DNA as a homodimer (R.M.H. and J.E.J., unpublished data). This is in contrast to Mash1 and Math1 that bind E-box DNA efficiently only as heterodimers with E-proteins. Thus, the dominant negative activity of the Mash1R-G mutant, which is probably the result of its ability to sequester the Class II bHLH heterodimer partners (E-proteins), would not affect the activity of the Ngn1 homodimer, and thus, dI2 interneurons would be unaffected.

In contrast to Mash1 activity in increasing dI3 neurons, Math1 overexpression resulted in an increase in the dI1 (Lhx2/9) neurons (Fig. 2G,I, red) (Gowan et al., 2001), at the expense of dI2 (Lhx1/5) and dI3 (Islet1) interneurons (Fig. 2G,H,K, green). These data complement parallel studies demonstrating that Ngn1 overexpression results in neuronal differentiation with an increase in dI2 (Lhx1/5) interneurons (Gowan et al., 2001) at the expense of dI1 (Lhx2/9) and dI3 (Islet1) (Gowan et al., 2001) (data not shown). Thus, although Mash1, Math1, and Ngn1 all induce neuronal differentiation, they have distinct activities in specifying dorsal interneurons (see diagram Fig. 2M).
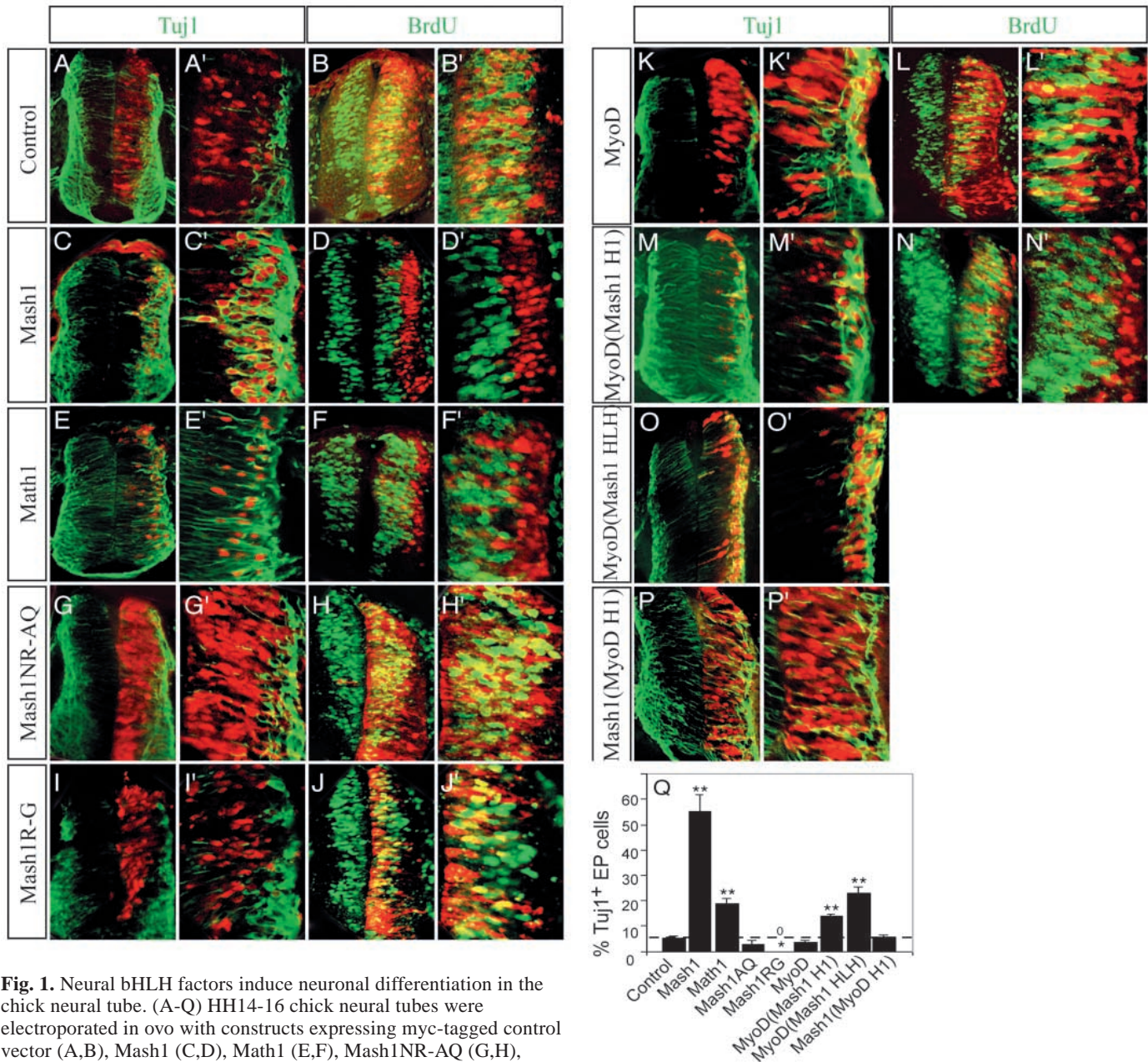
Fig. 1. Neural bHLH factors induce neuronal differentiation in the chick neural tube. (A-Q) HH14-16 chick neural tubes were electroporated in ovo with constructs expressing myc-tagged control vector (A,B), Mash1 (C,D), Math1 (E,F), Mash1NR-AQ (G,H), Mash1R-G (I,J), MyoD (K,L), MyoD(Mash1H1) (M,N), MyoD(Mash1HLH) (O), Mash1(MyoDH1) (P) and harvested 24 hours later. Cross sections through the neural tube are shown at 20× (A-P) and 40× (A′-P′). Immunofluorescence with anti-Mash1 or anti-myc antibodies indicates the expression of the transgene in the electroporated cells (A-P, red). Double labeling with the Tuj1 antibody was used to detect differentiating neurons (A,C,E,G,I,K,M,O,P, green), or with anti-BrdU to detect proliferating cells in the ventricular zone (B,D,F,H,J,L,N, green). (Q) The percentage of electroporated cells expressing Tuj1. (R) The percentage of electroporated cells incorporating BrdU. Expression of Mash1, Math1, MyoD(Mash1H1), and MyoD(Mash1HLH) caused cells to move laterally out of the ventricular zone, express the neuronal marker Tuj1, and exit the cell cycle. These activities require DNA binding since the DNA binding mutants Mash1NR-AQ and Mash1R-G lack these activities. Expression of the non-neural bHLH MyoD and Mash1(MyoDH1) also lacked these activities. The electroporated side is on the right in A-P. **$P<0.001$, *$P<0.01$.
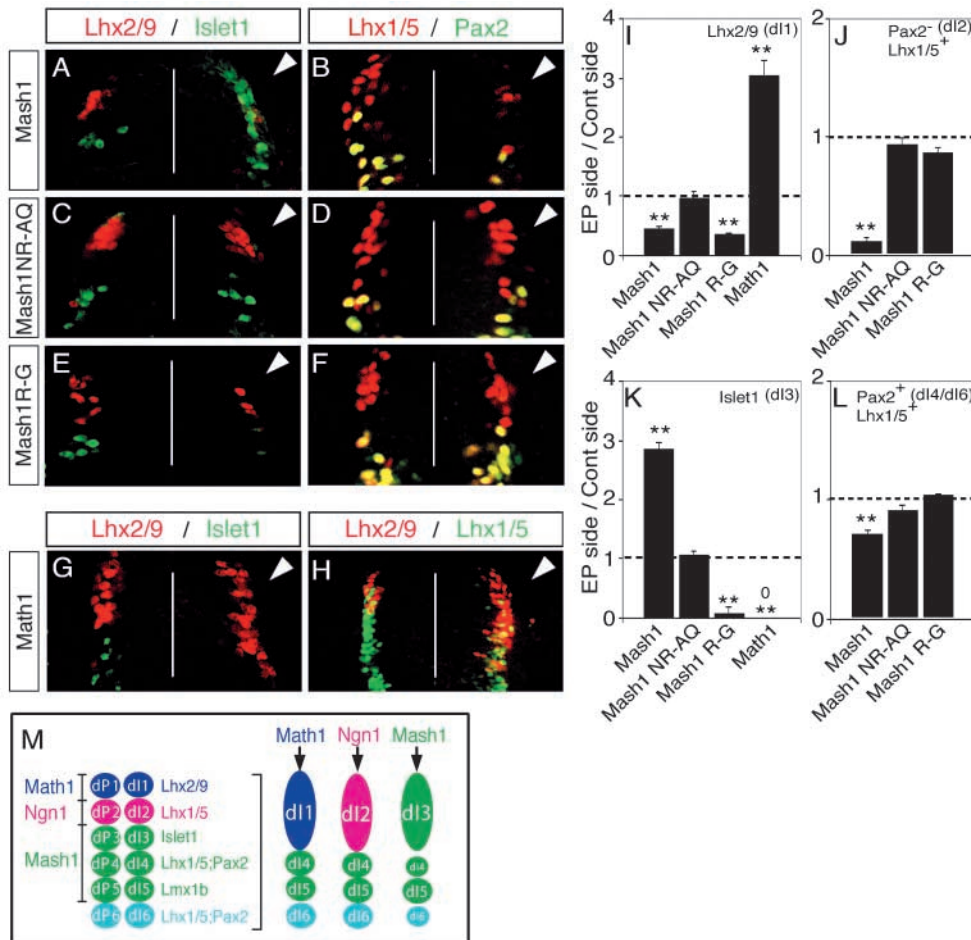
Fig. 2. Neuronal sub-type specification activity of the neural bHLH factors. (A-H) HH14-16 chick neural tubes were electroporated in ovo with expression constructs Mash1 (A,B), Mash1NR-AQ (C,D), Mash1R-G (E,F) and Math1 (G,H), and harvested at 24 hours. Immunofluorescence with antibodies to Lhx2/9, Islet1, Lhx1/5, or Pax2 as indicated illustrate dorsal interneuron populations dI1-dI3, dI4/6 (see diagram in M). dI2 cells are distinguished from dI4 and dI6 by their lack of Pax2 expression (B,D,F, red cells). In each case the electroporated side is on the right (arrowhead) and should be compared with the control side on the left. The white line indicates the midline. (I-L) Quantification of the data in A-H. Mash1 increases dI3 neurons (A) at the expense of dI1 (A) and dI2 (B) neurons. This activity of Mash1 requires Mash1 to bind DNA since the Mash1NR-AQ and Mash1R-G mutants, which lack DNA binding, do not have this phenotype (C-F). Math1 increases dI1 (G, H) at the expense of dI2 (H) and dI3 (G) neurons. (M) Diagram of different populations of progenitors (dP1-dP6 defined by bHLH expression) and interneurons (dI1-dI6 defined by LIM HD factors), and a summary of how the interneurons change in response to overexpression of the bHLH factors. **$P<0.001$.

