Development 122, 2449-2464 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV1079

Roles of cell-autonomous mechanisms for differential expression of region-specific transcription factors in neuroepithelial cells

Yasushi Nakagawa[1,2,†], Tadahiro Kaneko[1,3], Toshihiko Ogura[4], Taiga Suzuki[3], Masaaki Torii[3], Kozo Kaibuchi[3], Ken-ichi Arai[2], Shun Nakamura[1] and Masato Nakafuku[1,3,*]

[1]Division of Biochemistry and Cellular Biology, Institute of Neuroscience, National Institute of Neurology and Psychiatry, Kodaira, Tokyo, Japan
[2]Department of Molecular and Developmental Biology, The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo, Japan
[3]Division of Signal Transduction and [4]Metabolic Regulation in Animal Cells, Graduate School of Biological Sciences, Nara Advanced Institute of Science and Technology, Ikoma, Nara, Japan

*Author for correspondence at address 3 (e-mail: nakafuku@bs.aist-nara.ac.jp)
[†]Present address: Molecular Neurobiology Laboratory, The Salk Institute for Biological Studies, La Jolla, California, USA

SUMMARY

Although a number of genes have been found to have restricted expression domains in the embryonic forebrain and midbrain, it remains largely unknown how the expression of these genes is regulated at the cellular level. In this study, we explored the mechanisms for the differential expression of region-specific transcription factors in neuroepithelial cells by using both primary and immortalized neuroepithelial cells from the rat brain at embryonic day 11.5. We found that differential expression patterns of *Pax-3, Pax-5, Pax-6, Dlx-1, Dlx-2, Emx2, Otx1* and *Dbx* observed in vivo were maintained even when the cells were isolated and cultured in vitro, free from environmental influences. Furthermore, in response to Sonic hedgehog, which is a major inductive signal from the environment for regional specification, neuroepithelial cells that maintain distinct regional identities expressed different sets of ventral-specific genes including *Islet-1, Nkx-2.1* and *Nkx-2.2*. These results suggest that certain cell-autonomous mechanisms play important roles in regulating both environmental signal-dependent and -independent expression of region-specific genes. Thus, we propose that use of the in vitro culture systems we describe in this study facilitates the understanding of regulatory mechanisms of region-specific genes in neuroepithelial cells.

Key words: neural development, regional specification, neural stem cells, neuromere, homeobox gene, rat

INTRODUCTION

The mammalian central nervous system is the center of higher cognitive functions. Its generation involves a number of events including neural induction, patterning of the neural plate, commitment and differentiation of neurons and glia, and establishment of neuronal and glial connections. These events collectively contribute to the generation of neural cell diversity. However, the molecular mechanisms controlling this complex process have just begun to be elucidated.

An important aspect of early development of the brain is the regional specification of neuroepithelial cells (Jessell and Dodd, 1992; Ruiz i Altaba, 1994; Lumsden and Graham, 1995). For example, it is well established that the vertebrate hindbrain neuroepithelium is composed of discrete anatomical as well as functional units called rhombomeres, which serve as a framework for subsequent regional specification. In chick embryos, the cranial motor nerve nuclei are derived from adjoining pairs of rhombomeres (Lumsden, 1990), which indicates that neuroepithelial cells in each rhombomere are fated to give rise to particular types of neurons. Searches for possible molecular correlates of the rhombomeres have succeeded in identifying many putative regulatory genes that show segment-specific expression domains. These include the *Antennapedia* class of homeobox-containing (*Hox*) genes (Wilkinson et al., 1989a) and the zinc-finger gene *Krox-20* (Wilkinson et al., 1989b). The boundaries of these expression domains have been shown to restrict the mixing of neuroepithelial cells (Birgbauer and Fraser, 1994) as well as the intercellular movement of small molecules via gap junctions (Martinez et al., 1992).

Although there have long been controversies over the early organization of more anterior parts of the brain, i.e. the forebrain and midbrain, the recent discoveries of genes that are expressed in a region-specific manner along with refined morphological studies have revealed that the vertebrate forebrain, like the hindbrain, can also be divided into discrete domains (Bulfone et al., 1993; Figdor and Stern, 1993; Puelles and Rubenstein, 1993; Macdonald et al., 1994). Bulfone et al. (1993) compared the expression domains of the *Dlx-1*, *Dlx-2*, *Gbx-2* and *Wnt*-3 genes in the embryonic day (E) 12.5 mouse forebrain and concluded that the developing forebrain can be

divided into six transverse domains called prosomeres, and that each prosomere can be subdivided further into longitudinal domains. These proposed domain structures matched the neuromeric model of the forebrain organization based on morphological studies (Puelles et al., 1987). Analyses of expression domains of other genes from different families including the paired-domain-containing *Pax* family (Stoykova and Gruss, 1994) and the homeobox-containing *Otx*, *Emx* and *Nkx* families (Simeone et al., 1992a,b; Guazzi et al., 1990; Price et al., 1992; Shimamura et al., 1995) have provided further evidence for their hypothesis (see Boncinelli et al., 1993 and Rubenstein et al., 1994 for reviews). Studies of chick and zebrafish embryos revealed that such gene expression boundaries partially restrict cell mixing and are correlated with the positions of early generated neurons and their axonal tracts (Figdor and Stern, 1993; Wilson et al., 1993; Macdonald et al., 1994; Guthrie, 1995). Although the functions of these region-specific genes remain to be elucidated, some of them are expressed in specific populations of postmitotic neurons at later stages of development. Since many of these region-specific genes encode transcription factors, they are likely to serve as important regulators in the genetic network leading to the generation of particular neural cell types. Therefore, it is reasonable to expect that neuroepithelial cells expressing different combinations of region-specific transcription factors give rise to distinct types of neurons or glia.

In this respect, it is important to elucidate how the expression of region-specific genes is controlled in neuroepithelial cells. In particular, it has been a salient issue whether the patterns of gene expression and fate choices of neuroepithelial cells are defined by lineage-dependent cell-autonomous mechanisms or environment-dependent non-cell-autonomous regulation (Jessell and Dodd, 1992; Williams and Goldowitz, 1992; Lumsden et al., 1994; Johnson and Tabin, 1995; Simon et al., 1995). Several lines of evidence have suggested an important role for environmental regulation. Transplantation experiments of embryonic brain tissues and/or cells have revealed that neuroepithelial cells are plastic and can change their regional phenotypes dependent on their environments (Nakamura, 1988; Vicario-Abejón et al., 1995). In the chick spinal cord, the specification of dorsoventral identity of neuroepithelial cells is also altered by environmental signals: either removal or implantation of the notochord dramatically altered the dorsoventral expression patterns of *Pax-3* and *Pax-6* (Goulding et al., 1993). In this case, the secreted protein Sonic hedgehog (SHH), also termed Vhh-1 or Hhg-1, or its related proteins have been identified as signals from the notochord that control the restricted expression of these genes (Ekker et al., 1995; Liem et al., 1995). More recent studies have indicated that SHH is also involved in the induction of genes expressed in a subset of cells in the ventral forebrain including *Islet-1* (*Isl-1*), *Nkx-2.1*, *Nkx-2.2* and *Lim-1* (Ericson et al., 1995; Barth and Wilson, 1995; Lumsden and Graham, 1995). It has also been shown that members of the transforming growth factor-β superfamily (BMP-4, BMP-7 and dorsalin-1) are involved in specification of the dorsoventral polarity in the spinal cord by regulating the expression of particular region-specific genes such as *Pax-3* and *Msx1* (Basler et al., 1993; Liem et al., 1995). These studies have emphasized the important role of the environment in defining the expression of region-specific genes.

In contrast, some previous studies have also demonstrated the importance of cell-autonomous mechanisms in maintaining regional identity of neuroepithelial cells. In the chick embryonic hindbrain, a fate-tracing study of single neuroepithelial cells (Lumsden et al., 1994) as well as transplantation studies of particular rhombomere segments (Guthrie et al., 1992; Simon et al., 1995) revealed a role for cell lineage in cell-type determination and *Hox* gene expression. Consistently, the existence of such mechanisms has also been implicated in the forebrain region concerning the expression of the limbic system-associated membrane protein (LAMP) and the PC3.1 antigen (latexin; Arimatsu et al., 1992; Ferri and Levitt, 1993). Furthermore, it has been shown very recently that the expression of the *Otx2*, *Emx2* and *Dlx-1* genes was maintained in neuroepithelial cells cultured in vitro (Robel et al., 1995). These studies have suggested that differential expression of particular antigens and genes in restricted regions of the rodent cerebral cortex was maintained in neuroepithelial cells by certain cell-autonomous mechanisms. However, these studies were carried out using mixtures of cells which are likely to be heterogeneous in terms of the expression of region-specific genes even if they were isolated from restricted areas or domains. Thus, it still remains to be clarified whether cell-autonomy is really operating at the single-cell level in maintaining particular regional identities.

