INTRODUCTION

During the development of the vertebrate spinal cord, specific neuronal cell types are formed along the dorsoventral axis of the neural tube. In general, the dorsal half of the neural tube gives rise to cells of the roof plate, neural crest and multiple types of interneurons, whereas the ventral half gives rise to floor plate cells and motor neurons. The formation of these distinct cell types along the dorsoventral axis of the spinal cord involves both extracellular signals from neighboring cells and the intracellular response to these signals viewed as altered transcription factor activity within the cell. Defined extracellular signals from the notochord and floor plate dorsally (Basler et al., 1993; Chiang et al., 1996; Echelard et al., 1993; Ericson et al., 1995; Liem et al., 1995, 1997; Marti et al., 1995; Roelink et al., 1994, 1995; Tanabe et al., 1995) have been characterized, and the combined actions of these signals are thought to regulate cell identity and position in the dorsoventral axis of the neural tube. The molecular identity of the transcription factors that respond to these extracellular signals to activate or repress cell-type-specific gene expression is not known. Members of one family of transcription factors, the basic helix-loop-helix (bHLH) family, isolated in both invertebrates and vertebrates, have been shown to be essential in the formation of specific subsets of neurons during development (Campos-Ortega and Jan, 1991; Guillemot et al., 1993; Jan and Jan, 1993; Jarman et al., 1993, 1994, 1995; Kageyama et al., 1995; Lee et al., 1995; Ma et al., 1996; Sommer et al., 1995). Three bHLH transcription factors, MATH1, Mash1 and ngn1, have discrete expression patterns along the dorsoventral axis of the neural tube that are complementary and apparently non-overlapping within the proliferative zone that contains neural progenitor cells (Lo et al., 1991; Akazawa et al., 1995; Ben-Arie et al., 1996; Ma et al., 1996). These patterns of expression suggest that the different bHLH molecules might be important for the formation of distinct neuronal subtypes in the spinal cord.

MATH1 is a mammalian bHLH with 67% amino acid identity to the bHLH domain of the Drosophila proneural gene atonal and is transiently expressed in the developing central nervous system (Akazawa et al., 1995; Ben-Arie et al., 1996). MATH1 mRNA is most prominent in the dorsal region of the closed neural tube just adjacent to the roof plate, from the midbrain/hindbrain boundary caudally down the length of the neural tube. As development proceeds, MATH1 expression becomes restricted to the developing rostral hindbrain in the rhombic lip region. MATH1 expression continues in the external germinal layer (EGL) of the neural tube in the proliferative zone.

Summary

MATH1 is a neural-specific basic helix-loop-helix transcription factor. Members of this family of transcription factors are involved in the development of specific subsets of neurons in the developing vertebrate nervous system. Here we examine the cells expressing MATH1 with respect to their proliferative state and co-expression of cell-type-specific differentiation markers. We localize the MATH1 protein to the nucleus of cells in the dorsal neural tube and the external germinal layer (EGL) of the developing cerebellum. Using double-label immunofluorescence, we demonstrate that MATH1-expressing cells span both the proliferating and the differentiating zones within the dorsal neural tube, but within the EGL of the cerebellum are restricted to the proliferating zone. The early differentiating MATH1-expressing cells in the dorsal neural tube co-express TAG-1, DCC-1 and LH2, markers of dorsal commissural interneurons. In addition, transgenic mice with lacZ under the transcriptional control of MATH1-flanking DNA sequences express β-galactosidase specifically in the developing nervous system, in a manner that mimics subsets of the MATH1-expression pattern, including the dorsal spinal neural tube. Expression of the MATH1/lacZ transgene persists in differentiated dorsal commissural interneurons. Taken together, we demonstrate MATH1 expression in a differentiating population of neuronal precursors in the dorsal neural tube that appear to give rise specifically to dorsal commissural interneurons.

Key words: bHLH transcription factor, Mouse, Spinal cord, MATH1, Neuron, Cerebellum, Neural tube
ceases as the cells migrate and differentiate into mature granule cells. The movement of cells from the rhombic lip to the EGL and the restricted fate of these cells to granule cells of the cerebellum have been established (Alder et al. 1996; Gao and Hatten, 1994; Hallonet et al., 1990; Zhang and Goldman, 1996). From this detailed description of cerebellar development (Altman and Bayer, 1997), it is clear that MATH1 is expressed in the progenitors of cerebellar granule cells. However, the spinal cord contains many different types of neurons that exist in close proximity. Since these neurons are derived from an apparently uniform population of progenitors in the ventricular zone that undergo considerable cell movements (Leber and Sanes, 1995; Yaginuma et al., 1990), it has been difficult to predict the neuronal fate of the MATH1 progenitors in the dorsal spinal neural tube.

