Generation of neurons by transient expression of neural bHLH proteins in mammalian cells

Mohamed H. Farah1,2, James M. Olson3,4, Holly B. Sucic1, Richard I. Hume2,5, Stephen J. Tapscott6 and David L. Turner1,2,*

1Mental Health Research Institute, 2Neuroscience Program, and 5Department of Biology, University of Michigan, Ann Arbor, MI 48104-1687, USA
3Division of Pediatric Oncology, University of Washington, Seattle, WA 98105, USA
4Clinical Division and 6Division of Molecular Medicine, Fred Hutchinson Cancer Research Center, Seattle WA 98109, USA
*Author for correspondence (e-mail: dlteturner@umich.edu)

Accepted 29 November 1999; published on WWW 26 January 2000

SUMMARY

Basic helix-loop-helix (bHLH) transcription factors are known to function during mammalian neurogenesis. Here we show that transient transfection of vectors expressing neuroD2, MASH1, ngn1 or related neural bHLH proteins, with their putative dimerization partner E12, can convert mouse P19 embryonal carcinoma cells into differentiated neurons. Transfected cells express numerous neuron-specific proteins, adopt a neuronal morphology and are electrically excitable. Thus, the expression of neural bHLH proteins is sufficient to confer a neuronal fate on uncommitted mammalian cells. Neuronal differentiation of transfected cells is preceded by elevated expression of the cyclin-dependent kinase inhibitor p27Kip1 and cell cycle withdrawal. This demonstrates that the bHLH proteins can link neuronal differentiation to withdrawal from the cell cycle, possibly by activating the expression of p27Kip1. The ability to generate mammalian neurons by transient expression of neural bHLH proteins should create new opportunities for studying neurogenesis and devising neural repair strategies.

Key words: Basic-helix-loop-helix, Transcription, Neuron, p27, Mouse

INTRODUCTION

MASH1, neuroD, neuroD2, MATH1-3 and neurogenin1-3 (ngn1-3) are basic helix-loop-helix (bHLH) transcription factors expressed during mammalian neuronal determination and differentiation (Johnson et al., 1990; Bartholoma and Nave, 1994; Akazawa et al., 1995; Lee et al., 1995; Shimizu et al., 1995; Ma et al., 1996; McCormick et al., 1996; Sommer et al., 1996; Takebayashi et al., 1997). Targeted disruptions of MASH1, MATH1, ngn1, ngn2 or neuroD in mice lead to the loss of specific subsets of neurons (Guillemot et al., 1993; Ben-Arie et al., 1997; Fode et al., 1998; Ma et al., 1998; Miyata et al., 1999). Although the neural bHLH proteins encoded by these genes are essential for neuron formation, their functions during neurogenesis remain unclear. Forced expression of either neuroD or neuroD in Xenopus neural ectoderm (Lee et al., 1995) or MASH1 in rat neural crest stem cells (Lo et al., 1998) leads to premature neuronal differentiation, suggesting that the neural bHLH proteins may regulate the formation of neurons from neural precursors. In Xenopus, zebrafish or chicken embryos, ectopic expression of neuroD2, ngn2 or other neural bHLH proteins leads to the formation of neurons from a subset of non-neuronal cells (Turner and Weintraub, 1994; Lee et al., 1995; Ma et al., 1996; Blader et al., 1997; Perez et al., 1999), showing that neural bHLH proteins can impose a neuronal fate on some cells. In mammalian systems, however, expression of neural bHLH proteins has not led to the formation of neurons from non-neuronal cells (Johnson et al., 1990, 1992). Thus, it is not clear whether neural bHLH proteins are sufficient to direct mammalian cells to adopt a neural fate, or whether they can only regulate neuronal differentiation in mammals.

We have investigated the ability of neural bHLH proteins to direct neuron formation from pluripotent mouse P19 embryonal carcinoma cells. P19 cells were derived from a mouse embryo and have been used extensively as a model system for in vitro differentiation (McBurney, 1993; Bain et al., 1995). After treatment with retinoic acid (RA) and aggregation, P19 cells differentiate into neurons and glia while, under other conditions, these cells can form skeletal or cardiac muscle, endoderm or other cell types. Numerous genes are activated in P19 cells in response to RA treatment, including genes encoding several neural bHLH proteins (Johnson et al., 1992; McCormick et al., 1996; Boudjelal et al., 1997; Itoh et al., 1997). Here we show that transient expression of neuroD2, MASH1, ngn1 or related bHLH proteins is sufficient to convert P19 cells into differentiated neurons in the absence of RA or aggregation. Moreover, we find that expression of neural bHLH proteins can initiate cell cycle withdrawal, accompanied by elevated expression of the cyclin-dependent kinase inhibitor (CDKi) p27Kip1 (Polyak et al., 1994). These observations...
demonstrate that the neural bHLH proteins can impart a neuronal fate on mammalian cells and that these proteins can link neuronal differentiation to cell cycle regulation.