To address these questions, we employed in vitro systems in which the involvement of cell-autonomous mechanisms and environmental signals can be independently manipulated and evaluated. In this study, we first examined the expression of region-specific transcription factors including *Pax-3*, *Pax-5*, *Pax-6*, *Dlx-1*, *Dlx-2*, *Emx2* and *Dbx* in primary culture of neuroepithelial cells from three distinct regions of the developing (E11.5) brain; dorsal forebrain, ventral forebrain and caudal midbrain. Consistent with the results of previous in situ hybridization studies, each of the above genes was indeed differentially expressed in cell populations from distinct regions. We also found that such patterns were maintained when the cells were cultured for 5 days in vitro, suggesting that cell-autonomous mechanisms contribute to this phenomenon. To further explore this possibility, we utilized clonal neuroepithelial cell lines that we recently established from the E11.5 rat forebrain and midbrain (designated as MNS cell lines; Nakafuku and Nakamura, 1995). All of the five cell lines which we characterized shared properties of undifferentiated neuroepithelial cells and could give rise to neurons and glia under particular culture conditions. We demonstrate that each of these cell lines maintained expression of a specific combination of region-specific transcription factors not only during their clonal expansion in vitro, but also when the cells were induced to differentiate. Furthermore, we show that SHH induced different sets of ventral-specific genes in primary culture of distinct regions of the embryonic neuroepithelium as well as in distinct MNS cell lines. These results provide evidence that each neuroepithelial cell harbors some cell-autonomous mechanisms that direct the expression of a particular combination of region-specific genes, and that the same mechanisms also play an important role in regulating how the cell responds to inductive signals from the environment.

MATERIALS AND METHODS

Primary culture of neuroepithelial cells

Neuroepithelia of the forebrain and midbrain were dissected out from E11.5 Sprague-Dawley rats as described previously (Nakafuku and Nakamura, 1995). The day on which the copulatory plug was found was considered as E0.5. Isolation of neuroepithelial cells from specified areas was carried out as follows; the prospective cerebral cortex was cut out from the embryos and used as a dorsal forebrain preparation. From the remaining embryonic head, the ventral forebrain and caudal midbrain regions were dissected out, and surrounding mesenchymal tissues were removed from the primary neuroepithelia. The uppermost position of the optic vesicle and caudal edge of the forebrain vesicle were used as landmarks of the dorsal and caudal margins, respectively, of the ventral forebrain preparations. The rostral margin of the caudal midbrain preparations was defined as half way between the edge of the forebrain vesicle and the rhombencephalic fissure. We noticed that by means of the above method, it was difficult to completely eliminate the most anterior portion of the neural tube, the prospective septal region, from the dorsal forebrain preparation, since no morphological landmark for that region was apparent by inspection at this stage (see Results for details). To further eliminate contaminating non-neural tissues, isolated tissue pieces were incubated at 4°C for 20 minutes in a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 medium (DF; Sanko Junyaku) containing 0.05% (w/v) trypsin and 3 mM sodium ethylenediamine tetraacetic acid (EDTA) as described previously (Murphy et al., 1990). Subsequently, the samples were collected into test tubes and washed three times with DF medium. The preparations were then treated with DF medium containing 0.1% trypsin, 0.001% DNase I (Sigma) and 3 mM EDTA at 37°C for 15 minutes, and single-cell suspensions were made by repeated gentle pipetting. The dissociated cells were divided into two and the first half was directly collected (day 0 preparation). The other half was plated onto poly-D-lysine (10 μg/ml) -coated dishes at densities between 2 to 5×10^5 cells per cm^2 in a standard culture medium consisting of 10% fetal bovine serum (FBS; Sanko Junyaku), 5% horse serum (HS; Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin in DF medium, and was cultured in vitro for 5 days (day 5 preparation). Immunocytochemical studies showed that more than 98% of the isolated cells were positively stained by anti-nestin and the RC1 antibodies, indicating that the preparations were essentially free of non-neural cells. After 5 days in vitro, total cell numbers increased 2- to 3-fold, and the percentage of nestin-positive cells decreased to 65-70%, whereas MAP2-positive neurons and GFAP-positive astrocytes emerged at percentages of 10-20% and 1-5% of the total cells, respectively.

Establishment and maintenance of neuroepithelial cell lines

The neural stem cell lines used in this study, herein designated as MNS (multipotential neural stem) cell lines, were established and maintained as described previously (Nakafuku and Nakamura, 1995). Primary cultured neuroepithelial cells prepared from E11.5 rat forebrain and midbrain were immortalized by infection with the recombinant retrovirus from the Ψ2mycer cell-conditioned medium (Eilers et al., 1989). The retrovirus carries the c-*mycer* gene whereby c-Myc is fused to the ligand-binding domain of the estrogen receptor. With this system it is possible to conditionally activate the c-Myc protein by adding estrogen (e.g. β-E2) to the culture medium (Eilers et al., 1989). Since the retrovirus also carries the *neo*^{r} gene, infected cells were selected with G418. The clonality of the characterized cell lines was confirmed by Southern blot analysis of the provirus integration sites (data not shown). Each cell line was maintained in monolayer culture in the standard medium described above ('monolayer culture'). The medium was changed every 3 days. The detailed procedures to induce differentiation of the cells were described previously (Nakafuku and Nakamura, 1995). Briefly, cells were first allowed to form aggregates in suspension for 3 days in standard medium containing 20 ng/ml bFGF and 1 μM β-E2 ('aggregation culture'). Culture dishes were coated with poly [2-hydroxyethyl methacrylate] (poly HEMA; Sigma) to avoid cell attachment. Cell aggregates were then re-seeded onto poly-D-lysine (100 μg/ml) -coated dishes and were cultured in differentiation medium (standard medium without HS) for 3 or 4 days ('differentiation culture'). The cells cultured under each of the three different conditions were subjected to immunocytochemical studies and RNA preparation.

Expression of SHH and treatment of primary culture and MNS cell lines

CV1 cells stably expressing SHH (CV1SHH) were produced by transfection with a plasmid harboring the full-length chicken SHH cDNA (Ogura et al., 1996). The expression of the N-terminal cleavage product of SHH in CV1SHH cells and in their conditioned media was analyzed using the affinity-purified rabbit anti-SHH antibody (Ab 80) as described by Bumcrot et al. (1995), (a gift from Dr A. McMahon), in which cell pellets were directly lysed in Laemmli's sample buffer, whereas proteins in conditioned medium were concentrated 10-fold by precipitation with 10% trichloroacetic acid. In the case of primary culture of neuroepithelial cells, media conditioned for 48 hours by confluent monolayer of CV1SHH cells and the parental CV1 cells were used for the treatment with SHH and its control, respectively. Preparations of the dorsal and ventral forebrain were isolated and used exactly as described above. For the caudal midbrain, however, its ventral two-third was removed, and the remaining dorsal portion was used as a preparation of the dorsal midbrain. MNS cell lines were treated with SHH by culturing them in contact with a confluent monolayer of CV1SHH cells for 3 days. Control cells were cocultured with the parental CV1 cells in stead of CV1SHH cells. During this 3 day-culture period, no apparent differences in either growth property or viability were observed between the cells in contact with CV1 and those incubated with CV1SHH cells.

Immunostaining

The antibodies used for immunocytochemical studies were described previously in detail (Nakafuku and Nakamura, 1995) with the following exceptions: anti-Pax-6 mouse monoclonal antibody (mmAb; a generous gift from Drs H. Fujisawa and A. Kawakami, Nagoya University; Kawakami et al., unpublished data); anti-MAP2 polyclonal antibody (diluted 1:500; provided by Dr Y. Ihara, University of Tokyo); and the R24 anti-GD3 ganglioside mmAb (LeVine and Goldman, 1988, undiluted conditioned medium of hybridoma, from the ATCC Hybridoma Bank). Indirect immunocytochemical detection of various antigens was performed as previously described (Nakafuku and Nakamura, 1995). A2B5 and R24 antibodies specifically labeled cells in the oligodendrocyte lineage, but not MAP-2-positive neurons or GFAP-positive astrocytes under our experimental conditions. The immunoreactive cells were visualized using fluorescein isothiocyanate (FITC)- or texas red (TR)-conjugated species-specific secondary antibodies (diluted 1:50-100; Cappel or Amersham).

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were prepared from cells or tissues using the acid guanidinium-phenol-chloroform (AGPC) extraction method as described previously (Chomczynski and Sacchi, 1987). RNAs of primary neuroepithelial cells were prepared from day 0 and day 5 cultures (See above for detailed culture protocol). RNAs from MNS cell lines were prepared from three different types of culture: monolayer culture, aggregation culture and differentiation culture (see above). RNA was also prepared from tissues of E11.5 and E15.5 rat forebrain/midbrain

and E11.5 whole embryos deprived of forebrain/midbrain for use as control.

Relative expression levels of various genes in tissue and cultured cell samples were compared by quantitative RT-PCR analysis. cDNAs were synthesized from total RNA by MuMLV reverse transcriptase (Superscript II; Gibco) at 50°C for 1 hour. cDNA derived from 40 ng of total RNA was amplified in a 100 μl PCR reaction containing 5 units of Taq DNA polymerase (Boehringer Mannheim), 1× PCR buffer, 0.4 mM of each dNTP and 100 pmols of each primer (see Table 1) in a thermal cycler (Perkin Elmer, denaturation for 1 minute at 94°C, annealing for 1 minute at 56°C and extension for 2 minutes at 72°C). For *Wnt*-3 and *En-1* transcripts, annealing was carried out at 62°C and 58°C, respectively.