The experiments presented here probe the precise timing of MATH1 expression in the spinal neural tube and the EGL of the cerebellum relative to the differentiation state of the neural precursors. Localization of MATH1 alone and with markers of specific neuronal cell types have identified a specific fate of MATH1-expressing precursors in the dorsal spinal neural tube, the dorsal commissural interneurons. In addition, a transgenic mouse strain with a lacZ reporter gene under the transcriptional control of MATH1-flanking sequences is characterized, demonstrating transgene expression in specific subsets of commissural interneurons in the spinal neural tube.

MATERIALS AND METHODS

Generation of anti-MATH1 polyclonal antibody

To produce a rabbit polyclonal antibody against MATH1, a PCR fragment (5′ primer: CTGGATCCCTGATGAGCAGAAGAGTGG and 3′ primer: AAAGGATCCCTAAGGCGCTCATCAGA) containing the MATH1-coding region was inserted in-frame into the BamHI site of the glutathione-S-transferase fusion vector, pGEX-2T (Pharmacia). Bacterial protein was expressed and affinity purified from this construct according to established protocols (Smith and Johnson, 1988). MATH1-GST fusion proteins were purified further on an SDS-PAGE gel and this gel fragment was lyophilized and injected into rabbits with Freund’s Complete adjuvant (Sigma), followed by subsequent immunizations at 6 week intervals with Freund’s incomplete adjuvant. Anti-MATH1 polyclonal antibodies were purified from rabbit serum using standard procedures (Harlow and Lane, 1988). 293 human embryonic kidney cells were transfected with a construct containing the MATH1-coding region flanked by the RSV promoter and SV40 poly(A) addition sequence using the Tfx lipofection system (Promega). MATH1 transfected and untransfected cells were incubated for 48 hours and assayed for MATH1 expression using immunocytochemistry with the MATH1 antibody. Immunocytochemical conditions are described below.

Constructs and preparation of DNA fragments

Two overlapping MATH1-containing genomic clones from a Stratagene mouse λ129 library were isolated using a MATH1 bHLH probe. The MATH1 bHLH probe was originally isolated by PCR with degenerate primers designed using the bHLH region of Drosophila antennal (A. Leon and J. E. Johnson, unpublished data). The genomic sequences were subcloned into pBluescriptKS vectors and used to generate the fragments used here for injection into fertilized mouse eggs. The 5′ fragment is a 15 kb SpHlI fragment containing sequences 5′ of the MATH1-coding region. The 3′ fragment is a 6 kb BglII fragment containing a portion of the MATH1-coding region (~400 bp) and 3′ flanking sequences. The MATH1/lacZ fusion construct, which served as the middle fragment in these co-injections, replaces most of the MATH1-coding sequence with a 3.1 kb BamHI fragment from pmLacF (Mercer et al., 1991). The final MATH1/lacZ plasmid, pATOLacZ4.0, fuses the lacZ-coding sequence with no nuclear localization signals in frame with the MATH1-coding region just after the translation start site, and is flanked by 2.5 kb 5′ and 1.5 kb 3′ of the coding sequence. A 7 kb NcoI fragment was isolated from pATOLacZ4.0 for injection into mice. All fragments for injection were separated from vector sequences in a 1.0% SeaPlaque agarose gel, purified using Elutip columns (Schleicher & Schuell) and resuspended in injection buffer (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA). The 15 kb SpHlI fragment was injected at 1.5 ng/µl, the 7.0 kb MATH1/lacZ fusion NcoI fragment at 0.5 ng/µl and the 6 kb BglII fragment at 1 ng/µl.

Generation and screening of transgenic mice

Transgenic mice were generated by standard procedures (Hogan et al., 1986) using fertilized eggs from B6D2F1 (C57BL/6 × DBA) crosses. Transgenic founder animals were identified by standard tail DNA dot blots. The transgene integration in the MATH1/lacZ line was characterized by Southern blot analysis and indicated all three fragments had integrated. The correct recombination of the 5′ and middle fragments and the middle and 3′ fragments could be confirmed but several attempts to demonstrate the correct recombination of all three fragments in series was ambiguous (data not shown). A 2.5 kb PvuII fragment from lacZ was used to probe for transgene sequences. Founder animals were outbred with B6D2F1 animals for all studies. Embryos were staged based on the assumption that copulation occurred at E0, halfway through the dark cycle.

β-galactosidase staining of embryos

Staged transgenic embryos were dissected from the uterus in cold PBS and fixed for 30-60 minutes (depending on the age of the embryo) in 4% paraformaldehyde pH 7.2 at room temperature. Whole-mount β-gal staining of the embryos was carried out as described (Verma-Kurvari et al., 1996). For analysis of thin sections, fixed embryos were sunk in 30% sucrose overnight at 4°C, embedded in OCT and cryosectioned at 20-30 µm. Slides were stained for β-gal activity at 35°C for 4 hours to overnight.

Whole-mount in situ hybridization

For mRNA in situ hybridization, staged embryos were dissected from the uterus in cold PBS and fixed overnight at 4°C in 4% paraformaldehyde. Whole-mount in situ hybridization was carried out essentially as described (Rosen and Beddington, 1993) with minor modifications. A detailed protocol will be provided upon request.