MATERIALS AND METHODS

Expression plasmids

pCS2+MTneuroD (Lee et al., 1995), pCS2+MTXngn2 (pCS2+MTXNGNR1a) (Ma et al., 1996), pCS2+ngn1/neuroD3 (McCormick et al., 1996) and pCS2+ mouse MyoD (Rupp et al., 1994) have been described previously. cDNAs for other mouse neural bHLH proteins (see Introduction for references), rat MASH1 (Johnson et al., 1990), hamster BETAA3 (Peyton et al., 1996), mouse E12 (Walker et al., 1990) and EGFP (Clontech) were cloned into pCS2+ or pCS2+MT (for myc epitope tag fusions; Rupp et al., 1994; Turner and Weintraub, 1994). Details are available upon request. pCS2+ Hand1/Thing1, pCS2+Hand2/Thing2, and pCS2+Epicardin/Halo1 were provided by Stan Hollenberg (Hollenberg et al., 1995).

Expression vectors pCS2+ or pCS2+MT (for myc epitope tag fusions; Rupp et al., 1994; Turner and Weintraub, 1994). These residues are conserved in almost all bHLH proteins and are required for DNA binding in MyoD (Davis et al., 1990). An identical mutation changes E131R132 to A131Q132. The neuroD2 DBM mutation changes E131R132 to A131Q132. These residues are conserved in almost all bHLH proteins and are required for DNA binding in MyoD (Davis et al., 1990). For a 60 mm dish, BBS transfections included 7.5 g E12 vector and 5 gal (Turner and Weintraub, 1994) using the Tropix Dual Light system. Data shown is an average from three experiments.

Cell culture and transfections

P19 cells were cultured in MEM with 7.5% calf serum and 2.5% fetal bovine serum (HyClone) and maintained subconfluent prior to transfection (McBurney et al., 1988). For cells maintained under these growth conditions, we observed less than one spontaneous TuJ1-labeled cell per million untransfected P19 cells (0-5 TuJ1-positive cells per confluent 60 mm dish, 4 days after plating). For transfections, cells were plated on murine laminin (Gibco) or poly-L-lysine (Sigma)-coated dishes (Corning). Transfections were performed by the BES-buffered saline (BBS) CaP04 method (Chen and Okayama, 1987) with 5% CO2, or with FuGene6 (Roche) as directed by the manufacturer. For a 60 mm dish, BBS transfections included 7.5ng/ml neural bHLH vector, 2.5g/ml E12 vector and 5g/ml GFP vector. For FuGene transfections, one third as much DNA was used. When necessary, a vector that expresses a nuclear localized multimerized myc tag, pCS2+nlsMT (Rupp et al., 1994), was added to maintain a constant total amount of DNA. Cotransfection efficiency was at least 90%, based on a comparison of GFP with a cotransfected myc epitope-tagged protein (pCS2+MT).

For longer term culture of transfected P19 cells, media and serum were replaced 2 days after transfection with Neurobasal medium and B27 supplement (Gibco), without glutamine or glutamate, plus 15g/ml cytosine arabinoside (araC). 2 days later, the media was changed to Neurobasal medium and B27 supplement with 0.5 mM glutamine and 15g/ml arAC. Media was changed every 2 days thereafter. Most untransfected P19 cells die after 1-2 days in these conditions.

Immunochemistry and antibodies

Cells were fixed for 8-20 minutes with either 3.7% formaldehyde or 4% paraformaldehyde in phosphate-buffered saline (PBS). For amino acid neurotransmitter antibodies, 1% glutaraldehyde was included. Primary antibodies are listed followed by the antigen, with the dilution used in parentheses. TuJ1 neuron-specific tubulin (Babco, 1:1000). From Chemicon International, Inc.: AB1515 interxin (1:1000), MAB1621 neurofilament-M (1:1000), MAB 1615 neurofilament-L (1:250), MAB1623 neurofilament-H (1:300), AB1778 calbindin (1:1000), AB133 glutamate (1:200-500), AB141 GABA (1:200), AB1566 substance P (1:200-1000, preabsorbed on untransfected P19 cells), MAB363 NMDAR1 (1:200). From the Developmental Studies Hybridoma Bank (supernatants diluted 1:3; ascites 1:500): 5A5 NCAM, 40.2D6 islet1, 4F2 LIM1+2, RC2 and 2C3 radial glia, 2H3 neurofilament-M, and MF20 myosin heavy chain. Other antibodies: rabbit anti-GFAP (Sigma, 1:500), rabbit anti-synapsin 1 (1:500, gift of T. Ueda), M6 (1:10, gift of C. Lagena), and 16A11 HuC/D (1:500, gift of M. Marusich). For p72Kpl, similar results were obtained with three different antibodies (Santa Cruz sc397, 1:1000; Neomarkers DC527F6, 1:600; Transduction Laboratories K25020, 1:250).