Oligonucleotides used to amplify the cDNAs are listed in Table 1. The identities of the PCR products were confirmed by sequencing the subcloned fragments using an automated sequencer (A.L.F. Sequencer II, Pharmacia and ABI 373A DNA Sequencer, Applied Biosystems). For the *Otx* genes, we used degenerate primers that can detect both *Otx1* and *Otx2*. At the time of this study, the rat sequences were not available for the *Pax-3*, *Pax-5*, *Dlx-1*, *Otx1*, *Emx2*, *Dbx*, *Wnt-3*, *En-1*, *Hox-B1*, *Hox-B3*, or *Nkx-2.2* genes. For these genes, we used the mouse sequences for primer design and compared the similarity of the obtained PCR products with the mouse sequences. More than 97% identity in nucleotide sequence was observed in each clone, which gave sufficient information to distinguish different members of each family. Therefore, we concluded that the obtained cDNA clones were the cognate rat counterparts of the mouse cDNAs.

Relative quantification of gene expression by use of RT-PCR was carried out as follows. During each set of PCR reactions, 8 μl aliquots were collected from the reaction mixtures every 2-3 cycles, and 4 μl of the samples were electrophoresed in 1% agarose or 6% polyacrylamide gels. The gels were then stained with 0.01% SYBR Green I (Amersham) for 45 minutes and fluorescence intensity was measured using FluorImager SI (Molecular Dynamics). For each primer pair, amplified PCR products among different samples were quantified at various cycles within the range of exponential amplification. In control experiments, we confirmed that relative levels of cDNA for a given gene present in different samples could be quantified within the range 20 pg to 10 ng by means of this method (Schneeberger et al., 1995 and Y. N., unpublished data). In all experiments, amplification of β-actin cDNA was carried out alongside, and was used to normalize different cDNA samples. We repeated the above experiments three to five times for each gene using two to five independent preparations of tissue or cell samples, and the normalized mean values (±s.d.) are shown in figures and tables. Cycle numbers used for quantification are also shown in the legends to Figs 2, 4, 7 and 9, and Table 2. To

Table 1. PCR primers used in this study

Gene	Sequence	Reference
β-*actin*	Sense: 5'-TGC CCA TCT ATG AGG GTT ACG-3' Antisense: 5'-TAG AAG CAT TTG CGG TGC ACG-3'	Nudel et al. 1983
MAP2	Sense: 5'-GAA GGA AAG GCA CCA CAC TG-3' Antisense: 5'-CGT GGC GAT GGT GGT GGG-3'	Kindler et al. 1990
GFAP	Sense: 5'-CAA GCC AGA CCT CAC AGC G-3' Antisense: 5'-GGT GTC CAG GCT GGT TTC TC-3'	Lewis et al. 1984
CNP-II	Sense: 5'-CCG GAG ACA TAG TGC CCG CA-3' Antisense: 5'-AAA GCT GGT CCA GCC GTT CC-3'	Gravel et al. 1994
Pax-3	Sense: 5'-GCT GTC TGT GAT CGG AAC ACT-3' Antisense: 5'-CTC CAG CTT GTT TCC TCC ATC-3'	Goulding et al. 1991
Pax-5	Sense: 5'-GAG CGG GTG TGT GAC AAT GAC-3' Antisense: 5'-CGA GGC CAT GGC TGA ATA CTC-3'	Adams et al. 1992
Pax-6	Sense: 5'-AGT CAC AGC GGA GTG AAT CAG C-3' Antisense: 5'-AGC CAG GTT GCG AAG AAC TCT G-3'	Walther and Gruss 1991
Dlx-1	Sense: 5'-CAA GGC GGG GCA GCT CTG-3' Antisense: 5'-GGG AGA CGG GCA GGA AGC-3'	Price et al. 1991
Dlx-2	Sense: 5'-AGG ATG ACT GGA GTC TTT GAC-3' Antisense: 5'-TCG GAT TTC AGG CTC AAG GTC-3'	Porteus et al. 1992
Otx	Sense: 5'-TAT/C CCI GCI ACI CCI A/CGA/G AAA/G CA-3' Antisense: 5'-ACI AA/GT/C TGT/C TGI CT/GA/G CAT/C TTI GC-3'	Simeone et al. 1992a
Emx2	Sense: 5'-GTC CCA GCT TTT AAG GCT AGA-3' Antisense: 5'-CTT TTG CCT TTT GAA TTT CGT TC-3'	Simeone et al. 1992b
Dbx	Sense: 5'-GCA GA/CG A/GAA A/GGC/G C/GCT GGA GAA-3' Antisense: 5'-TA/GG AA/GT T/GCC GCC AC/TT TCA TC/GC-3'	Lu et al. 1992
Wnt-3	Sense: 5'-GAA GGC TGG AAG TGG GGC GGC-3' Antisense: 5'-ACG CAA TGG CAT TTC TCC TTC CG-3'	Roelink et al. 1990
En-1	Sense: 5'-GAC AGT GGC GGT GGT AGT G-3' Antisense: 5'-GAG GAG CCT GGA GGT GGC-3'	Joyner et al. 1987
Hox-B1	Sense: 5'-CCG GAC CTT CGA CTG GAT G-3' Antisense: 5'-GGT CAG AGG CAT CTC CAG C-3'	Wilkinson et al. 1989a
Hox-B3	Sense: 5'-GTC GAC GCA AAC TGC CAA GC-3' Antisense: 5'-GGG TCA TGG AGT GTA AGG CG-3'	Wilkinson et al. 1989a
Nkx2.1	Sense: 5'-GGC CAT CTC TGT GGG CAG C-3' Antisense: 5'-CTC AGG CGC GTC CCA CAT C-3'	Guazzi et al. 1990
Nkx2.2	Sense: 5'-GGG GGA NCGC AGG CAA GAA G-3' Antisense: 5'-TGT AGG CGG AAA AGG GGA TG-3'	Price et al. 1992
Isl-1	Sense: 5'-GCA GCA TAG GCT TCA GCA AG-3' Antisense: 5'-GTA GCA GGT CCG CAA GGT G-3'	Karlsson et al. 1990

visualize the difference in expression levels among different samples, direct printouts from the fluorescence image analyzer are shown in Figs 4A, 5 and 9C. In these cases, β-actin-normalized cDNA templates were amplified at fixed cycle numbers. For clearer visualization of PCR products, cycle numbers in some cases were 2-3 times larger than those used for quantification, but they were still within the range of exponential amplification (see figure legends for details).

RESULTS

Expression of region-specific genes in neuroepithelial cells in primary culture

To explore the mechanisms that control differential expression of region-specific genes among discrete domains of the forebrain and midbrain, we first examined the expression of a set of transcription factors in neuroepithelial cells cultured in vitro. Neuroepithelia of three distinct regions, including the dorsal forebrain, ventral forebrain and caudal midbrain were dissected out from embryonic rat brain at E11.5 (which roughly corresponds to E9.5-10.5 in mice). Immunocytochemical studies showed that more than 98% of the cells in all the preparations expressed nestin (Lendahl et al., 1990) and RC1 antigen (Edwards et al., 1990), both of which are specific markers for undifferentiated neuroepithelial cells (Fig. 1A-C). From these tissue samples, we isolated RNAs either immediately after dissociation (day of in vitro culture [DIV] 0) or after 5 days in culture (DIV 5), and compared the expression levels of *Pax-3*, *Pax-5*, *Pax-6*, *Dlx-1*, *Dlx-2*, *Emx2* and *Dbx* genes by quantitative RT-PCR analysis (Fig. 2).

We first focused on the three members of the *Pax* gene family, *Pax-3*, *Pax-5* and *Pax-6*, which encode transcription factors related to the *Drosophila* protein paired (Stuart et al., 1993). In situ hybridization studies have shown that *Pax-3*, *Pax-5* and *Pax-6* expressions are first observed by E8.5 in mice along the entire anterior-posterior axis of the neural tube. At later stages, however, their expression domains become restricted to particular areas within the forebrain and midbrain. The rostral limits of *Pax-3* (Goulding et al., 1991) and *Pax-5* (Asano and Gruss, 1992) expression domains retract caudally to dorsal and ventral midbrain, respectively, whereas strong expression of *Pax-6* remains in the dorsal telencephalon at E13.5. In addition, other areas such as ventral thalamus, epithalamus and the ventral midbrain also show significant *Pax-6* expression (Walther and Gruss, 1991). Consistent with these in vivo data, the levels of *Pax-3* and *Pax-5* mRNA were three to twenty times higher in DIV 0 cells of the caudal midbrain preparations than those in the ventral and dorsal forebrain, whereas *Pax-6* was expressed at comparable levels in all of these three regions (Fig. 2).

