Tohoku J. Exp. Med., 1997, **182**, 327-336

Gene Expression of MASH-1, MATH-1, neuroD and NSCL-2, Basic Helix-Loop-Helix Proteins, during Neural Differentiation in P19 Embryonal Carcinoma Cells

FUMIAKI ITOH, TOKIO NAKANE and SHIGETOSHI CHIBA

Department of Pharmacology, Shinshu University School of Medicine, Matsumoto 390

ITOH, F., NAKANE, T. and CHIBA, S. *Gene Expression of MASH-1, MATH-1, neuroD and NSCL-2, Basic Helix-Loop-Helix Proteins, during Neural Differentiation in P19 Embryonal Carcinoma Cells.* Tohoku J. Exp. Med., 1997, **182** (4), 327-336 —— We examined the gene expression of MASH-1, MATH-1, neuroD and NSCL-2 during neural differentiation of P19 embryonal carcinoma cells using reverse transcription-polymerase chain reaction and high performance liquid chromatography. These proteins are members of basic helix-loop-helix transcription factor family and their expressions are reported to be transient and restricted in the nervous system during early neurogenesis. Retinoic acid (RA; 1 μM)-treatment and aggregation for 4 days induced and greatly increased MASH-1, neuroD and NSCL-2 mRNA in P19 cells. The increases peaked at day 3, 4 and 5, respectively. RA-treatment increased MATH-1 mRNA slightly. mRNA of MAP2, a neural differentiation marker, were increased by RA-treatment and the increases reached to the plateau at day 5. The results indicate that the gene expression of MASH-1, MATH-1, neuroD and NSCL-2 during neural differentiation in P19 cells is transient and the order is similar to that in the mouse embryo nervous system as previously reported. ———— neural differentiation; helix-loop-helix protein; proneural gene; embryonal carcinoma cell; retinoic acid

The transcription factor family termed basic helix-loop-helix (bHLH) protein participates in a wide range of developmental processes such as neurogenesis (Ghysen and Dambly-Chaudiere 1989; Jan and Jan 1990; Campuzano and Modolell 1992; Jarman et al. 1993), myogenesis (Weintraub et al. 1991), hematogenesis (Aplan et al. 1990; Mellentin et al. 1989) and sex determination (Parkhurst et al. 1990). The mechanism of neural fate determination in mammalian remains to be elucidated. *Drosophila* proneural genes of the AS-C (*achaete*, *scute*, *lethal of scute*, and *asense*) and *atonal* encode proteins that play as positive neural fate determination factors and are members of bHLH protein

Received March 27, 1997; revision accepted for publication July 1, 1997.

Address for reprints: Tokio Nakane, M.D., Department of Pharmacology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390, Japan.

family (Ghysen and Dambly-Chaudiere 1989; Jan and Jan 1990; Campuzano and Modolell 1992; Jarman et al. 1993). Recent works have shown that *Drosophila* proneural genes are conserved in vertebrates. Mammalian homologues of *achaete-scute* (MASH-1 and MASH-2, Johnson et al. 1990) and *atonal* (MATH-1 and MATH-2, Akazawa et al. 1995) and other bHLH proteins (neuroD, Lee et al. 1995; NSCL-1, Begley et al. 1992; NSCL-2, Göbel et al. 1992) were cloned. The expression of MATH-1, neuroD and NSCL-2 is transient and restricted in differentiating neurons during mouse embryonic development (Göbel et al. 1992; Akazawa et al. 1995; Lee et al. 1995). Only MASH-1 seems to be expressed in mitotic neural precursor cells (Johnson et al. 1990).

P19 mouse embryonal carcinoma cells were derived from a teratocarcinoma formed following transplantation of a 7.5 day embryo into the testis (McBurney and Rogers 1982). P19 cells differentiate to neuron and glia when they are aggregated and treated with retinoic acid (RA, Jones-Villeneuve et al. 1982, 1983; McBurney et al. 1988). Neural induction in P19 cells is positively correlated with MASH-1, but not MASH-2 (Johnson et al. 1992). The expression of bHLH proteins other than MASH-1 and MASH-2 has not been analyzed during neural differentiation of P19 cells. Gene expression of MASH-1 and MASH-2 was constitutive and only moderately regulated during neural differentiation in PC12 and MAH cells (Johnson et al. 1990). There has been no report that the gene expression patterns of the bHLH proteins, whose expression is transient and restricted in the nervous system, were simultaneously examined in the same cell line during neurogenesis and neural differentiation. In this study, the induction of the gene expression of MASH-1, MATH-1, neuroD and NSCL-2 during neural differentiation in P19 cells were studied using the reverse transcription-polymerase chain reaction (RT-PCR) and quantified by high performance liquid chromatography (HPLC).