Math1 and Mash1 act as transcriptional activators in inducing neuronal differentiation and in specifying dorsal interneurons

Class II bHLH factors are classically reported to be transcriptional activators. To determine whether Math1 and Mash1 were acting as activators in the neuronal differentiation and cell-type specification functions, we generated chimeric proteins with the bHLH domains of Math1 or Mash1 fused to either the VP16 activation domain (Triezenberg et al., 1988) or the engrailed repressor (EnR) domain (Smith and Jaynes, 1996). Myc-tagged control vectors containing VP16 (Fig. 3A,B) or EnR (Fig. 3G,H), had no effect on neuronal differentiation or alterations in cell-type composition when electroporated into HH14-16 stage chick neural tubes. As a readout of neuronal differentiation, we measured the fluorescence intensity of electroporated cells in the lateral (differentiation zone) compared with that in the medial (proliferation zone) regions (Fig. 3M,N). However, when these activator or repressor domains were fused to the bHLH domains of Math1 or Mash1, specific phenotypes were observed. Math1bHLH-VP16 phenocopied the activity of wild-type Math1 in both inducing the lateral movement of cells and inducing specific cell-types to form (Fig. 3C,D,M,N). The electroporated cells were preferentially found lateral to the ventricular zone suggesting a bias towards neuronal differentiation (Fig. 3C,M), and there was a dramatic increase