Secondary antibodies were Cy3- or rhodamine-conjugated anti-rabbit or anti-mouse (1:500-1000, Jackson or Chemicon). Because of glutaeraldehyde-induced autofluorescence, GABA and glutamate expression was confirmed with alkaline phosphatase-conjugated goat anti-rabbit (Roche).

Cells were photographed with a 35 mm film camera or a color video camera and images were digitized. Double-label images were assembled in Adobe Photoshop. To determine the percentage of TuJ1-labeled cells (Table 1), digitized video images were superimposed in Photoshop and scored on a computer monitor.

Reporter assays

The reporter EB7Luc was cloned by inserting three copies of a double-stranded oligonucleotide AGAGAGTGCAGATTCGCAGCGGTG into the Smal site of pT81-Luc. This oligo contains an E-box (underlined) from the neuroD2 promoter (J. M. O. and S. J. T., unpublished observations). Reporter activity was assayed 2 days after transfection. To control for transfection efficiency, luciferase activity was normalized to β-galactosidase activity from cotransfected pCS2+βgal (Turner and Weintraub, 1994) using the Tropix Dual Light system. Data shown is an average from three experiments.

Electrophysiology

Tight-seal, whole-cell recordings from cells in culture 3-7 days after transfection were made using standard techniques. Transfected cells were identified by GFP fluorescence. The pipette solution contained (in mM) KCl 140, NaCl 5, MgCl2 1, Na2EGTA 10, Heps 10, pH 7.4. In voltage-clamp experiments in which the ionic dependence of the voltage-dependent current was tested, extracellular solutions were: normal sodium solution (in mM): NaCl 140, KCl 1, MgCl2 1, CaCl2 1, Heps 10, pH 7.4. choline solution: cholineCl 139, NaCl 1, KCl 1, MgCl2 1, CaCl2 1, Heps 10, pH 7.4. The choline solution was applied to the cell under study from a 0.9 mm diameter pipette placed close to the cell. The shift in reversal potential (see Results) indicated that the solution exchange was about 95% effective. In all other experiments, the external solution used contained (in mM) 132 NaCl, 5.3 KCl, 1.3 NaH2PO4, 1.7 MgSO4, 5.4 CaCl2, 12 Heps, 6.3 glucose and 0.3 phenol red (pH, 7.4). To assess excitability, voltage responses obtained under current clamp were classified as non-regenerative or regenerative. Constant current was passed so that the resting potential was near −80 mV; 2.5 msec depolarizing current pulses of increasing amplitude were given, until a regenerative response was elicited or until the cell was depolarized to 0 mV. A response was considered regenerative if it deviated by +2 mV or more from that predicted by passive decay.

Proliferation studies

For BrdU experiments, transfected P19 cells were fixed as described under Immunohistochemistry and antibodies. BrdU (final 15 ng/ml,
695bHLH proteins and neuronal differentiation

Sigma) was added from 24-36 or 48-60 hours after transfection. Rat anti-BrdU supernatant (Accurate Chemical & Scientific) was diluted 1:40 in PBS with 1 mM CaCl₂, 1 mM MgCl₂ and 100 units per ml DNase (Promega), and applied to the cells for 1 hour at 37°C (modification of Amersham BrdU-labeling kit protocol). Secondary antibody was Texas Red-conjugated donkey anti-rat (Jackson) at 1:200, with 2% calf serum.

RT-PCR analysis

Transfections included the GFP vector and transfection efficiency was monitored by observation of GFP in live cells prior to RNA harvest. RT-PCR was performed essentially as described (Rupp and Weintraub, 1991) with 0.5 μg of total RNA for each RT-PCR. Specific PCR primer pairs were designed for mouse neuroD, neuroD2, MATH2 and MATH3. Primer pairs generate products that span the first intron of each target gene (to facilitate the identification of mRNA-derived products). These primer pairs do not generate a PCR product from the corresponding neural bHLH expression vectors used for transfections (since bHLH coding regions in the vectors start in the second exon of each gene). The product of the control primer pair for the ubiquitous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA spans three introns. Primer sequences are available upon request. PCR was performed for 18 cycles for GAPDH and 28 cycles for neural bHLH genes.