Materials and Methods

Cell culture

P19 embryonal carcinoma cell line was obtained from American Type Culture Collection (CRL 1825; Rockville, MD, USA). P19 cells were grown in DMEM (Nikken BioMedical Laboratory, Kyoto) supplemented with 7.5% calf serum (Life Technologies, Gaithersburg, MD, USA) and 2.5% fetal bovine serum (Equitech-Bio, W. Ingram, TX, USA). P19 cells were treated with *all-trans* retinoic acid (1 μM; Sigma, St. Louis, MO, USA) as described by Rudnicki and McBurney (1987).

RNA isolation and PCR

Total cellular RNA was isolated from P19 cells by acid guanidine phenol chloroform method (Chomczynski and Sacchi 1987) using ISOGEN (Nippon Gene, Tokyo) and was treated with RQ1 RNase-free DNase (Promega, Madison, WI,

TABLE 1. *Oligonucleotide primers for studying gene expression in RA-treated P19 embryonal carcinoma cells by RT-PCR analysis*

Gene	5′ primer sequence	3′ primer sequence	PCR product (bp)
β-Actin	5′-GTGGGAATGGGTCAGAAGGA-3′	5′-CAGGGACAGCACAGCCTGGA-3′	296
G3PDH	5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′	5′-CATGTAGGCCATGAGGTCCACCAC-3′	983
MAP2	5′-GCAGCAAGTGGTGACTTGGCTC-3′	5′-GAGGAGTGCGGATGATGGCAAC-3′	420
MASH-1	5′-CTCGTCCTCTCCGGAACTGATG-3′	5′-CGACAGGACGCCCGCCTGAAAG-3′	303
MATH-1	5′-CACAGCTTCCTGCAAAAATG-3′	5′-GTTTTGCTGTTGTCCTCCTG-3′	311
neuroD	5′-TCAGCATCAATGGCAACT-3′	5′-TGACTCGCTCATGATGCGA-3′	212
NSCL-2	5′-CAGGATTTGAGCTTGGTGAGAC-3′	5′-GAGGGAGTGTAAACACTTGAC-3′	318

USA) at 37°C for 30 minutes (Davis et al. 1994). mRNA was isolated from ICR mouse (Japan SLC, Hamamatsu) embryo (E13) by FastTrack kit (Invitrogen, La Jolla, CA, USA). RT-PCR was done using RNA PCR kit (Perkin Elmer Japan, Urayasu). First strand cDNA synthesis was performed using 1 μg of total RNA (P19 cells) or 50 ng of mRNA (mouse embryo) in a reaction volume of 20 μl at 42°C for 20 minutes. Two-5 μl of first strand cDNA was amplified by PCR in a reaction volume of 25 μl. The reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP and dTTP, 0.15 μM of the appropriate primers and 0.625 units of AmpliTaq DNA polymerase (Perkin Elmer Japan). The reactions were carried out in an automated thermal cycler (MJ Research, Wartertown, MA, USA). The amplification sequence consisted of an initial denaturation at 95°C for 2 minutes, followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute except MATH-1 (30 cycles). A final extension at 72°C for 7 minutes was performed. We tested the amplification efficiencies in changing the amount of cDNA (correspond to 1, 10 and 100 ng total RNA per tube) and amplification cycle (25, 30, 35 and 40 cycles) using β-actin primers. The condition used in this study was within a linear relationship between the amount of template and the amplification cycle (data not shown). The contamination of genomic DNA was checked by 40 cycle PCR of the total RNA without reverse transcription using β-actin primers. PCR product was not detected in the reaction mixtures without reverse-transcriptase. Primers are listed in Table 1. Mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) primers were purchased from CLONTECH (Palo Alto, CA, USA). Genomic DNA was isolated from P19 cells by general method (Davis et al. 1994). One hundred ng of genomic DNA was amplified by PCR in a reaction volume of 25 μl. The composition of PCR solution and the amplification sequence were the same as those in RT-PCR except the first denaturation at 95°C for 5 minutes.