in dI1 (Lhx2/9) neurons at the expense of dI3 (Islet1) neurons (Fig. 3D,N). In addition, Math1bHLH-EnR inhibited cells from differentiating as seen by the electroporated cells being biased to the ventricular zone rather than being distributed uniformly as in controls (Fig. 3 compare I with G,M). Math1bHLH-EnR preferentially decreased dI1 (Fig. 3J,N). Mash1 also acts as an activator to induce neuronal differentiation and specify dI3 interneurons since Mash1bHLH-VP16 phenocopied full-length Mash1, albeit not as efficiently (Fig. 3E,F,M,N), and Mash1bHLH-EnR inhibited neuronal differentiation, just as seen with Math1bHLH-EnR (Fig. 3E,M). In addition to confirming a role for Math1 and Mash1 as transcriptional activators, these data demonstrate that sequences outside the Math1 and Mash1 bHLH domains are not required, but may modulate, their specificity of function in this assay.

Helix 1 of Mash1 is the critical domain for the neuronal differentiation activity

Of the Class II bHLH factors, only the neural bHLH factors, Mash1 and Math1 but not the non-neural bHLH factor MyoD, induced neuronal differentiation in chick neural tubes. To identify the important structural domain of the neural bHLH factors required for this neurogenic activity, we used chimeric proteins between Mash1 and the muscle-specific bHLH factor, MyoD. Full-length Mash1 or MyoD were modified such that
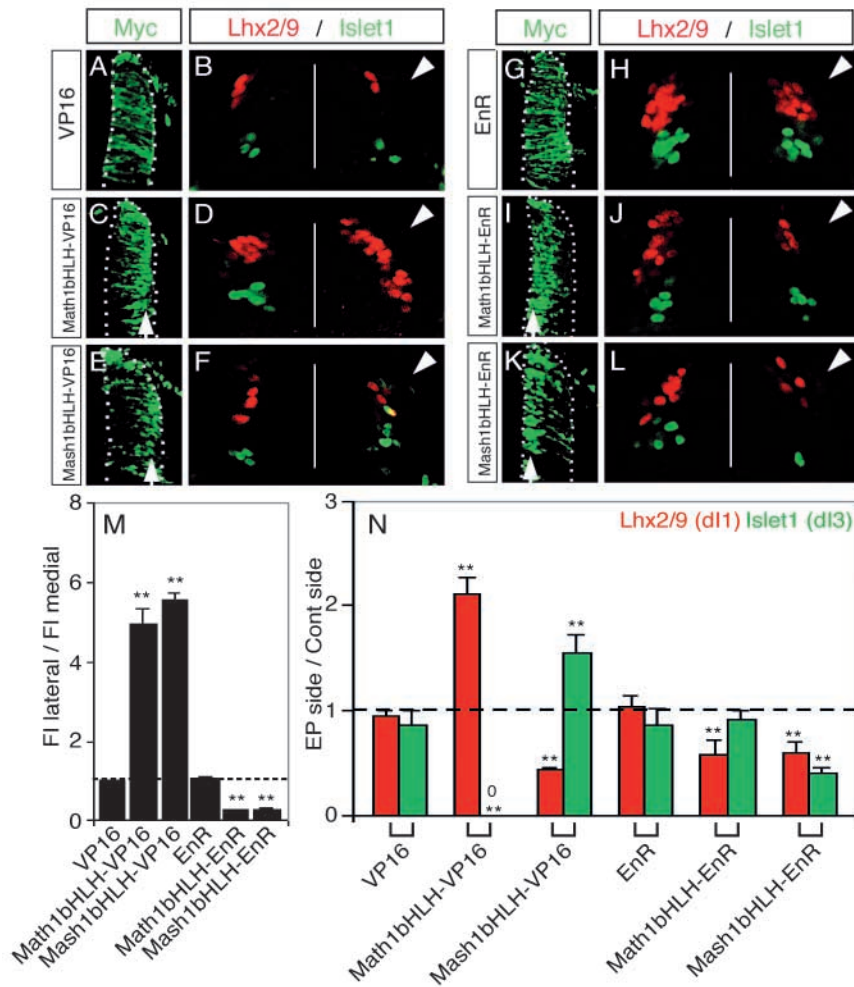
Fig. 3. Math1 and Mash1 are transcriptional activators in their neurogenic and neuronal cell-type-specific activities. (A-L) HH14-16 chick neural tubes were electroporated in ovo with expression constructs VP16 (A,B), Math1bHLH-VP16 (C,D), Mash1bHLH-VP16 (E,F), EnR (G,H), Math1bHLH-EnR (I,J) and Mash1bHLH-EnR (K,L), harvested at 24 hours. (A,C,E,G,I,K) Immunofluorescence using anti-myc antibodies to assay for expression of the transgene demonstrates the movement of the cells to the lateral neural tube where differentiating neurons reside. White dots outline the injected side of the neural tube and arrows indicate the lateral (C,E) or medial (I,K) position of the electroporated cells within the neural tube. (M) Quantification of these data shown as a ratio of fluorescence intensity (FI) in the lateral half versus the medial half of the neural tube (M). (B,D,F,H,J,L) Interneuron populations dI1 and dI3 were detected using anti-Lhx2/9 (red) or Islet1 (green). (N) Quantification of these data shown as a ratio of the number of labeled cells on the electroporated side (right, indicated by arrowhead) versus the number of labeled cells on the control side (left). VP16 and EnR on their own have no effect in these assays. Math1bHLH-VP16 and Mash1bHLH-VP16 approximate the activity of full-length Math1 and Mash1 in both the neuronal differentiation and cell-type specification activities. In contrast, Math1bHLH-EnR and Mash1bHLH-EnR have the opposite effect. **P<0.001.

subdomains of one protein were swapped with the comparable region from the other (Fig. 4A). The changes made are color coded in Fig. 4, with Mash1 amino acid residues in blue, MyoD amino acid residues in red, Math1 amino acid residues green, and basic region mutations in black. Each construct was Myc-tagged and cloned into the pMiwIII expression vector and electroporated into chick neural tubes with a CMV-GFP reporter for efficient detection of the electroporated cells. The lateral expression of the co-expressed GFP reporter was used as a simple qualitative assay for the neuronal differentiation phenotype. For quantification, fluorescence intensity was measured using Metamorph software comparing the lateral half with the medial half of the neural tube. These measurements cannot distinguish subtle differences in the activity of the constructs as detected by cell counts in Fig. 1, but rather serves to score the activity of each chimeric protein relative to Mash1 or MyoD. The data are summarized in Fig. 4A, representative sections are shown in Fig. 4B-M, and Fig. 4N and 4O show the quantification of these data.