Next, we examined the expression of three classes of homeobox genes including the *Dlx*, *Emx* and *Dbx* genes, which are vertebrate homologues of the *Drosophila Distal-less* (Boncinelli, 1994; Price, 1993), *empty spiracles*, (Boncinelli et al., 1993) and *H2*, respectively. In the developing mouse brain, *Dlx-1* and *Dlx-2* share most of their expression domains, which reside predominantly in the forebrain, particularly in ventral regions (Bulfone et al., 1993). At E9.5 to 9.75 in mouse embryos, strong expression of *Emx2* is restricted both in the dorsal telencephalon and ventral diencephalon (Simeone et al., 1992b; Shimamura et al., 1995). As development proceeds to E12.5, however, it becomes detectable also in other regions such as the dorsal midbrain (Simeone et al., 1992b). Strong expression of *Dbx* is observed in the midbrain and in some ventral regions of the forebrain, but is scarce in the dorsal forebrain (Lu et al., 1992; N. Takahashi, personal communication). The results of RT-PCR analysis matched the above observations; the levels of *Dlx-1*, *Dlx-2* and *Emx2* expression in the DIV 0 cells from the ventral forebrain were higher (34, 6 and 4 times, respectively) than those from the midbrain, whereas the *Dbx* expression was eight times stronger in the midbrain than in the dorsal forebrain. Comparison of the expression of the above genes between the dorsal and ventral forebrain regions, however, has raised several issues that should be addressed. The expression levels of *Emx-2* in the

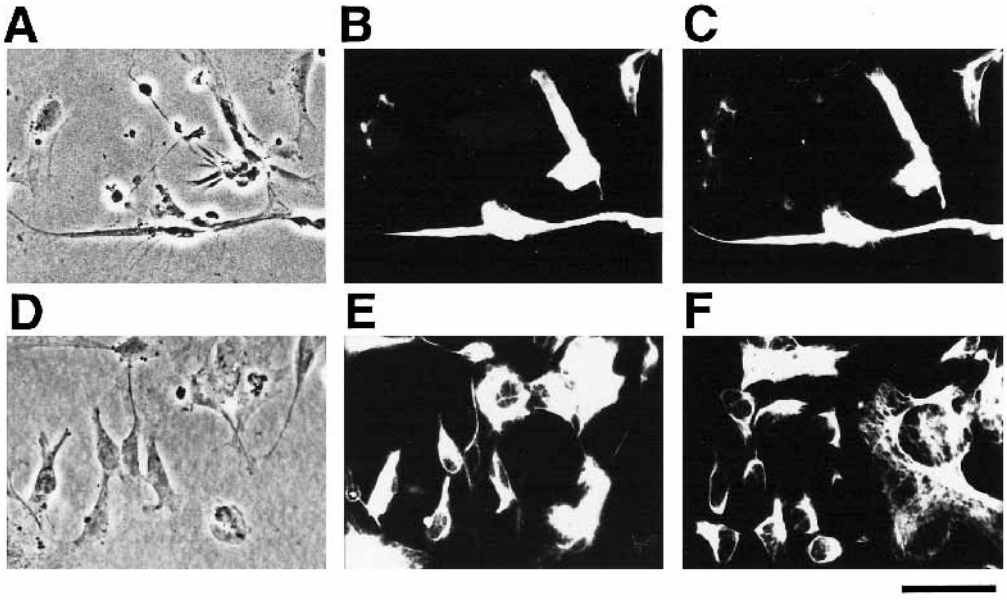

Fig. 1. Indirect immunocytochemical analysis for neuroepithelial cell antigens in primary neuroepithelial cells and MNS cell lines. Primary neuroepithelial cells from E11.5 rat forebrain and midbrain (A-C) and MNS-8 cells (D-F) were stained with antibodies that detect neuroepithelium-expressed antigens. (A) Phase contrast micrograph of primary neuroepithelial cells. The same population of cells was immunoreactive with anti-nestin (B) and the RC1 (C) antibodies. A-C show the same field. In our preparations of neuroepithelia from separate regions, more than 98% of the cells were always nestin/RC1-double positive at DIV 0 (data not shown). (D) Phase contrast micrographs of MNS-8 cells. Fluorescence micrographs showing the expression of nestin (E) and RC1 (F). D and E show the same field. Scale bar, 100 μm.

dorsal forebrain was about twice as high as that in the ventral forebrain, which is generally consistent with the results of previous in situ hybridization studies (Simeone et al., 1992b; Shimamura et al., 1995). In contrast, only three times higher expression of *Dlx-1* and *Dbx* was observed in the ventral than that in the dorsal forebrain, which was smaller than expected from previous studies (Bulfone et al., 1993; Lu et al., 1992). In addition, although the expression of *Dlx-2* is reported to be predominant in the ventral rather than in the dorsal forebrain, it was expressed at similar levels in our preparations of the two regions. One possible reason for these results is that a part of the prospective septal region, which also abundantly expresses *Dlx-1*, *Dlx-2* and *Dbx*, was included in our preparation of the dorsal forebrain. Yet, the overall profiles (also see below) suggest that the results shown here reflect some parts of the differential gene expression patterns between the dorsal and ventral sides of the forebrain.

Next, to test whether the selective expression profiles of the above genes are maintained upon proliferation and differentiation of neuroepithelial cells, we cultured these cells for 5 days in vitro. During this culture period, neuroepithelial cells underwent significant proliferation as well as differentiation into neurons and glia (data not shown), and the cells from each of the three regions maintained gene expression profiles typical of region-specific genes. For example, *Pax-5* expression remained very low in cells derived from the ventral and dorsal forebrain, whereas expression of *Dlx-1* and *Dlx-2* genes remained high in the ventral forebrain. Likewise, both low levels of *Dlx-1* and *Dlx-2* and high levels of *Pax-3*, *Pax-5* and *Dbx* were maintained in cells from the caudal midbrain after in vitro culture. We noticed several exceptions to this general feature: firstly, *Pax-3* was expressed at a significant level in the ventral forebrain, and was even upregulated after 5 days of culture; secondly, *Emx2* and *Dbx* showed marked upregulation in the DIV 5 preparations of caudal midbrain and ventral forebrain, respectively. These observations can be explained by the shift in their expression domains during development. As described above, *Pax-3* expression is initially observed along the entire anterior-posterior axis of the neural tube, and its retraction in the forebrain region occurs between E11- 12 in mice, which corresponds to E13-14 in rats (Goulding et al., 1991). Thus, it appears that our DIV 0 preparation of the ventral forebrain at E11.5 still contained a significant population of *Pax-3*-positive cells. The upregulation of *Emx2* and *Dbx* in DIV 5 cells is also consistent with the extension of their strong expression domains into the midbrain and ventral forebrain at E12.5 in mice as previously described in detail (Simeone et al., 1992b; Lu et al., 1992). In particular, the change in *Emx2* expression from a dorsally enriched to a ventrally enriched pattern after 5 days in culture is consistent with the previous observation that the expression of *Emx2* declines in the dorsal forebrain accompanying the differentiation of cortical neurons, whereas it persists in the ventral regions including hypothalamus even at E17.5 (Simeone et al., 1992b). Thus, we can consider that the expression patterns of the above genes in neuroepithelial cells cultured in vitro reflect, at least in part, their spatial and temporal dynamics observed in vivo.

Taken together, these results raise the possibility that in neuroepithelial cells, certain cell-autonomous mechanisms operate to maintain selective gene expression even after the cells are isolated from the original environment. It is notable that Robel et al. (1995) reported very recently that the expression of *Otx2*, *Emx1* and *Dlx-1* was maintained in telencephalon-derived neuroepithelial cells in vitro, which is consistent with our findings. However, primary cultures inevitably contain heterogeneous