PCR products quantification

PCR products were analyzed by HPLC (Henninger et al. 1993; Zeillinger et al. 1993). The HPLC system used in this study consisted of an LCSS-900 system controller, two PU-980 pumps, a DG-980-50 degasser, an HG-980-31 gradienter, an AS-950 autosampler, a UV-970 UV detector, a CO-965 column oven (JASCO, Tokyo) and a C-R5A chromatopack (Shimadzu, Kyoto). The analytical column was a TSK DEAE-NPR column (hydrophilic resin bonded with DEAE groups, 2.5-μm particle size, 4.6-mm ID, 35-mm long; TOSOH, Tokyo). The mobile phase was as follows: buffer A was 25 mM Tris-HCl (pH 9.0) and buffer B was 25 mM Tris-HCl (pH 9.0), 1 M NaCl. The sample was injected into the column equilibrated for 2 minutes with 70% buffer A+30% buffer B. A gradient program consisting of linear systems was employed: 30–100% buffer B in 12 minutes, 100% buffer B for 1 minute, and then 100–30% buffer B in 0.5 minute. The column was operated at 1 ml/min at 40°C and UV-extension was measured at

260 nm. The amount of a PCR product was normalized by that of β-actin.

Sequence analysis

The PCR products except G3PDH were directly sequenced using Dye Deoxy Termination cycle sequencing kit with an autosequencer (Applied Biosystems model 373A; Perkin Elmer) according to the manufacturer's instruction. The sequences of PCR products in this study were identical to those previously reported (Alonso et al. 1986; Lewis et al. 1986; Göbel et al. 1992; Franco del Amo et al. 1993; Akazawa et al. 1995; Lee et al. 1995).

Results

P19 cells were cultured in the bacteriological grade Petri dishes with the DMEM containing 1 μM RA and aggregated for 4 days. At day 4, P19 cells were transferred to the tissue culture grade Petri dishes with normal culture media. At day 5, a small number of cells differentiated to neuron and glia (Fig. 1A). Until day 8, many P19 cells differentiated to neuron and glia (Fig. 1B). The time course of the neural differentiation was the same as described previously (Rudnicki and McBurney 1987).

β-Actin mRNA decreased as P19 cells differentiated to neuron and glia, but not in P19 cell aggregates (Fig. 2A). G3PDH mRNA also changed within 3-fold and the lowest level was at day 5 (data not shown). mRNA of MAP2, a neural differentiation marker (Lewis et al. 1986), were detected in undifferentiated P19 cells and increased by about 11-fold by RA-treatment (Fig. 2A). The level of MAP2 mRNA reached to the plateau at day 5. MASH-1 and MATH-1 mRNA