RESULTS

Generation of neurons from P19 cells by transfection of neuroD2

P19 cells were transiently cotransfected with vectors for myc epitope-tagged neuroD2, E12 and GFP were fixed 4 days after transfection. (A) GFP fluorescence revealed that transfected cells adopted a neuronal morphology. (B) Most transfected cells expressed a neuron-specific β-tubulin isoform, detected by indirect immunofluorescence with the antibody TuJ1. (C) Combination of GFP fluorescence (green) and indirect immunofluorescence for TuJ1 (red). Overlapping signals are yellow or orange. (D,E) P19 cells cotransfected with E12 and GFP maintained the normal morphology of P19 cells as revealed by GFP fluorescence (D), and were not labeled by TuJ1 (E). (F) P19 cells transfected with neuroD2 and GFP without E12 adopted a neuronal morphology and were labeled by indirect immunofluorescence for TuJ1. Colors are the same as in C.

Table 1. Neuron formation and reporter activation in P19 cells transfected with bHLH plasmids

<table>
<thead>
<tr>
<th>bHLH class</th>
<th>Transfected bHLH cDNA(s)</th>
<th>TuJ1+/GFP+ (%)</th>
<th>E-box reporter (fold activation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuronal differentiation</td>
<td>neuroD2+E12</td>
<td>64</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>neuroD2</td>
<td>49</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>neuroD+E12</td>
<td>6</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>MATH2+E12</td>
<td>&lt;1</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>MATH3+E12</td>
<td>2</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>neuroD2DBM+E12</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Neural precursor</td>
<td>MATH1+E12</td>
<td>12</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>ngn1+E12</td>
<td>8</td>
<td>309</td>
</tr>
<tr>
<td></td>
<td>Xgn2+E12</td>
<td>&lt;1</td>
<td>266</td>
</tr>
<tr>
<td></td>
<td>ngn3+E12</td>
<td>&lt;1</td>
<td>149</td>
</tr>
<tr>
<td>Neural precursor</td>
<td>MASH1+E12</td>
<td>36</td>
<td>202</td>
</tr>
<tr>
<td>(achaete-scute family)</td>
<td>MASH1</td>
<td>31</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>D. m. scute+E12</td>
<td>&lt;1</td>
<td>36</td>
</tr>
<tr>
<td>Neuronal differentiation</td>
<td>NSCL1+E12</td>
<td>&lt;1</td>
<td>3</td>
</tr>
<tr>
<td>(other bHLH families)</td>
<td>Hand1+E12</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Repressor</td>
<td>BETA3+E12</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Non-neural</td>
<td>MyoD+E12</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Hand2+E12</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Epicardin+E12</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Dimerization partner</td>
<td>nlsMT+E12</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Vector Control</td>
<td>nlsMT</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Neuron formation was assessed by indirect immunofluorescence with TuJ1 4 days after transfection. The percentages of TuJ1 labeled cells (>1%) are from a representative experiment, with TuJ1 expression scored for 300-1100 GFP-labeled cells per vector. The E-box reporter column shows relative activation of a luciferase reporter by each neural bHLH vector (see text for details).

neuroD2DBM contains a mutation in the DNA-binding domain (see text).

Neuron formation was assessed by indirect immunofluorescence with TuJ1 4 days after transfection. The percentages of TuJ1 labeled cells (>1%) are from a representative experiment, with TuJ1 expression scored for 300-1100 GFP-labeled cells per vector. The E-box reporter column shows relative activation of a luciferase reporter by each neural bHLH vector (see text for details).

neuroD2DBM contains a mutation in the DNA-binding domain (see text).

Neuron formation was assessed by indirect immunofluorescence with TuJ1 4 days after transfection. The percentages of TuJ1 labeled cells (>1%) are from a representative experiment, with TuJ1 expression scored for 300-1100 GFP-labeled cells per vector. The E-box reporter column shows relative activation of a luciferase reporter by each neural bHLH vector (see text for details).

neuroD2DBM contains a mutation in the DNA-binding domain (see text).
expression and neuron formation (Fig. 1C) suggests that the generation of neurons after neuroD2 transfection is cell autonomous. We also tested a mutant of neuroD2 in which two essential residues in the DNA-binding basic region were altered (see Materials and Methods). Cotransfection of this mutant with the E12 plasmid into P19 cells did not generate neurons (Table 1), indicating that an intact DNA-binding domain is required for generation of neurons by the neuroD2 protein.