The basic region binds DNA and is required for the activity of bHLH transcription factors as shown here for Mash1. However, it is the Mash1 HLH domain (Fig. 4D) not the basic region (Fig. 4C) that is specifically required for Mash1 to retain its activity in this assay. Furthermore, the Mash1 HLH, not the basic region, confers onto MyoD the ability to induce cells to

translocate laterally (Fig. 4I,J). Math1 HLH also confers this activity to MyoD (Fig. 4A,O). These results indicate that the HLH domains of Mash1 and Math1 contain specific information necessary to induce the lateral movement of cells in the developing chick neural tube, suggesting their importance in neuronal differentiation.

To further define which subdomain within the Mash1 HLH region is required for this activity, individual helix 1, loop and helix 2 domain swaps between Mash1 and MyoD were tested (Fig. 4E-G,K-M). The data clearly demonstrate that helix 1 of Mash1 is required for the lateral movement of the cells from the ventricular zone (Fig. 4E-G,N), and is sufficient to confer this activity to MyoD (Fig. 4K-M,O). To verify that helix 1 of Mash1 was sufficient in the context of the MyoD protein to confer multiple aspects of the neuronal differentiation phenotype, we also determined that the electroporated cells were more likely to express the neuronal marker Tuj1 than controls (Fig. 1M,Q) and had reduced potential for incorporating BrdU (Fig. 1N,R). Although this chimeric protein was not as efficient as wild-type Mash1, it still conferred this activity to MyoD. Even the Mash1 HLH domain in MyoD did not completely recapitulate the level of activity of Mash1 seen in this assay (Fig. 1Q). Furthermore, Mash1 with its helix 1 replaced by MyoD helix 1 completely lost its ability to induce neuronal differentiation (Fig. 1P,Q). Together,

these data demonstrate the important domain for inducing neuronal differentiation is largely encoded in the fifteen amino acids of helix 1 of Mash1.

We also tested a subset of helix 1 amino acids for their role in this phenotype. We chose amino acids based on sequence comparison between neural bHLH factors and the non-neural MyoD bHLH. Five amino acids were tested (Fig. 4A), however, no specific amino acid was identified that was essential for the lateral movement activity of Mash1.

Helix 2 of Math1 is the critical domain for specifically inducing dI1 neurons

Helix 1 of Mash1 was identified as a necessary and sufficient domain for induction of neuronal differentiation, an activity shared between the neural bHLH factors. We next set out to determine if this domain, or a distinct domain, encodes the information for the neuronal subtype specificity function attributed to each neural bHLH factor using the dI1 and dI3 populations as the readout. Chimeras between Math1 and
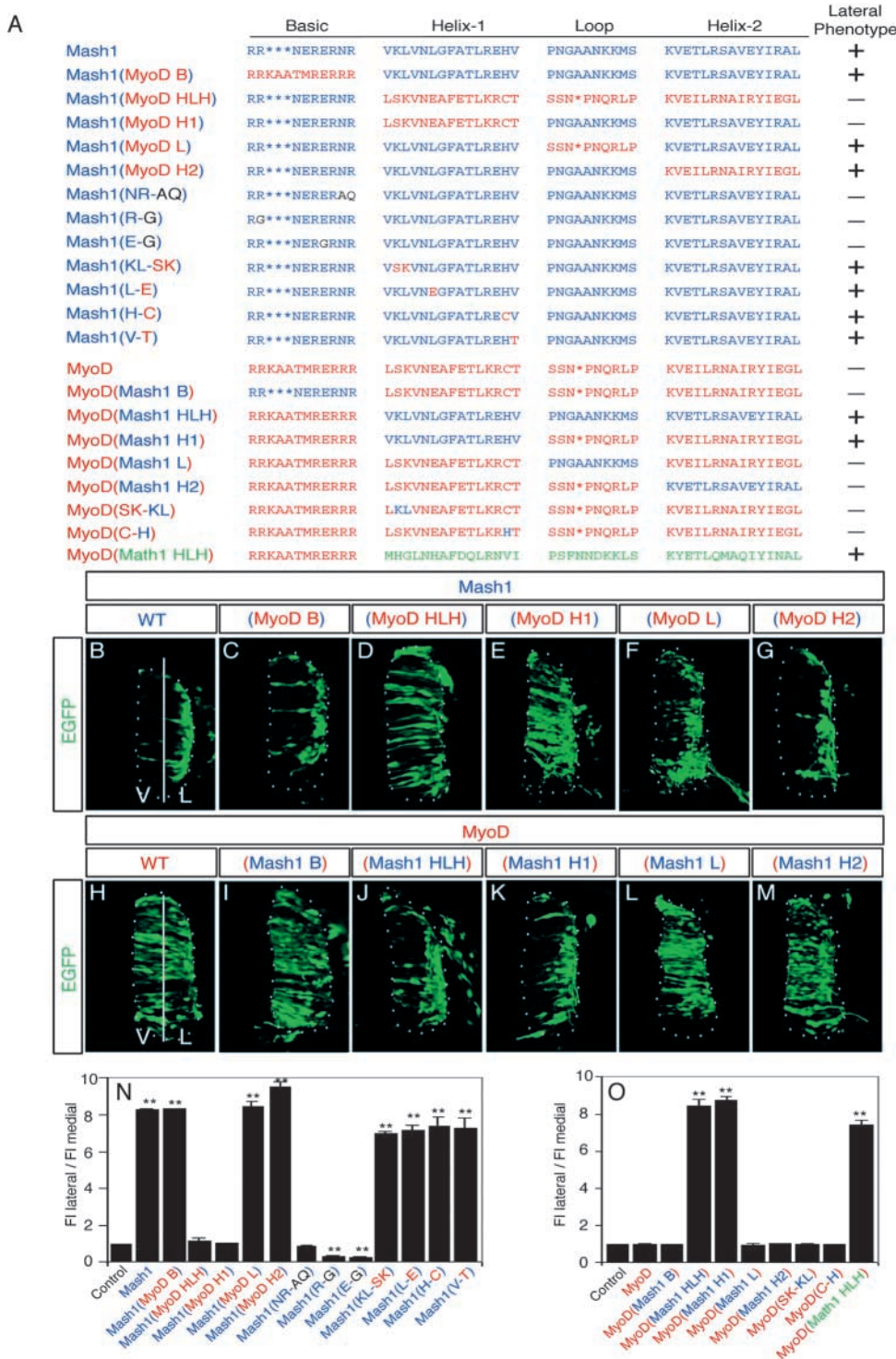