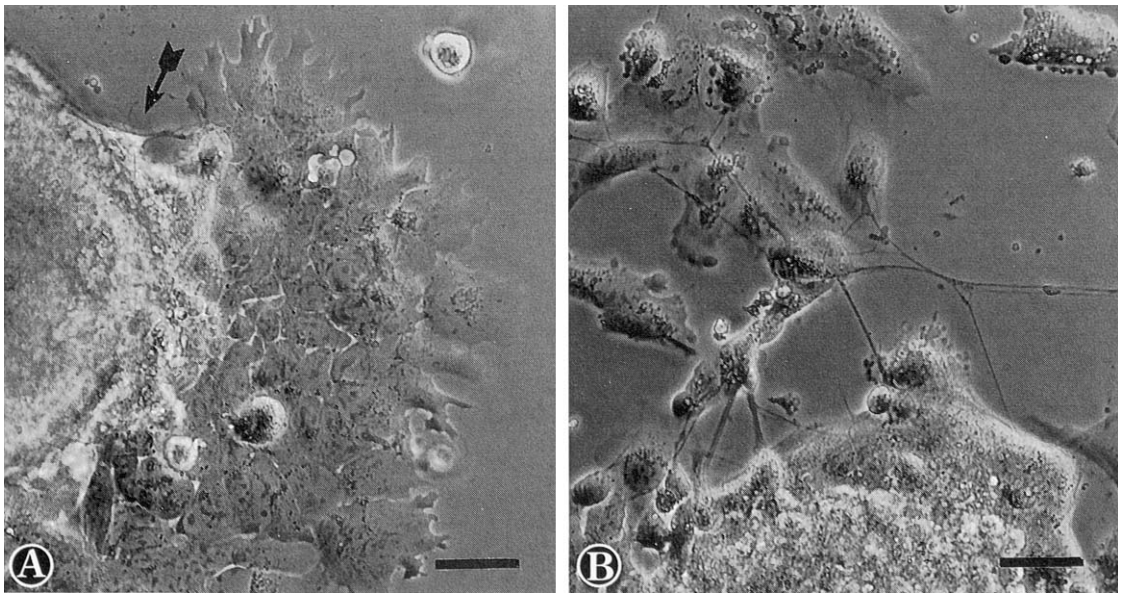

Fig. 1. Morphology of P19 cells treated with 1 μM RA. P19 cells were cultured and aggregated in the bacteriological grade Petri dish and transferred to the tissue culture grade Petri dish at day 4. P19 cells at day 5 (A) and day 7 (B). Arrow shows a neuron. Magnification bars equal 40 μm.

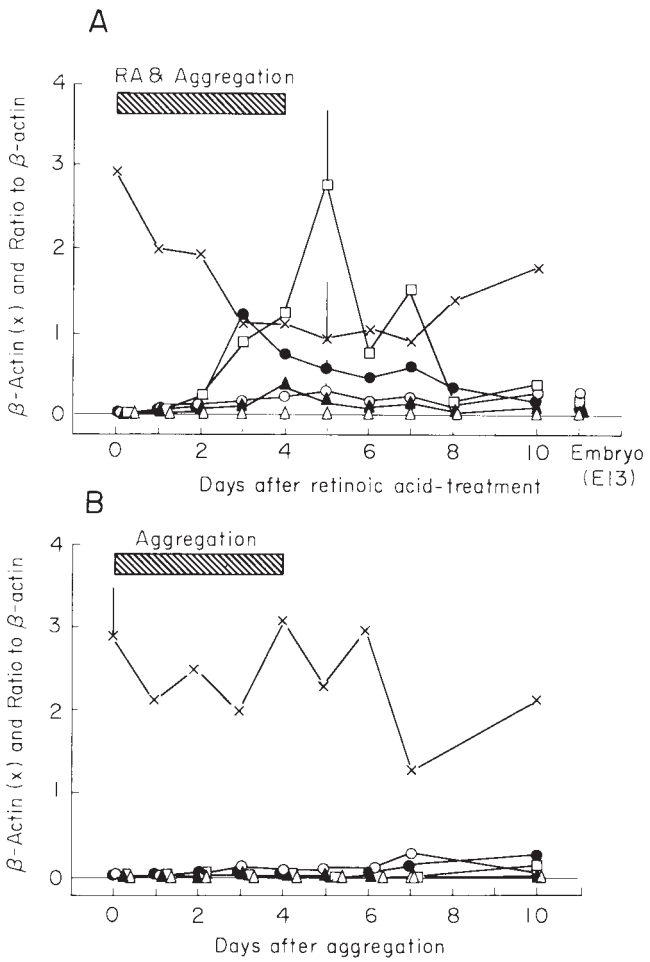

Fig. 2. Gene expressions of MAP2 and bHLH protein during neural differentiation of P19 cells. (A) P19 cells were treated with 1 μM RA and aggregated for 4 days in the bacteriological grade Petri dish. At day 4, the aggregates were transferred to the tissue culture grade Petri dish with normal culture media. E13 : E13 mouse embryo. (B) P19 cells were aggregated for 4 days in the bacteriological dish. At day 4, the aggregates were transferred to the tissue culture grade Petri dish containing normal culture media. Data represent the mean of 2–3 (A) or 1–3 (B) experiments and the error bars represent the standard error. β-Actin (arbitrary unit) per 1 μg total RNA of P19 cells: (×). Ratio to β-actin: (○), MAP2; (●), MASH-1; (△), MATH-1; (▲), NeuroD and (□), NSCL-2.

were not detected in undifferentiated P19 cells, but slight PCR products of neuroD and NSCL-2 mRNA were sometimes detected. MASH-1 and neuroD mRNA were detected at day 2 but not day 1. MASH-1, neuroD, and NSCL-2 mRNA were greatly increased by the RA-treatment and reached to the peak at day 3, 4 and 5, respectively (Fig. 2A). After the peak, NeuroD mRNA decreased near to zero (Fig. 2A). MAP2, MASH-1, neuroD, and NSCL-2 mRNA also increased in the aggregated P19 cells, but were much less than those in the P19 cells treated with RA (Fig. 2B). MATH-1 mRNA were not detected by 25 cycle PCR. Thus, we used 30 cycles for quantifying MATH-1 mRNA by HPLC. At 35 cycles, MATH-1 mRNA were detected in undifferentiated P19 cells and increased by RA-treatment