Since one hallmark of neurons is the ability to propagate electrical signals, we determined whether P19 cells transfected with neuroD2 and E12 vectors had the electrophysiological properties of neurons. Under current-clamp, transfected cells showed regenerative responses to depolarizing current steps in all cases (n=14; Fig. 2A). Cells transfected with only the E12 vector showed no regenerative responses (n=10; Fig. 2B). Under voltage-clamp with normal external sodium, all tested cells cotransfected with neuroD2 and E12 had rapidly activating, voltage-dependent, transient currents, which were inward at potentials negative to +40 mV (n=25; Fig. 2C), while none of ten E12 transfected control cells studied had fast activating inward currents (Fig. 2D). The rapidly activating currents were due to flow through voltage-dependent sodium channels, since the reversal potential decreased by 77±5 mV when extracellular sodium was replaced with choline (Fig. 2C). Cells transfected with E12 alone or with neuroD2 and E12 showed slowly activating, voltage-dependent, sustained outward currents (Fig. 2C,D). However, the conductance increase associated with the outward current was about seven times larger in cells transfected with neuroD2 and E12 (peak conductance of 7.0±1.1 nS, n=25, compared with 1.2±0.3 nS, n=10, for cells transfected with only E12). Thus, expression of neuroD2 and E12 confers P19 cells with the electrical signaling properties of neurons.

Other neural bHLH proteins can generate neurons from P19 cells

To determine whether neural bHLH proteins other than neuroD2 could generate neurons from P19 cells, we transfected vectors expressing the MASH1, neuroD, ngn1-3, MATH1-3 or NSCL1 (Begley et al., 1992) bHLH proteins, in combination with the E12 plasmid. All of these transfections led to the generation of TuJ1-positive neurons from a subset of the transfected cells (Fig. 3A-D; Table 1), although the percentage of transfected cells that differentiated as neurons varied more than 100-fold (Table 1 and data not shown). Interestingly, MATH2 and most of the other neural bHLH plasmids that generated neurons infrequently were able to activate a cotransfected multimerized bHLH DNA-binding site (E-box) reporter (Table 1), indicating that these proteins were expressed and functional. The ability to generate neurons in P19 cells is an evolutionarily conserved characteristic of the neural bHLH proteins, since vectors expressing Xenopus ngn2 or Drosophila melanogaster (D.m.) scute also produced neurons when cotransfected with the E12 vector (Table 1). As observed for neuroD2, cotransfection of E12 was not essential for neuron formation by any of the neural bHLH proteins, but it did increase the number of TuJ1-positive cells and these cells often had more extensive processes (Table 1 and unpublished observations).

In contrast to the neural bHLH proteins, transfection of expression plasmids for the myogenic bHLH protein MyoD (Weintraub et al., 1991), the non-neural bHLH protein epicardin (Robb et al., 1998), or the predominantly non-neural bHLH proteins Hand1 and Hand2 (Cserjesi et al., 1995; Hollenberg et al., 1995), each with the E12 plasmid, failed to generate neurons (Table 1). BETA3, a protein structurally related to neuroD but reported to function as a repressor (Peyton et al., 1996), also did not produce neurons. Although MyoD activated the E-box reporter, other non-neural bHLH proteins activated the reporter weakly, if at all. These proteins may prefer a different E-box, they may not be transcriptional activators (e.g. BETA3 and epicardin), or they may be expressed at lower levels. Nonetheless, our results indicate that the ability to generate neurons from P19 cells is specific to the neural-specific bHLH proteins and is unlikely to result from promiscuous bHLH dimerization or other nonspecific interactions.
Characterization and persistence of neuronal differentiation after transient neural bHLH expression

To further characterize the extent of neuronal differentiation of P19 cells transfected with neural bHLH vectors, we assessed the expression of various neuron-specific proteins. Pan-neuronal markers such as neurofilament-M, the HuC/D RNA-binding proteins (Wakamatsu and Weston, 1997), M6 (Yan et al., 1996) and synapsin I were present in most or all cells with neuronal morphology (Table 2; Fig. 3B,C,G). Moreover, subsets of the transfected cells were immunoreactive for the neurotransmitters GABA and glutamate (Fig. 3E), the GABA synthetic enzyme glutamic acid decarboxylase (GAD), the neuropeptide substance P (Fig. 3F), NMDA receptor 1, and the transcription factors Islet-1 or LIM1/2 (Table 2). At present, we do not know if the subsets of cells that express these proteins overlap. We have observed the same constellation of neurotransmitters, receptors and other markers of mature neuronal subtypes regardless of which neural bHLH vector was transfected. We did not detect expression of glial fibrillary acidic protein (GFAP), or markers of radial glia, in any transfected cells (Table 2).

Neurons generated by transient transfection of P19 cells with plasmids for MASH1, neuroD2 or ngn1 each cotransfected with the E12 plasmid, survived for at least 13 days after transfection (Fig. 3G) when cultured under conditions that eliminate most untransfected cells (see Materials and Methods). Cells maintained neuronal morphologies and continued to express neuron-specific proteins. Epitope-tagged versions of neuroD2 or MATH1 could be detected in the nucleus of most transfected cells 1 or 2 days after transfection, but were detectable in only a few cells at 4 days, and were undetectable at 10 days (data not shown). The cotransfected GFP marker also was undetectable by 10 days after transfection (data not shown). This suggests that the neural bHLH proteins initiate a stable program of neuronal gene expression that is maintained after the introduced bHLH proteins are no longer present.