An 800 bp MATH1 antisense probe was derived from a plasmid pLAI-HindIII containing 600 bp of the MATH1-coding region and approximately 200 bp of the 5′ flanking sequence.

Immunohistochemistry

Staged embryos were dissected from the uterus and washed in PBS at 4°C, followed by fixation in 4% paraformaldehyde at 4°C from 30 minutes to 6 hours. Fixed embryos were sunk in 30% sucrose overnight, embedded in OCT compound (Tissue Tek) and cryosectioned at 20-30 µm. Single-label immunohistochemistry for MATH1 was performed by incubation with a 1:100 dilution of affinity-purified rabbit anti-MATH1 in PBS/1% goat serum/0.1% Triton X-100, followed by either a goat anti-rabbit IgG Texas Red or Cy3 conjugate (Jackson ImmunoResearch). Double-label immunofluorescence was performed by simultaneous incubation with antibodies of interest, which included: 4D7 (anti-TAG-1), 4F2 (anti-Lim1/2), 39.4D5 (anti-Islet-1), Tuj-1 (anti-neuronal β-tubulin), anti-BruT (Brock-Dickinson), 40.1a (mouse monoclonal anti-beta-galactosidase), L1 (anti-LH-2B), Enhβ (anti-engrailed-1) and rabbit polyclonal anti-β-galactosidase (5 prime-3 prime, Inc). 39.4D5 (anti-
islet-1) and 40.1a (anti-β-galactosidase) were obtained from the Developmental Studies Hybridoma Bank, 4D7 antibody was a gift from J. Dodd, 4F2 and L1 were gifts from T. Jessell, Enhb antibody was a gift from A. Joyner and Tuj-1 monoclonal antibody was a gift from A. Frankfurter. Goat anti-mouse and goat anti-rabbit secondary antibodies conjugated to Texas Red, FITC and Cy3 (Jackson Immunoresearch) were used to detect double labelling. In experiments where anti-BrdU was used, pregnant mice were injected with 100 μg/g body weight BrdU 1 hour prior to killing. Sections from BrdU-injected embryos were treated with 2 N HCl for 15 minutes to denature DNA, followed by a 15 minute neutralization with 0.1 M sodium borate pH 8.5 prior to the addition of primary antibody. Immunofluorescence studies were carried out on a Leica DMR microscope equipped for fluorescence. Confocal analysis was carried out on a Bio-Rad MRC 1024 confocal microscope.

RESULTS

Localization of MATH1 protein

By mRNA in situ hybridization studies, it has been determined that MATH-1 is expressed in the developing nervous system, most prominently in the dorsal part of the neural tube, from the midbrain/hindbrain boundary caudally down the length of the tube (Akazawa et al., 1995; Ben-Arie et al., 1996). This expression begins at E9.5 in the dorsal neural tube in presumptive neuronal precursors located adjacent to the roof plate and in the cranial ganglia. As development proceeds, MATH1 expression remains constant along the length of the dorsal neural tube and metencephalon until E12.5. After E12.5, MATH1 is expressed mainly in the metencephalon, including prominent expression in the rhombic lip, the origin of the precursors of the EGL of the cerebellum. Expression continues in the EGL until well after birth.

In order to examine the expression of MATH1 at the protein level, we generated rabbit polyclonal antibodies against a full-length mouse MATH1-GST fusion protein. To confirm the specificity of the MATH1 antisera, we transiently expressed MATH1 under the control of the RSV promoter in 293 human embryonic kidney cells, which do not express MATH1 endogenously. MATH1 was detected only in the transfected cells with the affinity-purified anti-MATH1 antisera (Fig. 1A,B). Consistent with its role as a transcription factor, MATH1 protein is localized to the nucleus.

We used the MATH1 polyclonal antisera to analyze the expression pattern of MATH1 in the neural tube of E10.0 and E10.5 mouse embryos. At these stages, MATH1-immunoreactive cells are located in the most dorsal aspect of the neural tube (Fig. 1C,D), excluding the roof plate, similar to the pattern seen in MATH1 mRNA in situ hybridization studies (Akazawa et al., 1995; Ben-Arie et al., 1996). However, MATH1 protein is also detected in a small population of cells located at the lateral edges of the neural tube, just ventral to the larger MATH1 domain (Fig. 1C,D, arrows). This small population of MATH1+ cells was not detected in earlier in situ hybridization studies and the lateral position suggests that they are in a more differentiated state than the cells in the ventricular domain. MATH1 protein persists in this pattern in the spinal neural tube through E12.5 (data not shown). MATH1-immunoreactive cells were also detected as expected in the EGL of the postnatal cerebellum (Fig. 1E).