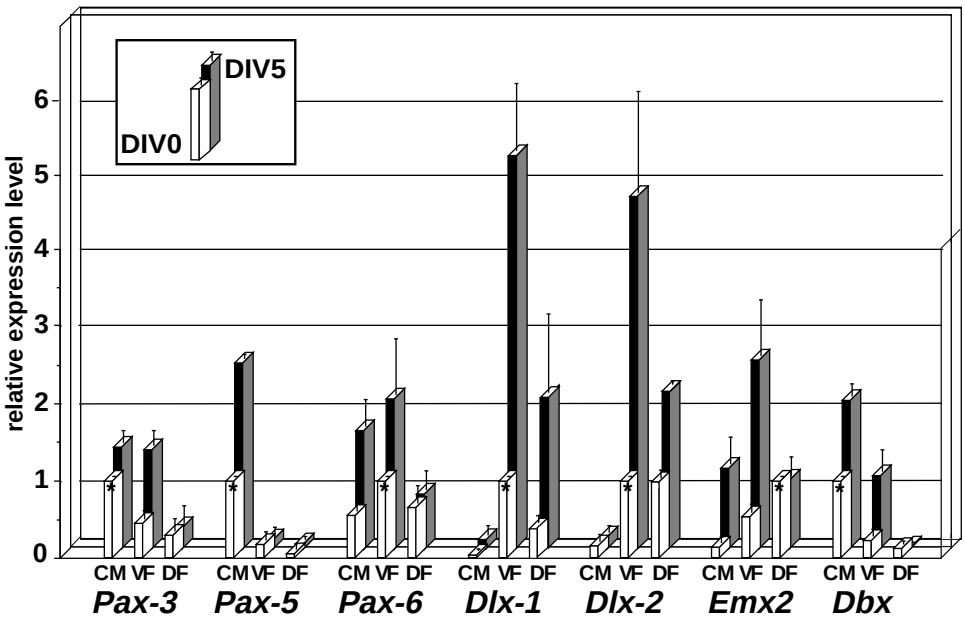

Fig. 2. Analysis of the expression of various region-specific genes in primary cultures of neuroepithelial cells. Quantitative RT-PCR analyses were carried out to examine the expression of the *Pax-3*, *Pax-5*, *Pax-6*, *Dlx-1*, *Dlx-2*, *Emx2* and *Dbx* genes in the primary cultures of neuroepithelial cells from different regions of E11.5 rat brain. Neuroepithelial cells were prepared from the caudal midbrain (CM), ventral forebrain (VF), and dorsal forebrain (DF) as described in Materials and Methods. Total RNA from these cells immediately after dissection (DIV 0; open bars) or after 5 days in vitro (DIV 5; hatched bars) was used for RT-PCR analyses. PCR products were electrophoresed, stained by SYBR Green I, and quantified by measuring the fluorescence intensity. For each primer pair, amplified PCR products among different samples were quantified at various cycles within the range of exponential amplification, and further normalized with the levels of β-actin transcript. For each gene, relative expression levels are shown as mean ± s.d. of three independent reactions, where the highest expression level among the DIV 0 samples from the three brain regions (marked with asterisks) was designated as 1. Essentially identical results were obtained for five independent preparations. Data shown are from one representative preparation. The cycle numbers of PCR reactions used were: 35 for *Pax-3*; 35 for *Pax-5*; 35 for *Pax-6*; 29 for *Dlx-1*; 30 for *Dlx-2*; 35 for *Emx2*; 35 for *Dbx*; and 20 for β-actin.

cell populations expressing different combinations of region-specific genes. Therefore, it is still possible that the maintenance of expression of particular sets of genes is due to non-cell autonomous interactions among cells with different regional identities. Furthermore, we could not examine whether a given single cell in culture continued to express the same sets of genes when it underwent proliferation and/or differentiation. To clarify these points more definitively, it is necessary to analyze clonal and homogeneous cell populations whose proliferation and differentiation can be manipulated in vitro. Therefore, we have established several clonally distinct neuroepithelial cell lines from the E11.5 rat forebrain and midbrain.

MNS cell lines exhibit the properties of multipotential neural stem cells

Detailed characteristics of one of the cell lines used in this study (designated as MNS cell lines) were described previously (Nakafuku and Nakamura, 1995). Here, we show that the established MNS cell lines represent the properties of cultured neuroepithelial cells. We first examined the expression of nestin and the RC1 antigen. MNS-8 cells, like MNS-57 cells that were characterized previously (Nakafuku and Nakamura, 1995), expressed both antigens (Fig. 1D-F). Other clonally distinct cell lines, including MNS-70, -71 and -92, showed similar antigenic phenotypes (data not shown). All these cell lines maintained in monolayer culture continued to express these antigens, indicating that they represent undifferentiated neuroepithelial cells.

We also examined the differentiation potentials of the MNS cell lines. By using the three-step culture protocol (monolayer, aggregation and differentiation culture; described in detail in Materials and Methods), we induced differentiation of the cells, and identified neurons, astrocytes and oligodendrocytes by specific antibodies against microtubule-associated protein 2 (MAP2), glial fibrillary acidic protein (GFAP) and galactocerebroside (GC), respectively. MNS-8 cells, like MNS-57 cells as described previously (Nakafuku and Nakamura, 1995), gave rise to these three cell lineages, whereas MNS-70, -71 and -92 cells generated only MAP2-positive neurons and GFAP-positive astrocytes, but not GC-positive oligodendrocytes under the conditions used in this study (Fig. 3 for MNS-8 and -70; data not shown for the other cells). However, the A2B5 (Gard and Pfeiffer, 1990) and R24 (LeVine and Goldman, 1988) antibodies, which recognize Gq and GD_3 gangliosides on the cell surface, respectively, specifically labeled oligodendrocyte precursor cells with typical unipolar or bipolar morphologies in the differentiated cultures of all the cell lines.

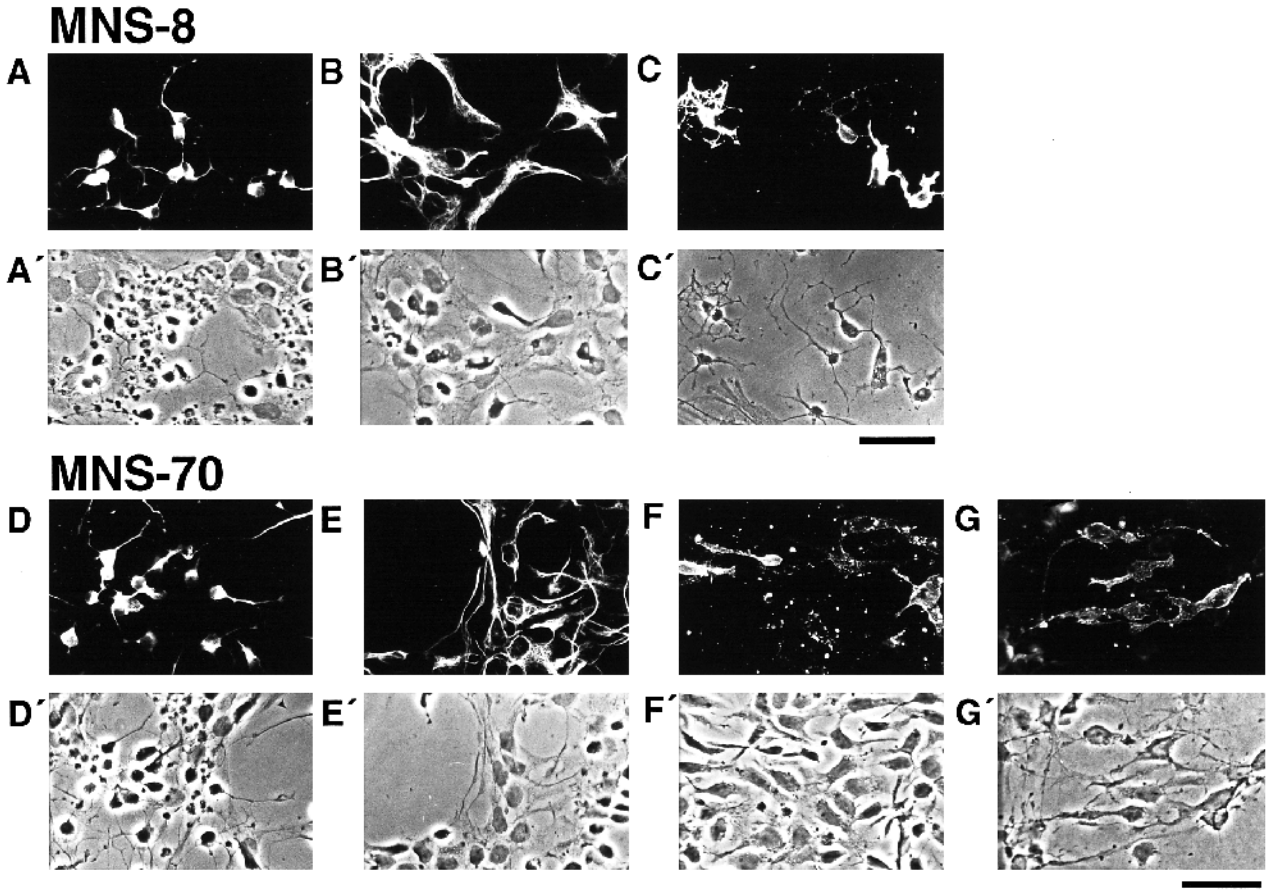

Fig. 3. Generation of both neurons and glia in cultures of MNS cells after induction of differentiation. The cells in 'differentiation culture' (for details, see Materials and Methods) of MNS-8 (A-C) and MNS-70 cells (D-G) were subjected to immunocytochemical analyses using antibodies against various neuron- and glia-specific antigens. Immunofluorescent (A-G) and phase-contrast (A′- G′) images of the same fields are shown side by side. The primary antibodies used were; A and D, anti-MAP2 antibody; B and E, anti-GFAP antibody; C, anti-GC antibody; F, A2B5 antibody; and G, R24 antibody. Scale bar, 50 μm.