Cell cycle withdrawal and elevated p27Kip1 expression in P19 cells transfected with neural bHLH vectors

During neurogenesis, neuronal differentiation is preceded by withdrawal from the cell cycle. We asked whether neurons generated by expression of bHLH proteins withdraw from the cell cycle by using bromodeoxyuridine (BrdU) incorporation to label proliferating cells. 3 days after transfection, 84% of the GFP-expressing control transfected cells incorporated BrdU (Fig. 4C). The degree to which transfected neural bHLH cDNAs also reduced the fraction of cells that incorporated BrdU (Fig. 4C). The degree to which different neural bHLH proteins reduced BrdU incorporation paralleled the ability of each bHLH protein to generate neurons. Since transfected cells can survive for more than 10 days in the presence of cytosine arabinoside, a nucleotide analog that kills dividing cells (Fig. 3G), it is likely that cell cycle withdrawal triggered by the neural bHLH proteins is permanent.

The myogenic bHLH protein MyoD also can initiate cell cycle withdrawal, and this is likely to be mediated in part by a direct activation of the CDKi p21 (Halevy et al., 1995; Otten et al., 1997). Recently, neuroD has been reported to inhibit cell cycle progression and activate p21 expression in a transfected non-neural cell line (Mutoh et al., 1998). We tested whether P19 cells transfected with the neural bHLH vectors and E12 expressed elevated levels of the CDKi proteins p21 or p27Kip1. We could not detect p21 expression in P19 cells after transfection of either neuroD or other neural bHLH cDNAs (data not shown). However, P19 cells transfected with any of the neural bHLH vectors with E12 expressed the CDKi protein p27Kip1, as assessed by indirect immunofluorescence with three different antibodies (Fig. 4D,E,H and data not shown; see Materials and Methods). In contrast, only occasional cells had detectable p27Kip1 expression in P19 cells transfected with a control vector (Fig. 4H).

To further characterize the relationship between p27Kip1 expression, cell cycle withdrawal and neuronal differentiation, we compared p27Kip1 expression with BrdU incorporation and TuJ1 expression after cotransfection of P19 cells with the neuroD2, E12 and GFP vectors. Most cells that expressed p27Kip1 did not incorporate BrdU (Fig. 4F,G). In addition, the percentage of transfected cells that expressed p27Kip1 was essentially the same as the percentage of cells that did not incorporate BrdU (Fig. 4H). These observations indicate that the transfected cells with elevated p27Kip1 did not enter the cell cycle. This is consistent with the possibility that the neural bHLH proteins drive cell cycle withdrawal by elevating the level of p27Kip1. We also observed that almost all cells labeled by TuJ1 expressed p27Kip1 (Fig. 4L-K), and the percentage of TuJ1 and p27Kip1 double-labeled cells increased with time (Fig. 4K). This suggests that p27Kip1 expression (and cell cycle

### Table 2. Neural antigens detected by indirect immunofluorescence after transfection of P19 cells with neural bHLH plasmids

<table>
<thead>
<tr>
<th>Antigen type</th>
<th>Antigen</th>
<th>Expression</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>panneuronal</td>
<td>HuC/HuD (16A11)</td>
<td>+++ 1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>internexin</td>
<td>+++ 1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>neurofilament-M</td>
<td>+++ 1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>neurofilament-L</td>
<td>+ 1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>neurofilament-H</td>
<td>- 1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>synapsin1</td>
<td>+++ 1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M6</td>
<td>+++ 1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NCAM (sialylated form)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Neuronal subpopulations

- calbindin: ++ 1
- glutamate: + 1.2
- GABA: + 1.2
- GAD: + 1.2
- substance P: + 1.2
- NMDA receptor 1: + 1.2
- islet1: + 1.2
- LIM1/2: + 1.2

Glial cells

- GFAP: - 3
- radial glia (RC2, 3CB2): - 1

Muscle

- myosin heavy chain: -
withdrawal) preceded neuronal differentiation in the transfected cells.