MATH1+ cells are found in both proliferating and differentiating populations of neuronal precursors

In the developing neural tube, proliferating precursors reside in the ventricular zone and, as the cells differentiate, they move to more lateral regions. To elucidate when during neural development MATH1 functions, we examined whether expression of MATH1 is restricted to either the proliferative or differentiating regions. The overlap of nuclear incorporation of bromodeoxyuridine (BrdU), which is only taken up in cells undergoing the S phase of the cell cycle, with MATH1-immunoreactive cells was examined to determine whether MATH1 cells are proliferating. Pregnant mice were injected with BrdU 1 hour before harvest of embryos. MATH1 expression and BrdU incorporation were examined at two stages of embryonic development, E10.5 and E11.5, corresponding to the peak of MATH1 expression in the neural tube. At E10.5, a subset of MATH1-expressing cells (~30%) are actively proliferating and thus identify a neural precursor population (Fig. 2A). However, by E11.5, a smaller number of MATH1+ cells (~20%) appear to proliferate, though there are still many MATH1+ cells in the ventricular zone (Fig. 2B). At both stages, MATH1+ cells at the lateral edges did not incorporate BrdU, consistent with the fact that postmitotic, differentiating neurons are located there (Altman and Bayer, 1984).

In the converse experiment, we examined the differentiation state of MATH1 neuronal precursors by evaluating the overlap of a general neuronal differentiation marker, neuronal β-tubulin (Tuj-1; Lee et al., 1990) with the expression of MATH1. At E10.5, a population of MATH1+ cells (~30%) are Tuj-1-positive. These MATH1/Tuj-1 co-expressing cells are found only at the lateral regions of the MATH1+ domain (Fig. 2C). Thus, MATH1 protein is not only expressed in proliferating neuronal precursors but is also maintained as they initiate differentiation at the lateral edges of the neural tube. At E11.5, a larger percentage of MATH1+ cells (~40%) are undergoing differentiation (Fig. 2D), as assessed by co-expression of Tuj-1. Taken together, these data demonstrate MATH1 expression in both proliferating and differentiating neural precursors in the dorsal spinal neural tube.

We next examined the differentiation state of MATH1+ neuronal precursors in the EGL of the postnatal cerebellum to evaluate the generality of this observation. The EGL of the cerebellum is a transient structure during the development of the mature cerebellum. The future granular layer of the mature cerebellum is formed when proliferative neural precursors located in the EGL differentiate and migrate to the internal granular layer (IGL), where further differentiation takes place and the mature granular layer is formed. Even within the EGL, there is an outer zone of proliferating (BrdU-positive) granule cell precursors and an inner premigratory zone of cells that have left the cell cycle (BrdU-negative) and are preparing for differentiation and migration into the IGL (Altman and Bayer, 1997). To examine where MATH1 is expressed relative to these two zones, we compared the co-localization of MATH1 with BrdU incorporation in the EGL of the cerebellum at postnatal day 7 (P7). At this stage, the MATH1+ cells are restricted exclusively to the proliferating (BrdU-positive) zone of the EGL (Fig. 2E-G). Thus, in contrast to what was seen in the spinal neural tube earlier in development, in the EGL of the
developing cerebellum, MATH1 is found only in proliferating granule cell precursors.

**MATH1 co-localizes with markers of dorsal commissural interneurons**

Expression of MATH1, like other bHLH transcription factors of this subclass, including Mash1, ngn1/2 (MATH4A/C) (Cau et al., 1997; Gradwohl et al., 1996; Lo et al., 1991; Ma et al., 1996) is transient in neural precursors. The lack of expression of these transcription factors in overtly differentiated neurons, which can be identified by cell-type-specific markers, position and axonal projections, makes it difficult to assign specific neuronal fates for these precursors. Immunofluorescence studies with the MATH1 antibodies demonstrate a small domain of MATH1 expression in the lateral, more differentiated regions of the spinal neural tube (Figs 1C,D, 2C,D). To identify the fate of these neuronal precursors, we asked whether they co-express markers of a specific neuronal subtype. Interneurons, including the dorsal commissural neurons, develop in the dorsal neural tube beginning at embryonic day 10.0 in the mouse (Altman and Bayer, 1984). We compared the pattern of MATH1 expression with specific markers of dorsal commissural neurons, TAG-1, DCC-1 and LH-2. TAG-1 is a transiently expressed axonal surface glycoprotein that is found specifically on early motor and commissural neurons in the developing neural tube (Dodd et al., 1988; Furley et al., 1990). DCC-1, or Deleted in Colorectal Cancer, is a transmembrane protein of the immunoglobulin family that has recently been identified as a mediator of the neurite outgrowth effects of netrin-1 on dorsal commissural axons (Keino-Masu et al., 1996). LH-2 (LIM homeobox 2) is a putative transcription factor containing two N-terminal LIM domains and one C-terminal Hox domain, and is also thought to be an early marker of dorsal commissural neurons (Liem et al., 1995; 1997; Xu et al., 1993). Double-label immunofluorescence was performed using antibodies to MATH1 and TAG-1 or DCC-1. At E10.5, a subset of MATH1+ cells (yellow), (C,D) Stained with anti-MATH1 (red) and anti-neuronal β-tubulin (Tuj-1) (green). Nuclear-stained MATH1-positive cells co-localize with cytoplasmic Tuj-1 staining at the dorsolateral edges of the neural tube. (E) Stained with anti-MATH1 (red); (F) BrdU-labeled cells (green) and (G) overlap of the images to show their co-localization (yellow) in the EGL of the cerebellum. Scale bar, 50 μm (A-D) and 100 μm (E-G).
cells at the lateral edges of the neural tube were both TAG-1 and DCC-1 positive (Fig. 3A,B). Thus, MATH1+ cells are not only undergoing differentiation, as defined by BrdU and Tuj-1 immunostaining, but a subset of these cells are differentiating into TAG-1/DCC-1-positive neurons. We were unable to compare directly MATH1 and LH-2 expression in the same section because both of antibodies were of the same subtype. However, in adjacent sections at E10.5, it appears that the lateral domain of MATH1 expression overlaps with the LH-2-expression domain (Fig. 3C,D). This overlap of MATH1 expression with markers of proliferation and differentiation, as well as the cell-type-specific markers of differentiation in dorsal neural tube suggests that MATH1 plays a role in the differentiation of dorsal commissural interneurons.