Fig. 4. Essential structural component of Mash1 required for its neuronal differentiation function is identified. (A) Schematic of the Mash1 and MyoD chimeric bHLH domains used to identify amino acids required for the neuronal differentiation activity of Mash1. Mash1 sequences are shown in blue and MyoD sequences are in red. The first ten constructs are in the context of Mash1 with the domain replaced by MyoD sequences shown in parentheses. The next ten constructs are in the context of MyoD with the domain replaced by Mash1 sequences shown in parentheses. One MyoD/Math1 chimeric protein was tested; the Math1 HLH sequence is shown in green. Mash1NR-AQ, Mash1R-G, and Mash1E-G (black) are DNA binding mutants. The ability of each chimeric protein to drive neuronal differentiation was assayed by the fluorescence intensity of GFP found preferentially in the lateral half of the neural tube (+). (B-M) Representative sections from chick embryos expressing the chimeric proteins. B-G are in the context of full-length Mash1, and H-M are in the context of full-length MyoD. The white dots outline the electroporated side of the neural tube. Ventral is on the left in each panel. Expression of each construct was verified using immunofluorescence with myc antibody (data not shown). (N,O) Quantification of these data shown as a ratio of fluorescence intensity (FI) in the lateral half versus the medial half of the neural tube. Helix 1 of Mash1 is necessary (E) and sufficient (in the context of MyoD) (K) for the neuronal differentiation phenotype. **$P<0.001$.

Mash1 were analyzed for their effect on the number of dI1 (Lhx2/9) and dI3 (Islet1) interneurons. Full-length Math1 or Mash1 were modified such that subdomains of one protein were swapped with the comparable subdomains from the other (Fig. 5A). The changes made are color coded in Fig. 5, with Math1 amino acid residues in green and Mash1 amino acid residues in blue. Each construct was Myc-tagged, cloned into the pMiwIII expression vector, and electroporated into chick neural tubes. The data are summarized in Fig. 5A and quantification of data is shown in the graph (Fig. 5B).

One prediction was that the basic region that interacts with DNA would confer specificity of function of each bHLH factor as suggested from studies of Atonal and Scute in *Drosophila* (Chien et al., 1996). However, just as with the neuronal differentiation activity of Mash1, the HLH is the critical domain, not the basic region (Fig. 5A). Math1 with the HLH from Mash1 has the Mash1 phenotype of increased dI3 (Islet1) and decreased dI1 (Lhx2/9), and conversely, Mash1 with the

HLH from Math1 has the Math1 phenotype of increased dI1 (Lhx2/9) and decreased dI3 (Islet1) (Fig. 5B).

Further dissection of the HLH revealed that helix 2 of Math1 is both necessary and sufficient (in the context of Mash1) to increase the number of dI1 neurons. Replacing Math1 helix 2 with Mash1 helix 2 resulted in a decrease rather than increase in dI1 neurons (Fig. 5). Furthermore, Math1 helix 2 in Mash1 resulted in an increase in dI1 neurons as predicted for a Math1 phenotype (Fig. 5). This increase in dI1 neurons was not as robust as that seen with full-length Math1 but it dramatically converted the Mash1 phenotype of repressing dI1 to increasing dI1. Exchanging helix 1 or the loop region of Math1 had no effect on the cell-type specification properties of the protein (Fig. 5). Thus, Math1 helix 2 appears to be a critical domain for Math1 effects on the dI1 interneuron population.