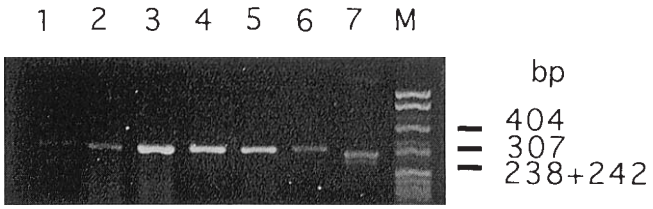

Fig. 3. RT-PCR of P19 cell total RNA by MATH-1 primers and P19 cell genomic DNA by MATH-1 and β-actin primers. Lane 1: untreated P19 cells. Lanes 2-5: day 1-4, RA (1 μM)-treated P19 cells. Reverse transcription mixtures were amplified at 35 cycles. Lanes 6-7: genomic DNA from P19 cells was amplified by MATH-1 and β-actin primers at 25 cycles, respectively. Lane M: pBR322 *Msp*I cut.

at day 1 (Fig. 3). The PCR primers for MATH-1 were as efficient as those for β-actin in amplifying genomic DNA (Fig. 3).

In E13 mouse embryo, MASH-1, MATH-1, neuroD and NSCL-2 mRNA were detected and the levels that are normalized by β-actin mRNA were almost the same as those of P19 cells at day 10 (Fig. 2A).

Discussion

P19 cells treated with 1 μM RA and aggregated for 4 days differentiated to neuron and glia at day 5 in this study (Fig. 1A). The time course was the same as that previously described (Rudnicki and McBurney 1987). The RA-treatment induced the gene expression of MASH-1, neuroD and NSCL-2 before day 4 when aggregates of P19 cells were transferred to tissue culture grade Petri dishes (Fig. 2A). MASH-1, neuroD and NSCL-2 mRNA peaked at day 3, 4, and 5, respectively, in this study. RA-treatment greatly induced the gene expression of NSCL-2. NSCL-2 mRNA gradually decreased from day 5, but the level of NSCL-2 mRNA at day 10 were still higher than that in undifferentiated P19 cells. NSCL-2 mRNA were detected in developing brain and the weak expression was observed in adult brain (Lipkowitz et al. 1992). RA-treatment induced gene expression of MATH-1 before day 4 and increased MATH-1 mRNA (Fig. 3). The signals of MATH-1 PCR products were much weaker than those of MASH-1, neuroD and NSCL-2 in this study. However, MATH-1 PCR primers had a good efficiency when genomic DNA were amplified (Fig. 3). MATH-1 mRNA might be low abundant even in differentiating P19 cells. Thus, the gene expression of MASH-1, MATH-1, neuroD and NSCL-2 is induced and reaches to the peak before P19 cells begin to differentiate to neuron morphologically. In mouse embryo, MASH-1 mRNA were detected in the ventricular zone of neuroepithelium in mid and hind brain at E8.5 (Guillemot and Joyner 1993). NeuroD mRNA were detected in the trigeminal ganglion at E9.0-9.5 (Lee et al. 1995). MATH-1 mRNA were detected in cranial ganglions and the dorsal part of the central nervous system at E9.5 (Akazawa et al. 1995). NSCL-2 mRNA were detected at E11.5-12 in the subependymal layer of the central nervous system and the dorsal root ganglion

(Göbel et al. 1992). Gene expression of MATH-1, neuroD and NSCL-2 was transient in differentiating neurons of mouse embryo (Göbel et al. 1992; Akazawa et al. 1995; Lee et al. 1995). Therefore, the order and pattern of the gene expression of MASH-1, MATH-1, neuroD and NSCL-2 during neural differentiation of P19 cells were similar to those in mouse embryo (Göbel et al. 1992; Guillemot and Joyner 1993; Akazawa et al. 1995; Lee et al. 1995), although there is no report that showed the gene expression of these bHLH proteins in the same region of the embryo nervous system during neurogenesis and we did not observe the gene expression in the same P19 cell during neural differentiation in this study.