**MATH1-flanking sequences drive expression of a lacZ reporter in commissural neurons**

In studies to identify upstream regulators of MATH1 expression, we generated a line of transgenic mice that express lacZ under the control of MATH1-flanking DNA sequences. The transgenic embryos used in these experiments were made by co-injecting three overlapping fragments of DNA that contain 15 kb 5′ and 6 kb 3′ of the MATH1 sequence flanking the coding region. The co-injected fragments are shown in Fig. 4A. The 7 kb MATH1/lacZ fusion construct used as the central fragment replaces most of the MATH1-coding region with the lacZ gene, retaining both the MATH1 5′ and 3′ untranslated regions. Expression of the lacZ transgene was first characterized in transgenic embryos at E10.5-11.5. 10 embryos with independent transgene integrations were identified by dot-blot analysis of yolk sac DNA with a lacZ probe. (A) Diagrammatic representation of the genomic structure of the MATH1 gene, the MATH1/lacZ fusion construct and the 15 kb and 6 kb fragments used for injection. B, BglII; N, NcoI; S, SphI. (B) Whole-mount in situ hybridization of an E10.5 mouse embryo with a MATH1 antisense probe. (C) Side view and dorsal view of whole-mount β-gal-stained transgenic embryos at E10.5. The arrows in B and C indicate the rostral extent of staining at the midbrain/hindbrain boundary and the arrowhead indicates the lack of dorsally restricted expression. (D-F) 150 μm vibratome sections of whole-mount β-gal-stained embryos from E10.0 (D), E10.5 (E) and E11.5 (F). At E10.5 and E11.5, β-gal activity is seen in fibers that extend ventrally (arrows) and cross the floor plate (asterisk). Scale bar, 1.0 mm (B); 1.2 mm (C) and 60 μm (D-F).
demonstrated the presence of all three fragments but, due to complex integration of these fragments, the presence of the properly recombined transgene cannot be confirmed (data not shown). However, this MATH1/lacZ line expresses the transgene in a pattern that reflects endogenous MATH1 expression (detailed below). Although we cannot conclude that all 21 kb of MATH1-flanking DNA is required for this pattern, the middle fragment alone, which contains 2.5 kb of 5′ flanking sequence and 1.5 kb of 3′ flanking sequence, does not result in lacZ expression (0/8 embryos, A. W. Helms and J. E. Johnson, unpublished data).

Analysis of the lacZ gene product, β-gal, in the MATH1/lacZ transgenic line shows expression specifically in the developing neural tube from the midbrain/hindbrain boundary caudally to the tail, mimicking the precise rostrocaudal boundaries of MATH1 expression from E9.5 to E12.5 (Fig. 4B,C). We detect expression of the transgene in the neural tube in whole embryos as early as E9.5, the time at which MATH1 expression begins (data not shown) and the strongest staining is detected in embryos from E10.5-E11.5 (Fig. 4C), correlating with the strongest expression of MATH1 mRNA detected by northern analysis and in situ hybridization (Fig. 4B) (Akazawa et al., 1995; Ben-Arie et al., 1996). The MATH1/lacZ transgene is also expressed at later stages of neural development specifically in the EGL of the postnatal cerebellum, just as endogenous MATH1 (data not shown). Surprisingly, expression of the transgene is not detected in the cranial ganglia at E9.5, as is expected from the endogenous MATH1-expression pattern.