**Endogenous bHLH gene expression in P19 cells transfected with neural bHLH vectors**

Sequential activation of neural bHLH gene expression has been demonstrated during vertebrate neurogenesis, suggesting that these genes function in a cascade (Lee et al., 1995; Ma et al., 1996; Cau et al., 1997). We used RT-PCR analysis (Rupp and Weintraub, 1991; Boudjelal et al., 1997) to examine whether transfection of P19 cells with vectors for the neural bHLH proteins activated transcription of the endogenous neural bHLH genes. We focused on neuroD, neuroD2, MATH2 and MATH3, since these genes are expressed during neuronal differentiation. P19 cells did not express detectable MATH3 after transfection of a control vector and E12. However, MATH3 mRNA was present 2 or 5 days after the transfection of vectors expressing either precursor- or differentiation-specific neural bHLH proteins (Fig. 5 and data not shown). P19 cells transfected with a control vector and E12 expressed detectable neuroD mRNA, consistent with a previous report that undifferentiated P19 cells express a low level of neuroD mRNA (Boudjelal et al., 1997). However, P19 cells transfected with neural bHLH vectors and E12 had increased levels of neuroD mRNA relative to control transfected cells at 2 days after transfection (Fig. 5). Interestingly, introduced MATH3 activated the endogenous MATH3 gene and introduced neuroD increased the expression of the endogenous neuroD gene modestly, suggesting that these proteins can autoactivate their own transcription. We did not detect expression of MATH2 or neuroD2 mRNA 2 days after transfection with any of the neural bHLH vectors, but neuroD2 mRNA was detectable at 5 days after transfection of MASH1 or ngn1 and E12 (data not shown). These results indicate that the introduced bHLH proteins activate the expression of one or more endogenous neural bHLH genes.

**Expression of neuronal markers in other cell types after transfection of neural bHLH vectors**

To determine whether mammalian cell types other than P19 could generate neurons in response to neural bHLH protein expression, we transfected the neuroD2, MASH1 or ngn1 vectors, together with the E12 and GFP vectors, into several cell lines and primary rat astrocytes. Most cell lines, including NIH3T3, CHO, NT2, PC12 and HaCAT, were not labeled by TuJ1 after transfection (unpublished observations). However, 10T1/2 fibroblasts and rat astrocytes were immunoreactive for TuJ1 after cotransfection of any of the three neural bHLH vectors with E12 (Fig. 3H,I and data not shown). Although we occasionally observed one or two TuJ1-labeled 10T1/2 cells or astrocytes per dish with a neuronal morphology, most transfected cells did not adopt a neuronal morphology (Fig. 3H,I), nor did they express other neuronal markers or detectable p21 or p27Kip1 proteins (unpublished observations). Thus, efficient generation of neurons by transfection of neural bHLH vectors was limited to P19 cells.
Our results demonstrate that transient expression of neural bHLH proteins is sufficient to initiate a stable program of neuronal differentiation in pluripotent mouse P19 cells. This program includes alterations in morphology, neurite extension, expression of neuron-specific proteins, cell cycle withdrawal and the capacity for electrical signaling. Thus, the neural bHLH proteins can function as master regulators for mammalian neurogenesis, in that they are sufficient to initiate a program of neuronal differentiation in non-neural cells. The only other mammalian proteins known to have similar abilities in cell culture are the MyoD family of bHLH proteins, which can direct myogenesis (Weintraub et al., 1991; Molkentin and Olson, 1996), and PPARα, which can direct adipogenesis (Tontonoz et al., 1994). Studies of MyoD and related bHLH proteins in cell culture have led to considerable progress in understanding the regulation of myogenesis. The ability of the neural bHLH proteins to generate neurons in culture from P19 cells should similarly facilitate studies on the molecular regulation of neurogenesis.

Neural bHLH proteins link neuronal differentiation, CDKi expression and cell cycle withdrawal

Neuronal differentiation of P19 cells transfected with neural bHLH vectors was preceded by cell cycle withdrawal, providing direct evidence that the neural bHLH proteins can link neuronal differentiation and cell cycle regulation. These findings are consistent with the observation that expression of neuroD from a retroviral vector introduced into rat retinal progenitors reduced clone sizes in vivo (Morrow et al., 1999). The expression of p27Kip1 CDKi protein in the transfected P19 cells suggests that the neural bHLH proteins may direct cell cycle withdrawal by elevating the level of p27Kip1 or other CDKi proteins. Such a mechanism would be analogous to that proposed for the myogenic bHLH proteins during muscle formation (Halevy et al., 1995). Although we did not observe increased expression of the CDKi p21 in the transfected cells,
MASH1 to activate neuron formation. Although cotransfection of the P19 cells cooperate more efficiently with neuroD2 and cotransfected reporter efficiently. Possibly, cofactors present in neurons were expressed and functional, since they activated E12. This difference is intriguing since the MA TH2 and neuroD2 proteins have similar amino acid sequences and both exist in some cells in embryos, since expression of endogenous MA TH3 and neuroD proteins each activated the expression of the corresponding endogenous gene. It has been proposed that the neural bHLH proteins participate in a cascade of gene expression to regulate sequential determination and differentiation steps during neuron formation (Lee et al., 1995; Ma et al., 1996; Cau et al., 1997). A similar stepwise process may occur in the transfected P19 cells, since introduction of precursor-specific bHLH proteins such as MASH1, MA TH1 or MASH3 may enhance neuronal differentiation by augmenting limited amounts of endogenous E12 or related proteins.