Table 2. Expression patterns of various region-specific genes in the five MNS cell lines

	Tissue			MNS cell lines				
	E11.5		E16					
	F/M brain	High expression	F/M brain	8	57	70	71	92
Pax-3	1.00	7.91±0.54 (CM)	0.88±0.14	0.03±0.02	0.03±0.03	0.03±0.02	3.64±0.59	<0.01
Pax-5	1.00	5.93±1.00 (CM)	1.18±0.21	<0.01	0.43±0.07	0.01±0.01	0.01±0.01	0.01±0.01
Pax-6	1.00	7.07±1.33 (VF)	2.32±0.73	0.52±0.08	2.37±0.44	2.40±0.39	0.28±0.11	0.04±0.02
Dlx-1	1.00	17.11±6.53 (VF)	12.88±2.93	0.18±0.03	3.65±1.02	0.82±0.19	8.88±1.96	0.73±0.025
Dlx-2	1.00	7.65±1.38 (VF)	3.23±0.65	0.07±0.04	1.96±1.13	1.46±0.30	3.59±1.32	0.01±0.00
Otx1	1.00	ND	0.85±0.24	0.40±0.10	2.97±0.69	0.20±0.10	0.76±0.20	0.10±0.02
Emx2	1.00	14.4±1.80 (DF)	2.45±0.58	0.41±0.10	1.87±0.35	0.58±0.24	0.13±0.03	1.22±0.32
Dbx	1.00	18.23±2.63 (CM)	1.29±0.46	0.02±0.02	0.64±0.36	8.94±4.46	0.66±0.50	0.05±0.02

In all cases, β-actin was used as an internal control and the expression level of each gene in the control E11.5 forebrain and midbrain tissue sample (F/M brain) was designated as 1.00. All the data shown are mean and s.d. values of three to five independent experiments. The highest level of each gene among the three distinct regions examined in Fig. 2 (indicated in parentheses) are also shown (High Expression). The cycle numbers of PCR used for quantification were: 31 for Pax-3; 35 for Pax-5; 35 for Pax-6; 30 for Dlx-1; 32 for Dlx-2; 33 for Otx1; 32 for Emx2; 35 for Dbx; and 21 for β-actin. DF, dorsal forebrain; VF, ventral forebrain; CM, caudal midbrain; ND, not determined.

In addition to these immunological studies, RT-PCR analyses also demonstrated the expression of lineage-specific genes upon differentiation of the MNS cell lines. Representative results for MNS-8 cells are shown in Fig. 4A and B, in which levels of mRNAs for MAP2, GFAP and 2′,3′-cyclic nucleotide 3′-phosphodiesterase isoform II (CNPII, a marker for oligodendrocytes; Scherer et al., 1994) increased upon induction of differentiation. Based on the above results, we concluded that the MNS cell lines share properties of multipotential neural stem cells present in original preparations of E11.5 forebrain/midbrain neuroepithelium.

The MNS cell lines show distinct expression profiles of region-specific genes

Using these cell lines, we performed intensive analyses of the expression of a series of genes that are restricted to specific domains of the developing forebrain and midbrain, from which all the MNS cell lines originated. First, monolayer culture of each cell line was subjected to quantitative RT-PCR analysis. As shown in Fig. 5A, a high level of *Pax-3* expression was detected only in MNS-71 cells, which was about four times higher than that in the mixture of forebrain and midbrain neuroepithelium and comparable to that in the caudal midbrain-enriched preparations (see Table 2). In contrast, the levels of the *Pax-3* transcript in the other four cell lines were less than 3% of the control. Likewise, a significant level of *Pax-5* expression was detected only in MNS-57 cells. Although *Pax-6* expression was observed in all the cell lines, the levels were highly variable among them. For example, a 60 times higher level of *Pax-6* transcript was detected in MNS-70 than in MNS-92 cells (see Table 2 for results of quantification).

We also examined the expression of four families of the homeobox genes (Fig. 5B). With respect to the *Dlx* family, all the cell lines expressed detectable levels of *Dlx-1* and *Dlx-2* transcripts. However, more than 100-fold differences were observed among different cell lines; MNS-71 cells expressed the highest level (nine times higher than the E11.5 control tissue for *Dlx-1*, and four times for *Dlx-2*), whereas MNS-8

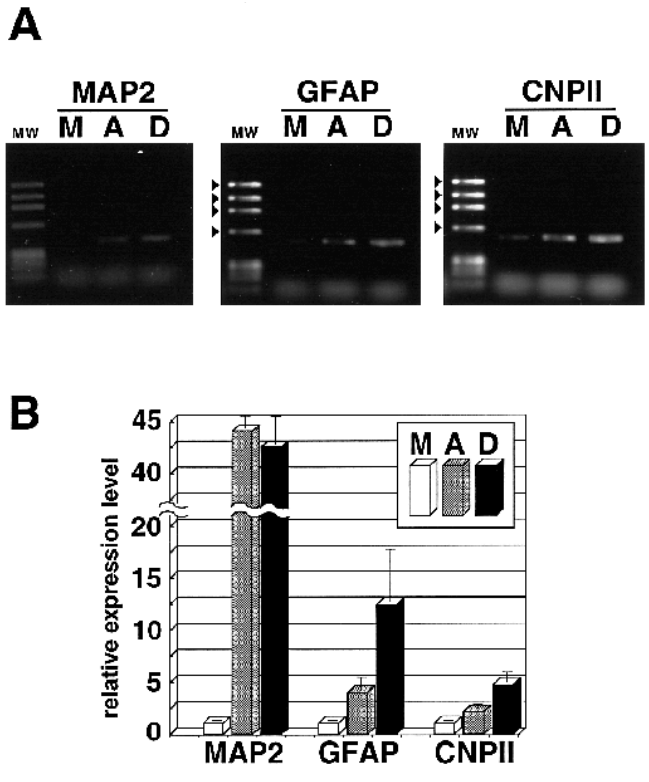

Fig. 4. Induction of neuron- and glia-specific marker genes upon induction of differentiation of MNS cell lines. RT-PCR analysis of the induction of neuronal (MAP2), astroglial (GFAP), and oligodendroglial (CNPII) markers in MNS-8 cells. A shows photographs of PCR products stained with 0.01% SYBR Green I. The positions of molecular size markers (MW) are shown on the left, where arrowheads indicate the ladder of ΦX174 DNA digested with *Hae*III (from the top, 1078 bp, 872 bp, 603 bp, and triplet of 310/281/271 bp). The sizes of specific PCR products are: 405 bp for MAP2; 508 bp for GFAP; and 450 bp for CNPII (see Table 1). Broad bands at the bottom of each figure represent the unincorporated PCR primers. Values for fold-induction of these genes were measured as described in Fig. 2 and are shown in B, where the level in monolayer cells was designated as 1.0. Open bars, monolayer culture (M); hatched bars, aggregation culture (A); and closed bars, differentiation culture (D). The cycle numbers of PCR reactions used for visualization (A) and quantification (B) were: 32 for MAP2; 30 for GFAP; and 32 for CNPII.

cells expressed less than one fifth of the control (Table 2). As shown in Fig. 2, *Dlx-1* and *Dlx-2* transcripts were most abundant in the ventral forebrain, and their levels were 8 to 17 times higher than those in our mixed preparation of E11.5 forebrain and midbrain cells (Table 2). Therefore, levels of *Dlx-1* and *Dlx-2* in MNS-57 and MNS-71 cells are comparable to those in '*Dlx*-high cells' present in the ventral forebrain in vivo. Moreover, it is notable that *Dlx-1* and *Dlx-2* showed parallel expression patterns among different cell lines, which coincides well with their in vivo expression patterns (Bulfone et al., 1993).

Members of the *Otx*, *Emx* and *Dbx* families also showed differential expression patterns among the five cell lines. RT-PCR and sequencing analyses revealed that among the members of these families, expression of only *Otx1*, *Emx2* and *Dbx* could be detected in the MNS cell lines. As shown in Fig. 5B and Table 2, *Otx1* and *Emx2* transcripts were detectable in all the cell lines, but there were marked differences in expression levels. The *Dbx* gene was expressed only in MNS-57, -70 and -71 cells. Again, quantitative comparison with primary tissue samples established that the expression levels of these genes in MNS cell lines were within the range of physiological levels.

Although all the genes described above have restricted expression domains in the developing forebrain and midbrain, some are also expressed in more caudal regions of the neural tube. Thus, we also examined the expression of four other genes that are expressed only in the caudal regions of the brain; *Wnt-3* (Bulfone et al., 1993; Salinas and Nusse, 1992), *En-1* (Davis et al., 1991), *Hox-B1* and *Hox-B3* (Wilkinson et al., 1989a; Lumsden, 1990). Transcripts of these genes were all undetectable in the MNS cell lines (Fig. 5C).

In summary, distinct MNS cell lines expressed different repertoires of region-specific genes, but they were restricted to those found in specific domains of the forebrain and/or midbrain in vivo. Thus, the overall expression patterns are consistent with the forebrain/midbrain origins of the cell lines. Table 2 summarizes the expression profiles of region-specific genes in the five MNS cell lines. It should be noted that each cell line was derived from a single neuroepithelial cell and was clonally expanded. We confirmed that the gene expression pattern observed in each cell line was strictly maintained during repeated passages of the cells. Thus, it is concluded that MNS cell lines possess certain cell-autonomous mechanisms that maintain the expression of specific sets of region-specific genes in vitro even in the absence of environmental signals.