Math1 helix 2 in the context of Mash1 was not sufficient, however, to induce a decrease in the dI3 population as was the case when the whole Math1 HLH domain was tested in Mash1.
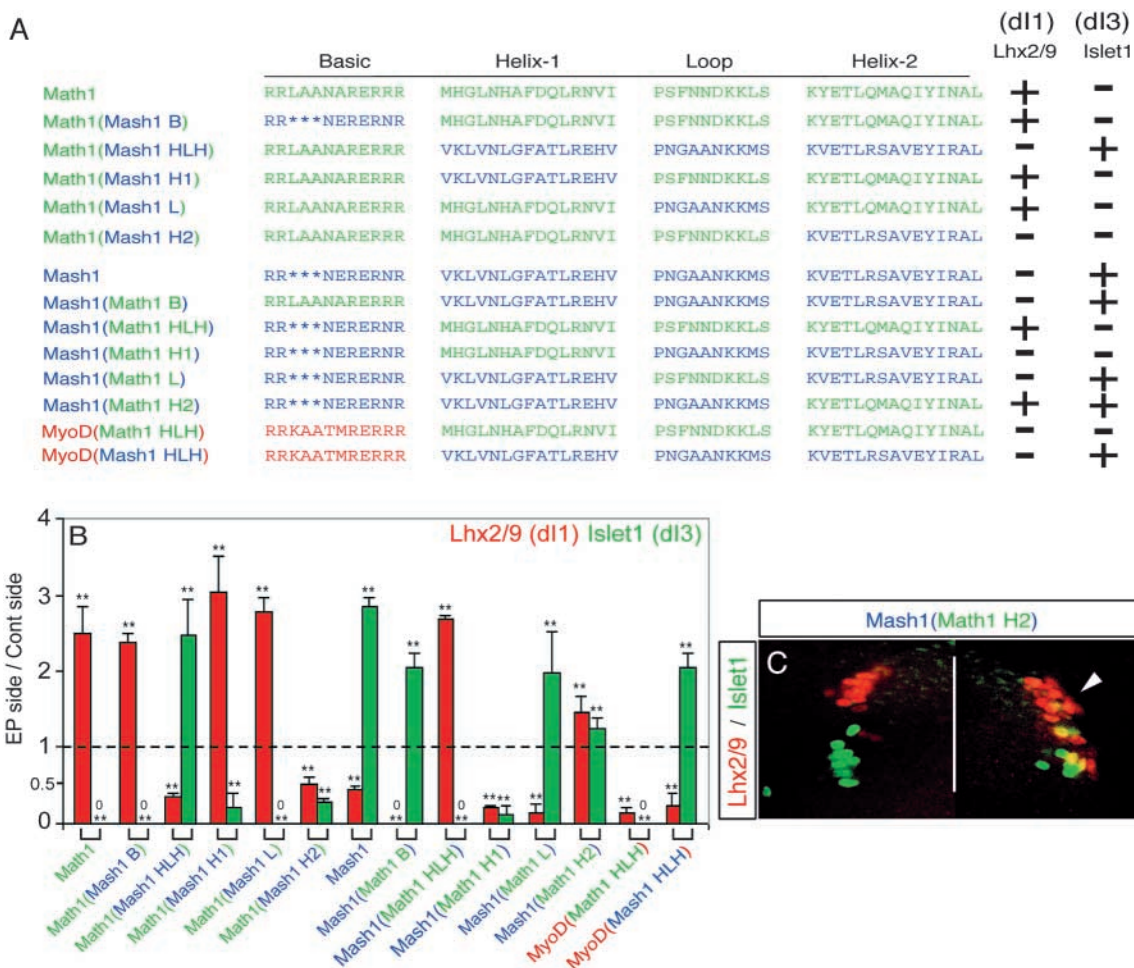

Fig. 5. Essential structural components of Math1 and Mash1 required for their neuronal specification properties are identified. (A) Schematic of the Math1 and Mash1 chimeric bHLH domains used to identify amino acids required for their neuronal specification properties. Math1 sequences are shown in green and Mash1 sequences are in blue. The first six constructs are in the context of Math1 with the domain replaced by Mash1 sequences shown in parentheses. The next six constructs are in the context of Mash1 with the domain replaced by Math1 sequences shown in parentheses. Math1 HLH (green) and Mash1 HLH (blue) were also tested in the context of MyoD (red). The effect of each chimeric protein, in increasing (+) or decreasing (–) dI1 and dI3 interneuron populations as described in Fig. 2, is shown. (B) Quantification of Lhx2/9- and Islet1-expressing cells in the electroporated side versus the control side for each construct. **$P<0.001$. (C) Double immunofluorescence with Lhx2/9 (red) and Islet1 (green) showing the aberrant co-expression (yellow) of these HD factors in neural tubes electroporated with Mash1 with the helix 2 of Math1.
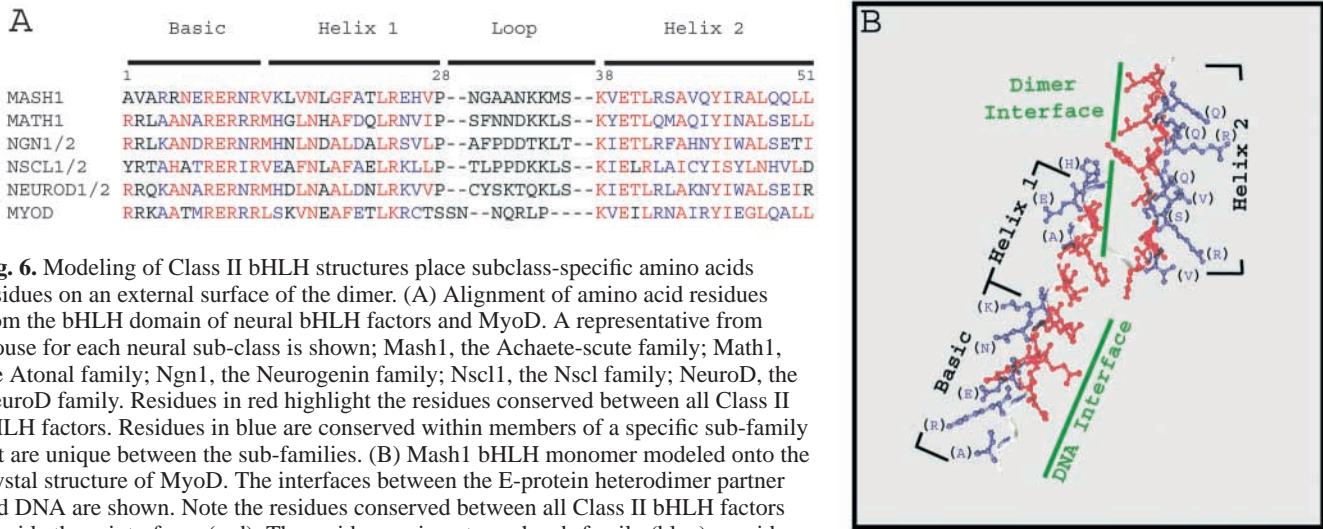
A

	Basic	Helix 1	Loop	Helix 2

Fig. 6. Modeling of Class II bHLH structures place subclass-specific amino acids residues on an external surface of the dimer. (A) Alignment of amino acid residues from the bHLH domain of neural bHLH factors and MyoD. A representative from mouse for each neural sub-class is shown; Mash1, the Achaete-scute family; Math1, the Atonal family; Ngn1, the Neurogenin family; Nscl1, the Nscl family; NeuroD, the NeuroD family. Residues in red highlight the residues conserved between all Class II bHLH factors. Residues in blue are conserved within members of a specific sub-family but are unique between the sub-families. (B) Mash1 bHLH monomer modeled onto the crystal structure of MyoD. The interfaces between the E-protein heterodimer partner and DNA are shown. Note the residues conserved between all Class II bHLH factors provide these interfaces (red). The residues unique to each sub-family (blue) provide surfaces for additional interactions.