Neurons generated from P19 cells by the neural bHLH proteins maintained neuronal characteristics for at least 13 days after transfection. Since cells did not express detectable levels of protein from the transfected vectors by 10 days after transfection, it seems likely that the introduced neural bHLH proteins activated endogenous regulatory factors, which maintained the program of neuronal gene expression. Such factors could include neural bHLH proteins encoded by endogenous genes. Consistent with this, we observed increased levels of mRNA from the endogenous MA TH3, neuroD and neuroD2 genes after the transfection of various neural bHLH vectors with E12. Expression of the MA TH3 and neuroD genes may be maintained in the transfected cells by autoactivation, since the MA TH3 and neuroD proteins each activated the expression of the corresponding endogenous gene. It has been proposed that the neural bHLH proteins participate in a cascade of gene expression to regulate sequential determination and differentiation steps during neuron formation (Lee et al., 1995; Ma et al., 1996; Cau et al., 1997). A similar stepwise process may occur in the transfected P19 cells, since introduction of precursor-specific bHLH proteins such as MASH1, MA TH1 or MASH3 may enhance neuronal differentiation by augmenting limited amounts of endogenous E12 or related proteins.

Fig. 5. RT-PCR analysis of endogenous MA TH3 and neuroD expression in P19 cells transfected with neural bHLH, E12 and GFP vectors. MA TH3 mRNA expression was detected in P19 cells 2 days after transfection of neural bHLH vectors, but not in P19 cells transfected with a control vector (pCS2+nlsMT) and E12. While neuroD mRNA was present in cells transfected with the control vector, its expression increased after transfection of neural bHLH vectors. The ubiquitous GAPDH mRNA was used as an internal control.

Specificity and persistence of bHLH-mediated neuron formation

The ability to generate neurons from P19 cells was restricted to bHLH proteins expressed primarily in the nervous system. All of these proteins are known to function as transcriptional activators. For neuroD2, we demonstrated an intact DNA-binding domain was required for neuron formation. These observations strongly suggest that neuroD2 and the other neural bHLH proteins initiate neuronal differentiation in P19 cells by activating the expression of specific target genes, rather than by titrating a dimerization partner or another protein. A potential application of the P19 transfection assay is the identification of target genes for the neural bHLH proteins.

While expression of any of the neural bHLH proteins led to the formation of neurons from the transfected P19 cells, the percentage of transfected cells that differentiated as neurons varied, depending on which bHLH protein was introduced. For example, neuroD2 or MASH1, in combination with E12, produced at least 100-fold more neurons than MA TH2 and E12. This difference is intriguing since the MA TH2 and neuroD2 proteins have similar amino acid sequences and both are expressed during RA-induced P19 differentiation (Turner and Weintraub, 1994; Lee et al., 1995). The observations presented here suggest that either the bHLH-activated neuronal differentiation program is dependent on cofactors preferentially expressed in P19 cells, or it is repressed by factors present in other cells.

Comparison of P19 cells with cell types that fail to support neuronal identity and bHLH proteins

The role of neural bHLH proteins in determining the identities of specific neuronal subtypes remains unclear. MASH1 is required for noradrenergic differentiation of many CNS and PNS neurons. However, expression of MASH1 from a retroviral vector did not confer noradrenergic properties on neurons, although it did activate expression of the Phox2a homeobox protein, which is associated with noradrenergic differentiation (Hirsch et al., 1998; Lo et al., 1998). In Xenopus and mouse retinas, as well as chick neural tube, expression of neural bHLH proteins can bias cells toward specific neuronal fates (Kanekar et al., 1997; Morrow et al., 1999; Perez et al., 1999; Mutoh et al., 1996; Cau et al., 1997). A similar stepwise process may occur in the transfected P19 cells, since introduction of precursor-specific bHLH proteins such as MASH1, MA TH1 or MASH3 may enhance neuronal differentiation by augmenting limited amounts of endogenous E12 or related proteins.

Restrictions on neural bHLH protein function

Transfection of cell types other than P19 with neural bHLH vectors did not lead to the formation of neurons at a significant frequency. The activation of a neuron-specific tubulin in 10T1/2 cells and astrocytes indicates that the neural bHLH proteins are functional in these cell types, despite their inability to activate other aspects of the neuronal differentiation program. Similar restrictions on neural bHLH function may exist in some cells in Xenopus embryos, since expression of neural bHLH proteins in these embryos only converts a subset of ectodermal cells to a neuronal fate (Turner and Weintraub, 1994; Lee et al., 1995). The observations presented here suggest that either the bHLH-activated neuronal differentiation program is dependent on cofactors preferentially expressed in P19 cells, or it is repressed by factors present in other cells.
REFERENCES