We further analyzed transgene expression in the spinal neural tube on 150 μm vibratome sections of whole-mount β-gal-stained embryos from E10.0 to E11.5 (Fig. 4D-F). Since no subcellular localization signal was included in the lacZ transgene, β-gal staining is observed throughout the cell bodies, including the nucleus and processes extending from these cells. Early at E10.0, expression of the transgene is essentially identical to MATH1 expression (compare Fig. 4D with Fig. 1D). However, unlike endogenous MATH1, at E10.5, the β-gal staining extends further down the lateral edges of the neural tube into more ventral regions (Fig. 4E). As development progresses, more β-gal staining is seen at E11.5 outside the ventricular zone in more ventrolateral regions of the neural tube, regions that do not express endogenous MATH1 (Fig. 4F). In addition, at E10.5 and E11.5, β-gal protein is seen in fibers that extend from the dorsal regions and cross the floor plate (Fig. 4E,F; arrows). Contralateral axonal projections of this type identify these neurons as dorsal commissural neurons (Altman and Bayer, 1984).

These apparent discrepancies between the expression of endogenous MATH1 and expression of the MATH1/lacZ transgene in the dorsoventral axis of the spinal neural tube suggests that either cis-acting regulatory elements responsible for restricting expression dorsally are missing from the transgene, or there is an artifactual persistence of the reporter gene, lacZ. However, in separate studies, we have determined that the persistence of lacZ expression in the dorsoventral axis of the neural tube is not simply due to the stability of the β-gal reporter since an epitope-tagged MATH1 reporter gene driven by the same elements is also expressed in the same ventrolateral pattern (A. W. Helms and J. E. Johnson, unpublished data). The pattern of lacZ expression seen in the dorsal commissural interneurons in this transgenic line is consistent with a dorsal commissural neuron fate of MATH1+ neuronal precursors in the dorsal spinal neural tube. In addition, the data presented above demonstrate that cis-acting regulatory information for much but not all of the MATH1-expression pattern lies within 15 kb 5′ and 6 kb 3′ of the MATH1-coding region.

Identity of the neuronal populations marked in the MATH1/lacZ transgenic line

It is clear from the vibratome sections of the transgenic embryos that the expression of the MATH1/lacZ transgene in the neural tube is not restricted to the MATH1 domain after E10.0 (compare Fig. 1C, D with Fig. 4D-F). We further characterized the lacZ-expressing neurons by co-localization of β-gal with neuronal cell-type-specific markers to better evaluate the relationship of these neurons with MATH1-expressing neural precursors. In addition, the MATH1/lacZ transgenic line described here may be useful for researchers studying these specific neuronal populations whether or not expression of the MATH1/lacZ transgene reliably reflects endogenous MATH1 expression. For this reason, we have characterized the lacZ-expressing neurons in some detail.

First, we confirmed the identity of a subset of these neurons as dorsal commissural interneurons by co-localization of β-gal immunoreactivity with markers for these neurons: TAG-1, DCC-1 and LH-2 (Fig. 5A-E). At E10.5, β-gal staining co-localizes with TAG1 staining in the cell bodies and axons of the commissural neurons (Fig. 5A,B). Double immunofluorescence analysis with DCC-1 and β-gal demonstrate that, at E10.5 and E11.5, β-gal-positive axons are co-localized with a subset of DCC-1-expressing axons (Fig. 5C,D, arrows, and data not shown). In addition, while the comparison of endogenous MATH1 and LH-2-expression domains was in adjacent sections (Fig. 2C,D), β-gal and LH-2 expression could be co-localized, showing extensive but not complete overlap between β-gal and LH-2 immunoreactivity at E10.5 (Fig. 5E). The LH-2 antibody recognizes both LH-2A and LH-2B proteins and, thus, β-gal may be co-expressed in cells expressing only one of these. The identification of axonal projections that extend ventrally to the floorplate and cross the midline, together with co-localization of TAG-1, DCC-1 and LH-2 in the β-gal-expressing cells identifies these neurons as dorsal commissural interneurons.

In addition to the dorsal commissural neurons characterized above, it is possible that the MATH1/lacZ-expressing cells are marking an additional dorsal interneuron population since multiple, distinct cell types are found in the dorsal spinal cord (Liem et al., 1997). To address this question, we compared β-gal immunoreactivity to additional markers defining different interneuron populations including lim1/2, islet-1 and engrailed-1 (Barnes et al., 1994; Davidson et al., 1988; Davis and Joyner, 1988; Ericson et al., 1992; Fujii et al., 1994; Pfaff et al., 1996). Lim1/2 and islet-1 are limb homeodomain proteins that mark distinct populations of sensory and motor interneurons in the developing neural tube. At E10.5 and E11.5, these markers did not co-localize with β-gal expression even upon careful analysis of confocal images (Fig. 5F,G). Engrailed-1 marks a distinct population of interneurons that is located adjacent to the developing motor neuron pools. At E11.5, the engrailed-1-positive cells also did not overlap with...
β-gal-expressing cells (data not shown). Thus, none of these additional markers of interneuron populations in the dorsal neural tube co-localize in cells that express β-gal in the transgenic embryos.