Rather, Math1 helix 2 in Mash1 caused a slight increase in the number of dI3 neurons on the electroporated side (Fig. 5). Since dI1 and dI3 both increased, we examined whether the Islet1 and Lhx2/9 populations remained in distinct populations as in controls. Using double immunofluorescence we detected interneurons inappropriately co-expressing these LIM HD factors, a situation that is not found normally (Fig. 5C). Thus, the increase in dI1 and decrease in dI3 phenotypes seen with Math1 overexpression are not always coupled, and in this case, have been separated as cells inappropriately express both markers. Taken together, although helix 2 of Math1 is the critical domain for the activity of increasing the dI1 interneuron population, the whole HLH domain of Math1 is necessary to completely switch Mash1 activity to Math1. Importantly, the HLH domain of Math1 is not sufficient in the context of the non-neural Class II bHLH MyoD to confer the complete neuronal specification program since with this chimeric protein the number of dI1 interneurons decrease instead of increase (Fig. 5).

Both helix 1 and helix 2 of Mash1 are required for controlling the number of dI3 neurons

The HLH but not the basic region of Mash1 is necessary for the increase in dI3 neurons, and the decrease in the dI1 neurons (Fig. 5), and furthermore, the HLH of Mash1 is sufficient in the context of Math1 and in the context of MyoD to alter the phenotype to the Mash1 phenotype (Fig. 5). Further dissection of functional domains revealed that the dI3 phenotype depends on both helix 1 and helix 2 of Mash1. Mash1 helix 1 is necessary for the increase in the dI3 neurons but not the decrease in dI1 neurons, and it is not sufficient in the context of Math1 to cause the increase in dI3. Likewise, Mash1 helix 2 is necessary for the normal increase in the dI3 population seen with wild-type Mash1, but it is not sufficient in the context of Math1 for this phenotype. Changes in the loop region are inconsequential to the specification function of these factors. Together these data suggest that information encoded in both helix 1 and helix 2 is required for the specification activities of Mash1.

Modeling of Class II bHLH structures place subclass specific amino acids residues on an external surface of the dimer

In order to gain insight into how the bHLH region may function in neural differentiation and specification, we aligned the bHLH region of known neural bHLH proteins and MyoD. This alignment included all known species homologs and is shown in Fig. 6A using the mouse sequence to represent each sub-family. The alignment highlights amino acids that are conserved globally in all Class II bHLH factors (red), and those that are conserved only within a specific sub-family (blue). The conserved amino acids were then mapped onto an to a computer generated 3D structure of Mash1, modeled on the 3D crystal structure of MyoD (Ma et al., 1994) (see Materials and methods). This reveals a clear difference in the spatial distribution of the two types of conserved amino acids. The globally conserved residues are positioned at either the DNA or E-protein dimerization interface (Fig. 6B). In contrast, the conserved residues specific to a sub-family, project away from these interfaces creating surfaces that are accessible to potential co-factors.

Discussion

Neural bHLH transcription factors play essential roles in nervous system development. The technical innovation of in ovo electroporation in chick embryonic neural tube (Muramatsu et al., 1997) has provided an assay to distinguish functions of the neural bHLH proteins in vivo not obvious from DNA binding activities in vitro. Using this assay, we have defined shared and distinct activities for Mash1 and Math1 when over-expressed in spinal cord development. Further, we have used the distinct activities of Mash1 and Math1 to identify structural components of the proteins that are necessary for the shared neuronal differentiation activity versus the distinct activities in neuronal cell-type specification. The domains required for the neural specific functions map not to the basic region important for DNA binding but rather the HLH domain that is involved in protein-protein interactions. Unique

functions for helix 1 and helix 2 have been identified and their placement within the bHLH domain suggest the importance of co-factors in conferring specific functions to each neural bHLH protein.

Shared versus distinct functions of the neural bHLH factors in inducing neuronal differentiation and cell-type specification in the spinal neural tube

Mash1, Math1 and Ngn1 are expressed with similar timing in ventricular zone cells in the developing neural tube. However, their expression spatially is in non-overlapping patterns in these progenitor cells along the dorsoventral axis (Gowan et al., 2001; Ma et al., 1997). Consistent with these expression characteristics, we have identified both shared and distinct functions for Mash1, Math1 and Ngn1 (Gowan et al., 2001). Their common function is in their ability to induce neuronal differentiation when each neural bHLH factor is over-expressed in ventricular zone cells within the chick neural tube. This over-expression biases the cells to exit the cell cycle, move laterally out of the ventricular zone, and initiate expression of neuronal-specific genes within 24 hours. This function in inducing neurogenesis has been previously ascribed to these neural bHLH factors using multiple paradigms including mis-expression in retina and cortex (Cai et al., 2000; Cepko, 1999), in cultured cortical progenitors (Sun et al., 2001), and in P19 embryonal carcinoma cells (Farah et al., 2000). Together with in vitro binding data that show the neural bHLH factors can bind similar DNA sequences (Bertrand et al., 2002), it is probable that there are shared downstream targets to carry out this function in neuronal differentiation. Indeed, shared downstream targets, whether direct or indirect, have recently been shown for the *Xenopus* homologs Xash1 and Xngnr1 (Talikka et al., 2002).