MAP2 mRNA were detected in undifferentiated P19 cells and increased in the aggregates of P19 cells treated with RA in this study. The increases reached to the plateau (about 11-fold) at day 5. Dinsmore and Solomon (1991) reported that Northern blot did not detect MAP2 mRNA in undifferentiated P19 cells. There is the possibility that total RNA were contaminated by genomic DNA. PCR products of β-actin were not detected when total RNA were not reverse-transcribed. Thus, this discrepancy may be due to the sensitivities of detection methods, RT-PCR vs. Northern blot. In this study, MAP2 mRNA slightly increased in the aggregates of P19 cells untreated with RA. This might be due to the variations of β-actin mRNA as an internal standard (Fig. 2B) or to the spontaneous differentiation (Rudnicki and McBurney 1987). Thus, MAP2 mRNA increases parallels to the time course of neural differentiation in P19 cells.

β-Actin mRNA is used as an internal standard in P19 cells. PCR primers used in this study have different efficiencies for amplifying DNA. We can not quantify the absolute amounts of mRNA in P19 cells. β-Actin mRNA decreased as P19 cells differentiated to neuron and glia after RA-treatment (Fig. 2A) and the absolute values of PCR products of β-actin in this study varied within 3 fold. PCR products of G3PDH also changed within 3 fold like those of β-actin. The peak values of MASH-1, MATH-1, neuroD and NSCL-2 PCR products were at least 41-fold more than those in the aggregates of P19 cells. Thus, we can show the changes of the bHLH protein mRNA amounts during neural differentiation of P19 cells.

In this study, we showed that 1) the gene expression of MASH-1, MATH-1, neuroD and NSCL-2 (bHLH proteins) during neural differentiation of RA-treated P19 cells is transient, but that of MAP2 (a neural differentiation marker) is maintained in the high level after neural differentiation; and that 2) the time courses are similar to those reported in mouse embryo. Thus, we can use P19 cell line for studying the roles of bHLH proteins in neural differentiation. To clarify the physiological roles of bHLH proteins in P19 cell differentiation, we need to observe their expressions using antibodies in future.

References

1) Akazawa, C., Ishibashi, M., Shimizu, C., Nakanishi, S. & Kageyama, R. (1995) Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system. *J. Biol. Chem.*, **270**, 8730–8738.
2) Alonso, S., Minty, A., Bourlet, Y. & Buckingham, M.E. (1986) Comparison of three actin-coding sequences in the mouse; evolutionary relationships between the actin genes of warm-blooded vertebrates. *J. Mol. Evol.*, **23**, 11–22.
3) Aplan, P.D., Begley, C.G., Bertness, V., Nussmeier, M., Ezquerra, A., Coligan, J. & Kirsch, I.R. (1990) The SCL gene is formed from a transcriptionally complex locus. *Mol. Cell. Biol.*, **10**, 6426–6435.
4) Begley, C.G., Lipkowitz, S., Göbel, V., Mahon, K.A., Bertness, V., Green, A.R., Gouch, N.M. & Kirsch, I.R. (1992) Molecular characterization of NSCL, a gene encoding a helix-loop-helix protein expressed in the developing nervous system. *Proc. Natl. Acad. Sci. USA*, **89**, 38–42.
5) Campuzano, S. & Modolell, J. (1992) Patterning of the *Drosophila* nervous system: The *achaete-scute* gene complex. *Trends Genet.*, **8**, 202–208.
6) Chomczynski, P. & Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159.
7) Davis, L.G., Kuehl, W.M. & Battey, J.F. (1994) In: *Basic Methods in Molecular Biology*, 2nd ed., Appleton & Lange, Norwalk.
8) Dinsmore, J.H. & Solomon, F. (1991) Inhibition of MAP2 expression affects both morphological and cell division phenotypes of neuronal differentiation. *Cell*, **64**, 817–826.
9) Franco del Amo, F., Gendron-Maguire, M., Swiatek, P.J. & Gridley, T. (1993) Cloning, sequencing and expression of the mouse mammalian *achaete-scute* homolog 1 (MASH1). *Biochim. Biophy. Acta*, **1171**, 323–327.
10) Ghysen, J.A. & Dambly-Chaudiere, C. (1989) Genesis of the *Drosophila* peripheral nervous system. *Trends Genet.*, **5**, 251–255.
11) Göbel, V., Lipkowitz, S., Kozak, C.A. & Kirsch, I.R. (1992) NSCL-2: A basic domain helix-loop-helix gene expressed in early neurogenesis. *Cell Growth Differ.*, **3**, 143–148.
12) Guillemot, F. & Joyner, A.L. (1993) Dynamic expression of the murine *Achaete-Scute* homologue *Mash-1* in the developing nervous system. *Mech. Dev.*, **42**, 171–185.
13) Henninger, H.P., Hoffman, R., Grewe, M., Schulze-Specking, A. & Decker, K. (1993) Purification and quantitative analysis of nucleic acids by anion-exchange high-performance liquid chromatography. *Biol. Chem. Hoppe Seyler*, **374**, 625–634.
14) Jan, Y.N. & Jan, L.Y. (1990) Genes required for specifying cell fates in *Drosophila* embryonic sensory nervous system. *Trends Neurosci.*, **13**, 493–498.
15) Jarman, A.P., Grau, Y., Jan, L.Y. & Jan, Y.N. (1993) *atonal* is a proneural gene that directs chordotonal organ formation in the Drosophila peripheral nervous system. *Cell*, **73**, 1307–1321.
16) Johnson, J.E., Birren, S.J. & Anderson, D.J. (1990) Two rat homologues of *Drosophila achaete-scute* specifically expressed in neuronal precursors. *Nature*, **346**, 858–861.
17) Johnson, J.E., Zimmerman, K., Saito, T. & Anderson, D.J. (1992) Induction and repression of mammalian achaete-scute homologue (MASH) gene expression during neuronal differentiation of P19 embryonal carcinoma cells. *Development*, **114**, 75–87.
18) Jones-Villeneuve, E.M.V., McBurney, M.W., Rogers, K.A. & Kanins, V.I. (1982) Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial

cells. *J. Cell Biol.*, **94**, 253–262.

19) Jones-Villeneuve, E.M.V., Rudnicki, M.A., Harris, J.F. & McBurney, M.W. (1983) Retinoic acid induced neural differentiation of embryonal carcinoma cells. *Mol. Cell Biol.*, **3**, 2271–2279.

20) Lee, J.E., Hollenberg, S.M., Snider, L., Turner, D.L., Lipnick, N. & Weintraub, H. (1995) Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science*, **268**, 836–844.

21) Lewis, S.A., Villasante, A., Sherline, P. & Cowan, N.J. (1986) Brain-specific expression of MAP2 detected using a cloned cDNA probe. *J. Cell Biol.*, **102**, 2098–2105.

22) Lipkowitz, S., Göbel, V., Varterasian, M.L., Nakahara, K., Tchorz, K. & Kirsch, I.R. (1992) A comparative structural characterization of the human NSCL-1 and NSCL-2 genes. Two basic helix-loop-helix genes expressed in the developing nervous system. *J. Biol. Chem.*, **267**, 21065–21071.

23) McBurney, M.W. & Rogers, B.J. (1982) Isolation of male embryonal carcinoma cells and their chromosome replication patterns. *Dev. Biol.*, **89**, 503–508.

24) McBurney, M.W., Reuhl, K.R., Ally, A.I., Nasipuri, S., Bell, J.C. & Craig, J. (1988) Differentiation and maturation of embryonal carcinoma-derived neurons in cell culture. *J. Neurosci.*, **8**, 1063–1073.

25) Mellentin, J.D., Smith, S.D. & Cleary, M.L. (1989) *lyl-1*, A novel gene altered by chromosomal translocation in T cell leukemia, codes for a protein with a helix-loop-helix DNA binding motif. *Cell*, **58**, 77–83.

26) Parkhurst, S.M., Bopp, D. & Ish-Horowicz, D. (1990) X: A ratio, the primary sex-determining signal in *Drosophila*, is transduced by helix-loop-helix proteins. *Cell*, **63**, 1179–1191.

27) Rudnicki, M.A. & McBurney, M.W. (1987) Cell culture methods and induction of differentiation of embryonal carcinoma cell lines. In: *Teratocarcinomas and Embryonic Stem Cells a Practical Approach*, edited by E.J. Robertson, IRL Press, Oxford, Washington DC, pp. 19–49.

28) Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T.K., Turner, D., Rupp, R., Hollenberg, S., Zhaung, Y. & Lasser, A.B. (1991) The MyoD gene family: Nodal point during specification of muscle cell lineage. *Science*, **251**, 761–766.

29) Zeillinger, R., Schneeberger, C., Speiser, P. & Kury, F. (1993) Rapid quantitative analysis of differential PCR products by high-performance liquid chromatography. *Biotechniques*, **15**, 89–95.