An additional population of lacZ-expressing neurons is apparent in a more ventral region of the neural tube at E11.5 (Fig. 4F). A confocal image of β-gal immunoreactivity in this region clearly identifies axons projecting horizontally, contrasting with the vertically projecting axons of the commissural neurons (Fig. 5H). These neurons, which first express β-gal at E11.5, also co-express DCC-1 (Fig. 5I) but not TAG-1 (Fig. 5J). The timing, location and axonal projection pattern of this population of neurons first suggested that they were a population of ipsilaterally projecting sensory neurons. These neurons are characterized by horizontal axons that project ipsilaterally to the lateral funiculus, turn and project rostrally to the brain (Altman and Bayer, 1984). However, a longitudinal view of whole-mount, X-gal-stained spinal cord preparations clearly reveals contralaterally projecting fiber tracts in the ventral funiculus (Fig. 5K, arrow) but no ipsilaterally projecting fiber tracts in the lateral funiculus (Fig. 5K). Thus, this second neuronal population is a distinct, later population of commissural neurons with axons that most likely turn and project contralaterally to the floor plate (Silos-Santiago and Snider, 1992). The two populations of commissural interneurons marked in the MATH1/lacZ line are distinguished by the location of their cell bodies in the dorsoventral axis, but share similar properties including axonal projections that cross the floor plate and the expression of DCC-1.

**DISCUSSION**

bHLH transcription factors have critical roles in multiple developmental processes (Jan and Jan, 1993; Kageyama et al., 1995; Lee, 1997). MATH1, a neural-restricted member of this family, is likely to play a critical role in the development of the cells in which it is expressed. We have localized MATH1 protein to specific neuronal progenitors in the dorsal neural tube and to the EGL of the developing cerebellum. A close examination of the proliferative state of these neuronal precursors demonstrates the MATH1 domain in the dorsal neural tube spans both proliferating and differentiating zones but, in the cerebellum, is restricted to the proliferative zone. Because MATH1 persists in early differentiating cells in the dorsal neural tube, we were able to demonstrate that MATH1-expressing neural precursors give rise to a specific neuronal cell type, the dorsal commissural interneurons. Given the essential role demonstrated for other neural-specific members of this family in the development of specific neuronal cell types (Guillemot et al., 1993), it is likely that MATH1 is involved in the development of at least these two neuronal populations; dorsal commissural interneurons and granule cells of the cerebellum.

**MATH1 function in neural development**

Neural-specific members of the bHLH family can be classified into two main subfamilies based on the timing of their expression relative to the stage of neuronal development (Lee, 1997): (1) those that appear to be expressed in proliferating neural precursors based on expression in ventricular zones of the neural tube and (2) those that initiate expression in differentiating precursors subsequent to becoming postmitotic and are more laterally located in the marginal zone. Some of the latter subtype even maintain expression in mature neurons in the adult animal (Bartholoma and Nave, 1994; McCormick et al., 1996; Shimizu et al., 1995). Due to the location of MATH1 in the ventricular zone of the dorsal neural tube, it is classified with the early expressed members of the family, which also include Mash1, ngn1/MATH4C and ngn2/MATH4A (Gradwohl et al., 1996; Lee, 1997; Lo et al., 1991; Ma et al., 1996; Sommer et al., 1996). However, closer examination of the MATH1 population with respect to markers of proliferation and differentiation revealed that MATH1 expression overlaps with both these developmental states (Fig. 2). From E10.0-E11.5 in development, MATHI-expressing cells in the spinal neural tube appear to undergo a shift from an actively proliferating neuronal precursor population to a postmitotic neuronal β-tubulin-positive state. This pattern of expression is consistent with a role for MATH1 in the dynamic process of maturation as these dorsally restricted proliferating cells undergo differentiation.

MATH1 expression in the developing EGL of the cerebellum has characteristics that differ from MATH1 in the dorsal spinal neural tube. An interesting aspect of cerebellar development is that precursors of the granule cells are first located embryonically in the rhombic lip in the dorsal hindbrain (Altman and Bayer, 1997; Miale and Sidman, 1961). They migrate to form the EGL of the cerebellum where postnatally they undergo massive proliferation before they differentiate and migrate into the underlying cerebellar cortex to form the internal granule cell layer (Altman and Bayer, 1985, 1997; Miale and Sidman, 1961). MATH1 is expressed early in the rhombic lip region of the dorsal hindbrain and persists as these cells migrate to form the transient EGL (Akazawa et al., 1995) and Fig. 1E, 4B). MATH1 persists in the proliferative zone as this layer of cells undergoes its massive proliferation (Fig. 2E-G). During this time, the cells in the EGL also begin to exit the cell cycle and move medially to exist in a postmitotic, premigratory zone within the EGL before they migrate into the underlying cerebellar cortex (Altman and Bayer, 1997; Miale and Sidman, 1961). Interestingly, MATH1 expression is extinguished as the cells begin to differentiate, restricting its expression exquisitely to the proliferative layer (Fig. 2E-G). This restriction of MATH1 expression is in contrast to its expression in the dorsal spinal neural tube, which spans both the proliferating and early differentiating populations. However, since cells migrating from the rhombic lip to the EGL are already specified to a granule cell fate (Alder et al., 1996), these cells can no longer be considered stem cells but represent an intermediate proliferating precursor to the granule cells of the cerebellum.