The expression of region-specific genes is maintained during proliferation and differentiation of MNS cell lines

We expected that if cell-autonomous mechanisms are operating independent of environmental signals, a particular set of genes expressed in undifferentiated MNS cells would be maintained even when the cells undergo proliferation and differentiation. To

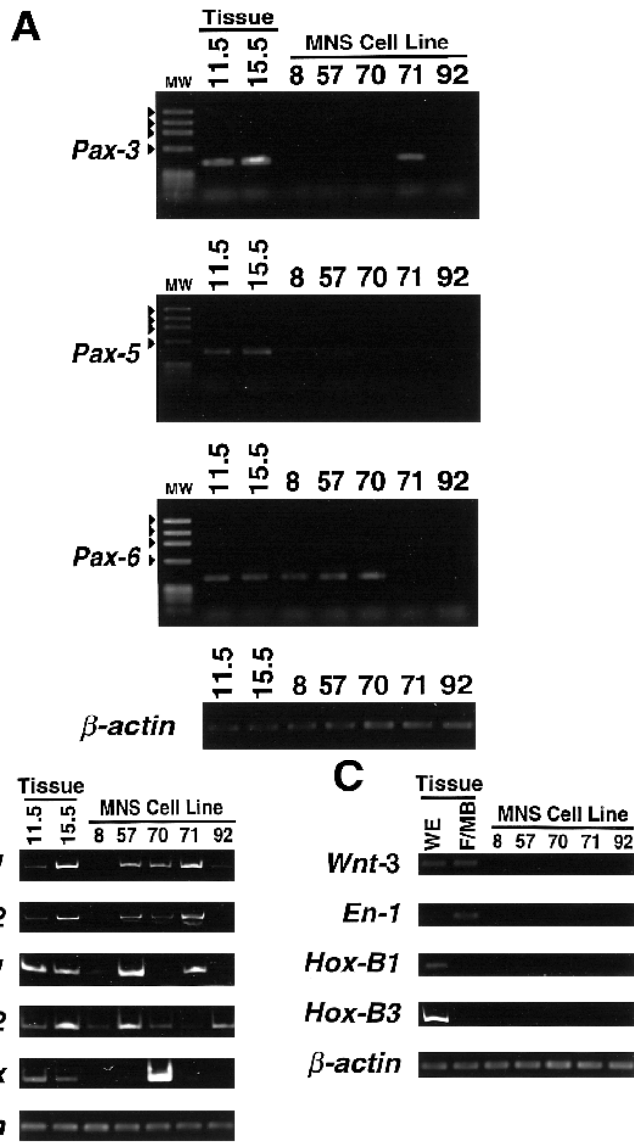

Fig. 5. Differential expression of various region-specific genes in monolayer cultures of MNS cell lines. The levels of expression of the *Pax* genes (A) and the four families of homeobox genes (B) were examined among different MNS cell lines cultured in monolayer as described in detail in Materials and Methods. Expression levels of each gene in the mixed preparation of the forebrain and midbrain tissues from E11.5 and E15.5 embryos are also shown as controls. In panel A, the positions of molecular size markers (MW) are shown by arrowheads on the left as described in Fig 4A. The sizes of specific PCR products are: 418 bp for *Pax-3*; 447 bp for *Pax-5*; and 405 bp for *Pax-6*. Broad bands at the bottom of each figure represent the unincorporated primers. In panel B, weakly stained and faster migrating bands in the lanes for *Dlx-2* show minor nonspecific PCR products, which was confirmed by subcloning and sequencing. See Table 2 for the results of the quantification and PCR cycle numbers used for each gene. In the experiments in Fig. 5, for the clearer visualization of the products, we used cycle numbers 2-3 times larger than those used for quantification. Photographs were obtained directly from the image files generated in the fluorescence image analyzer. The data shown are representative of three to five independent experiments. (C) Results of RT-PCR analyses of *Wnt*-3, *En-1*, *Hox-B1* and *Hox-B3* expression are shown. In all experiments, total RNA from monolayer culture of each cell line was used. The control cDNAs were prepared from E11.5 whole embryos deprived of the forebrain and midbrain (WE) and E11.5 forebrain and midbrain (F/M B), respectively. Note that transcripts of these genes were undetectable in MNS cell lines after 40 cycles of PCR amplification, and their levels were estimated to be at least 100 times lower than those in the control samples.

examine this possibility, we carried out RT-PCR analyses using RNA from cells cultured under three different (monolayer, aggregation and differentiation) conditions. As described above, monolayer culture represents conditions under which undifferentiated MNS cells undergo clonal expansion. In aggregation culture, cell growth was stimulated by the growth factor bFGF and β-E2, an activator of the c-MycER protein, and we assume that during this period the cells undergo commitment to become neurons and glia. In differentiation culture, differentiated neurons and glia were generated, and there were no overall increases in cell number. Representative growth properties of MNS-71 cells are shown in Fig. 6. Similar growth and differentiation patterns were observed in all the MNS cell lines examined in this study (for more detailed description, see Nakafuku and Nakamura, 1995). Furthermore, kinetics of the expression of lineage-specific marker genes clearly showed that differentiation of the MNS cell lines can be conditionally induced (Fig. 4). Thus, this three-step culture protocol allowed us to examine the regulation of region-specific genes in association with proliferation and differentiation of MNS cell lines.

In MNS-71 cells, a high level expression of *Pax-3* remained almost unchanged when proliferation was stimulated in aggregation culture (Fig. 7C). However, its expression level was about three times higher in differentiation culture, where the cells differentiated and ceased proliferation (Figs 6C, 7C). With respect to the *Pax-6* gene, strong (more than 20-fold) induction of expression was observed upon differentiation of MNS-8 cells (Fig. 7A). Weaker but significant upregulation of *Pax-6* was also evident in MNS-70 and -71 cells. Previous studies in mice (Walther and Gruss, 1991) and zebrafish (Macdonald et al., 1994) have revealed that *Pax-6* is expressed in a subset of differentiated neurons. Thus, we asked whether neurons generated from MNS cells express *Pax-6*. As shown in Fig. 8, specific antibodies identified Pax-6 proteins in MAP2-positive neurons in differentiation culture of MNS-70 cells. The expression of two homeobox genes, *Dlx-1* and *Otx1*, was also examined in MNS cell lines. In the three cell lines which we examined, both *Dlx-1* and *Otx1* expression was maintained and their levels increased upon proliferation and differentiation of the cells (Fig. 7). Robel et al. (1995) recently reported that bFGF upregulates the expression of *Otx2* in primary culture of neuroepithelial cells. In our study, proliferation of MNS cell lines were also stimulated by bFGF in aggregation culture, which in many cases, led to the upregulation of the mRNA levels of most of the above genes. Thus, these results may suggest some common regulatory mechanisms for various region-specific genes related to the growth of neuroepithelial cells.

In contrast to the above results, genes that were not expressed in monolayer cultures did not show detectable induction during aggregation or differentiation culture of MNS cell lines. For example, *Pax-3* expression, which was undetectable in monolayer cultures of MNS-8 or -70, was not induced at detectable levels even after aggregation or differentiation (Fig. 7A,B). Similar results were obtained for all the other genes examined in this study (data not shown). Furthermore, it is notable that although the expression of various region-specific genes examined were all upregulated upon induction of differentiation, the overall expression profile in monolayer culture of each cell line was conserved in this process. For example, in MNS-71 cells, high levels of expression of *Pax-3* and *Dlx-1* were maintained among three culture conditions, while the levels of *Pax-6* and *Otx1*, which were relatively lower than