In addition to the shared function in inducing neuronal differentiation, distinct functions for Mash1, Math1 and Ngn1 have been identified. Each factor specifies a different neuronal sub-type in chick neural tube in this assay. This activity is best distinguished by following the three most dorsal interneuron populations dI1, dI2 and dI3 (Helms and Johnson, 2003). Math1 induces and is required for the formation of dI1 neurons (Bermingham et al., 2001; Gowan et al., 2001). Ngn1 induces, and with Ngn2, is required for the formation of dI2 neurons (Gowan et al., 2001). And finally, although some dI3 neurons can form in the absence of Mash1 (A.W. Helms and J.E.J., unpublished data), Mash1 can induce dI3 neurons. In each case, the increase in one population appears to occur at the expense of the other two. This may reflect the fact that each bHLH represses the expression of the other bHLH factors in the dorsal neural tube (Gowan et al., 2001). Specificity in function has been demonstrated for neural bHLH factors in multiple regions of the developing nervous system including the forebrain (Fode et al., 2000; Parras et al., 2002), the neural crest (Perez et al., 1999) and retina (Perron et al., 1999). In addition, downstream targets have been identified for the Xngnr1 pathway that are not shared with Xash1 (Talikka et al., 2002). The distinct functions demonstrated for Mash1, Math1 and Ngn1 suggest that each factor has distinct downstream targets, and since these factors have different activities in different regions of the nervous system, these targets are likely context dependent.

Mash1 progenitor cells are located adjacent to the dI3, dI4

and dI5 interneuron populations, and it has been supposed that there is a lineage relationship between these cells. In this context, it was surprising that over-expression of Mash1 resulted in a moderate decrease in dI4/6 interneurons as defined by Lhx1/5 and Pax2 expression. Since dI4 and dI6 are difficult to distinguish by markers at this time, it is not clear what the effect is specifically on dI4. However, the absence of an increase in dI4 may suggest the progenitor/interneuron relationship between Mash1-expressing progenitor cells and dI4 interneurons should be revisited.

Protein-protein interaction domains of Mash1 and Math1 are required for their specific activities

Mash1 and Math1 are class II bHLH factors that characteristically have tissue-specific expression, and bind E-box DNA (CANNTG) as heterodimers with class I bHLH factors such as E12, E47, HEB and E2-2 (Massari and Murre, 2000). Crystal structures of a non-neural class II bHLH factor MyoD, or a class I bHLH factor E47, demonstrated that the basic region interacts with DNA and the HLH forms an amphipathic helix that is involved in protein-protein interactions in the formation of homo- or heterodimers (Ellenberger et al., 1994; Ma et al., 1994). Studies with other bHLH factors have demonstrated the importance of the basic region for specific functions (Chien et al., 1996; Davis and Weintraub, 1992; Dezan et al., 1999). More recently, however, studies of the *Xenopus* neural bHLH factors Xash1 (Mash1 homolog) and Xngnr1 (Ngn homolog) identified the HLH domain as the region encoding information for induction of specific downstream targets (Talikka et al., 2002). Our findings are similar to these latter experiments, demonstrating that the HLH domain, and not the basic region, encodes the necessary information for the specific functions in neurogenesis of the neural bHLH factors Mash1 and Math1 in the chick neural tube.

Since the HLH domain functions in protein-protein interactions, it is reasonable to propose that specificity of function may be conferred by interactions with specific co-factors that vary between the different bHLH proteins. It is known that in vitro, DNA binding activity is most efficient with heterodimers of the neural bHLH with an E-protein such as E12 (Akazawa et al., 1995; Helms et al., 2000; Johnson et al., 1992). There are no reports that the individual E-proteins (E2a, HEB, E2-2) can confer specificity of function on the neural bHLH/E-protein heterodimer, and knockout studies with the E-protein genes suggest they have a high level of functional redundancy (Zhuang et al., 1998; Zhuang et al., 1996). Thus, it may be that context-dependent co-factors that form higher order complexes with bHLH heterodimers are important for specific functions. Further support for the importance of cellular context for bHLH factor function is provided by the chick electroporation experiments. For example, electroporation of pMiWIII-Math1 results in overexpression of Math1 along the extent of the dorsoventral axis, however, the increase in the dI1 interneurons is biased to the dorsal regions. This is in contrast to the neuronal differentiation phenotype that is seen throughout the dorsoventral axis. Invoking context-dependent protein-protein interactions to explain the specificity of bHLH function along the dorsoventral axis of the spinal neural tube is also helpful in explaining how bHLH factors are required for different types of neurons in different regions of

the nervous system. Consistent with the involvement of specific co-factor interactions is the presence of conserved surface amino acids within each neural bHLH sub-family that are distinct between the different sub-families (Fig. 6).

Besides the E-proteins, a number of other co-factors have been described that form complexes with Class II bHLH factors. In *Drosophila*, Chip, a LIM homeodomain binding protein (homolog of Ldb factors in vertebrates), has been shown to form a complex with Achaete (Mash1 homolog) and Daughterless (E12 homolog), possibly as an adapter protein bringing another transcription factor, Pannier, in to form a higher order transcriptional complex (Ramain et al., 2000). In studies of Tal1, a class II bHLH factor important in hematopoiesis, a LIM only factor (LMO) and Ldb factors were seen to form a complex with Tal1 and E12 (Wadman et al., 1994; Wadman et al., 1997). In the neural tube, Pfaff and colleagues have demonstrated that higher order complex formation of homeodomain factors and bHLH factors control ventral cell fates. In this case, the homeodomain factor Lhx3 alone is not sufficient to generate motor neurons, but in combination with Ldb it specifies V2 interneurons. When co-expressed with islet1, a higher order complex is formed, resulting in different DNA binding characteristics, and the specification of motor neurons rather than V2 interneurons (Thaler et al., 2002). Furthermore, two bHLH factors, Ngn2 and NeuroM, transcriptionally synergize with the homeodomain complex to specify the motor neurons (Lee and Pfaff, 2003). This type of combinatorial interactions of transcription factors is an attractive hypothesis for the context-dependent functions seen with the bHLH factors studied here for dorsal neural tube development. The identity and role of co-factors in forming higher order transcriptional complexes with Mash1 and Math1 is yet to be determined.

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