These detailed co-localization studies of MATH1 with markers of proliferation and differentiation suggest that MATH1 marks cells in both the dorsal spinal neural tube and the dorsal hindbrain that appear to be undergoing a transition from stem cell precursor to early differentiating precursor. MATH1 is not the first neural bHLH transcription factor that defines an intermediate precursor state. Similarly, the hypothesis that Mash1 is functioning in a population of actively differentiating neural precursors has support in multiple neural
systems including the developing sympathetic neurons, olfactory neurons and ventral forebrain neurons (Cau et al., 1997; Gordon et al., 1995; Porteus et al., 1994; Sommer et al., 1995).

It may be worthwhile to consider a role for a bHLH transcription factor like MATH1 in regulating a common set of properties in differentiating subsets of neurons. For example, are there any common properties shared by the dorsal commissural interneurons and granule cells in the cerebellum that could be regulated by MATH1? Both types of neurons function as interneurons and both express two of the markers used here, TAG-1 and DCC-1 (Furley et al., 1990; Keino-Masu et al., 1996). While none of these similarities individually provide a compelling rationale for understanding why certain bHLH factors are expressed in certain neural precursors and not others, the combination of subtle common properties, even transient properties during development, may provide a framework to begin to understand the role of these molecules in neural development.

Regulation of MATH1 expression and the MATH1/lacZ transgenic line

The 21 kb sequence flanking the MATH1-coding region included in the injected fragments of the transgene (Fig. 4A) is sufficient to drive expression of the reporter gene lacZ in a subset of the cells that normally express MATH1. This is most dramatically illustrated in the restriction of lacZ expression to the dorsal neural tube at E10.0 with the precise rostral boundary of expression at the midbrain/hindbrain junction, respected (Fig. 4B,C). In addition, we have observed that
transgene expression at different stages in different neural tissue mimics MATH1 expression. Most notable is the EGL in the postnatal cerebellum (A. W. Helms and J. E. Johnson, unpublished data), the other neural-specific domain of MATH1 expression (Akazawa et al., 1995; this report) (Fig. 1). Thus, multiple domains of MATH1 expression are regulated by elements within 21 kb flanking the MATH1-coding region.

It is clear, however, that not all regulatory elements are present and/or functioning in the 21 kb MATH1lacZ transgene. The absence of lacZ expression in the cranial ganglia, the first domain of MATH1 expression seen at E9-E9.5 (Akazawa et al., 1995), suggests the absence of a positive element for driving expression to this region. In addition, the strong expression seen at E11.5 in the neural tube in at least two populations of overtly differentiated neurons indicates that the transgene is also lacking repressor element(s). At E10.0, lacZ expression closely reflects the dorsal restriction of endogenous MATH1 expression (compare Fig. 1D and Fig. 4D). At E10.5, lacZ expression is detectable in axons extending to the floorplate, identifying some of these cells as dorsal commissural interneurons. Expression in these commissural axons is not unexpected since we demonstrated MATH1 expression in this population of neurons by co-localization with specific markers and the transgenic line provides a more visible means of demonstrating expression in these axons. However, the first indication that a repressor element is missing from the transgene is evident in the high levels of lacZ expression that persist in these cells through E11.5. This repressor element may regulate the precise temporal control of MATH1 expression. The expression of the transgene in a second population of commissural interneurons found in the mid-dorsorventral region is suggestive of yet another repressor element absent from the transgene. The relationship, if any, of this second population of commissural interneurons to MATH1-expressing progenitors is unknown. However, the specificity of the ectopic expression in this population of neurons, which appear to be related to the dorsal commissural interneurons, at least in the axonal projections to the floorplate and the shared marker DCC-1, suggests a shared molecular background at some point in their developmental histories.

We gratefully acknowledge Drs J. Dodd, T. Jessell, A. Joyner, A. Frankfurter and the Developmental Studies Hybridoma Bank for antibodies. The Developmental Studies Hybridoma Bank is maintained by The University of Iowa, Department of Biological Sciences, under contract NO1-HD-7-3263 from the NICHD. We also acknowledge Drs K. Zimmerman and A. Furley for critical comments and discussions of early versions of the manuscript and Dr H. Krämer for critical comments and discussions of a later version of the manuscript; Dr M. Tessler-Lavigne for whole-mount spinal cord dissections; A. Leon for the original PCR cloning of MATH1; and D. Smith for technical assistance in generating the transgenic mice. A. W. H. was supported by NIH training grant #T32GM07062. This work was supported in part by the Council for Tobacco Research, the Perot Foundation and the Kent Waldrep National Paralysis Foundation. J. E. J. is an Established Investigator for the American Heart Association.

REFERENCES


Guillemot, F., Lo, L. C., Johnson, J. E., Auerbach, A. anderson, D. J. and
Sommer, L., Ma, Q. and Anderson, D. J. (1996). neurogenins, a novel family of atonal-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. Molecular and Cellular Neuroscience 8, 221-241.