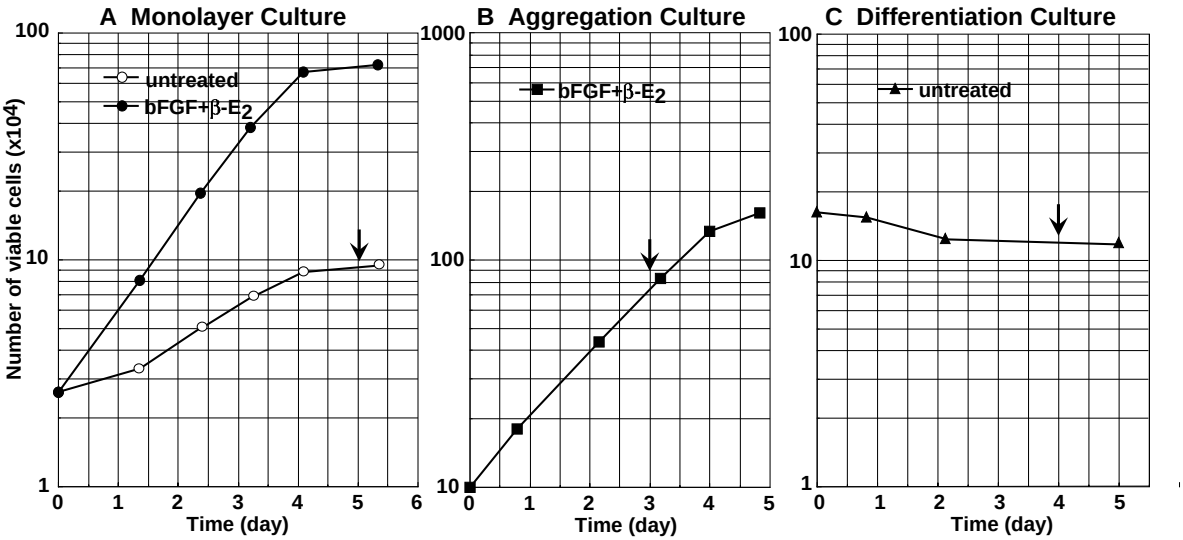

Fig. 6. Growth properties of MNS-71 cells under different culture conditions. MNS-71 cells were cultured under the three different conditions (monolayer, aggregation, and differentiation culture, for details, see Materials and Methods), and the growth of the cells was examined. For the monolayer culture (panel A), the cells were seeded at a density of 1×10^4 cells per ml in DF medium containing 10% FBS and 5% HS. Twenty-four hours later, the medium was replaced by the above medium supplemented with (closed circles) or without (open circles) 20 ng/ml bFGF and 1 μM β-E_2, and the subsequent increase in cell number was monitored daily. For the aggregation culture (B), cell suspensions at a density of 1×10^5 cells per ml were seeded onto poly HEMA-coated dishes in the above medium plus bFGF and β-E_2. For the differentiation culture (C), the cells aggregated in the presence of bFGF and β-E_2 for 3 days were re-seeded onto poly-D-lysine-coated dishes, and incubation was continued in DF medium plus 10% FBS without bFGF or β-E_2. The arrow in each panel indicates the time when the cells were harvested and subjected to RNA preparation.

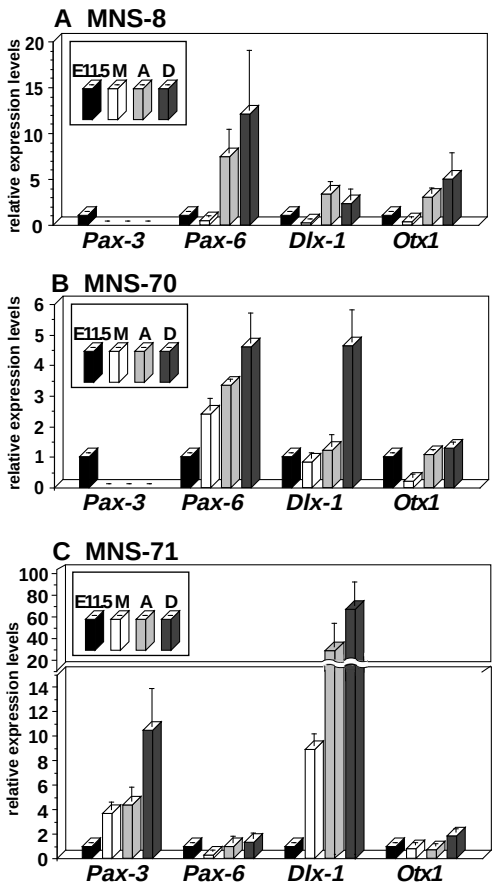

Fig. 7. Regulation of *Pax-3*, *Pax-6*, *Dlx-1* and *Otx1* expression upon differentiation of MNS cell lines. MNS cell lines were cultured under three different conditions as described in Fig. 3. Total RNAs were isolated, and relative expression levels of region-specific genes in MNS-8 (A), MNS-70 (B) and MNS-71 (C) were quantified as described in Fig. 2. In each set of genes and cell lines, the expression level in the forebrain and midbrain neuroepithelia from E11.5 embryos (E11.5; solid bar) was designated as 1.0, and those in monolayer (M, open bars), aggregation (A, light shaded bars) and differentiation (D, dark shaded bars) cultures were shown as mean (±s.d., *n*=3-5) values. The cycle numbers used for quantification were the same as those described in Table 2.

those in other cells, remained low under different culture conditions. Likewise, MNS-70 maintained the pattern of high expression of *Pax-6* and *Dlx-1* and low expression of *Pax-3* and *Otx1* under all culture conditions. This general feature is consistent with the data obtained in primary culture of neuroepithelial cells shown in Fig. 2, in which undifferentiated neuroepithelial cells at DIV 0 underwent significant proliferation and differentiation during 5 days in culture. Thus, the most important conclusion from these results is that the differential expression patterns of region-specific genes in undifferentiated MNS cell lines are maintained even after induction of their proliferation and differentiation.

SHH induces differential expression of ventral marker genes in MNS cell lines

We next asked whether distinct cell-autonomous properties among different neuroepithelial cells influence the responsiveness to environmental signals. For this purpose, we examined their responses to SHH, which is one of the best characterized signaling molecules involved in regional specification. It has been shown that SHH induces the expression of a series of ventral cell-specific transcription factors including *HNF-3β*, *Isl-1*, *Nkx-2.1*, *Nkx-2.2* and *Lim-1*, thereby playing a crucial role in specification of the ventral phenotype of neuroepithelial cells (Echelard et al., 1993; Roelink et al., 1994; Martí et al., 1995; Ericson et al., 1995; Barth and Wilson, 1995). In this study, we utilized CV1 cells which express chicken SHH at high levels and secrete its N-terminal cleavage product in the conditioned medium (Ogura et al., 1996; Fig. 9A). We first isolated primary cultures of neuroepithelial cells from three distinct regions, and compared their expression levels of the *Isl-1*, *Nkx-2.1* and *Nkx-2.2* genes (Fig. 9B). Consistent with previous in situ studies in chick and mouse embryos (Ericson et al., 1995; Shimamura et al., 1995), *Nkx-2.1* expression was restricted in the ventral forebrain, and its levels in the dorsal forebrain and midbrain were less than 0.3% and 0.1% of that in the ventral forebrain, respectively. The expression of *Nkx-2.2* and *Isl-1* was detected both in ventral forebrain and midbrain preparations, but they were much weaker in the dorsal forebrain (1.3% and 4.5 % of that in the ventral forebrain for *Nkx-2.2* and *Isl-1*, respectively). Upon incubation with the medium conditioned by CV1SHH cells, the levels of the all

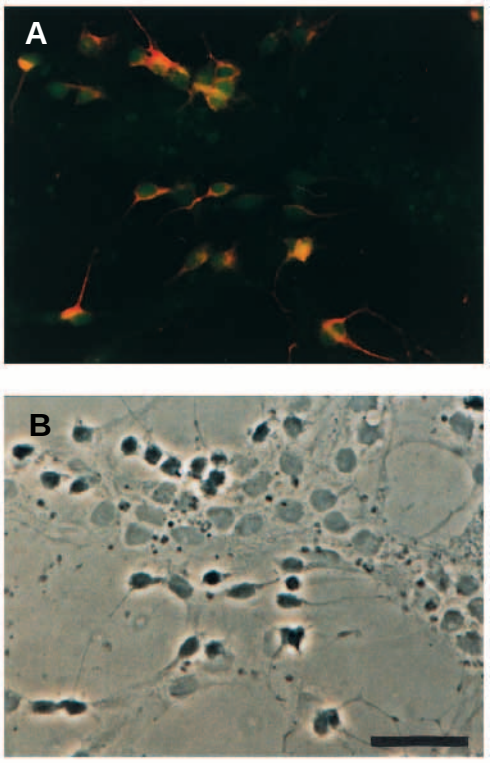

Fig. 8. The Pax-6 protein was expressed in MAP2-positive neurons generated from MNS-70. MNS-70 cells grown under differentiation culture conditions were analyzed by double immunostaining with anti-Pax-6 and anti-MAP2 antibodies. Secondary antibodies used were FITC-conjugated anti-mouse IgG for Pax-6 and TR-conjugated anti-rabbit IgG for MAP2. (A) Fluorescence micrograph showing the coexpression of Pax-6 (green) and MAP2 (red). Cells that expressed both proteins are shown in yellow. (B) Phase contrast micrograph showing the same field as A. Scale bar, 100 μm.

three genes in the dorsal forebrain was dramatically elevated and were close to those of the ventral forebrain (Fig. 9B). These results are consistent with the idea that SHH has the ability to ventralize the dorsal neuroepithelium (Ericson et al., 1995; Lumsden and Graham, 1995). In the ventral forebrain, which endogenously expressed much higher levels of *Nkx-2.2* and *Isl-1*, they remained almost unchanged after the treatment with exogenous SHH. This is probably due to the presence of large numbers of SHH-expressing cells in this preparation. Dorsal midbrain cells expressed *Nkx-2.1* and *Nkx-2.2* at very low levels, and exogenous SHH did not increased the levels of either genes significantly. In particular, the absence of *Nkx-2.1* induction in the midbrain and its remarkable induction in the dorsal forebrain coincided with its forebrain-specific expression in vivo (Shimamura et al., 1995). It is also consistent with a previous report describing that SHH could induce *Nkx-2.1*-positive cells in explant culture of the forebrain neuroepithelium but not of the hindbrain (Ericson et al., 1995). These results indicate that neuroepithelial cells in distinct regions possess distinct properties in terms of the expression of ventral-specific genes and their responsiveness to SHH.

Next we asked how clonal MNS cell lines respond to SHH. Among the monolayer cells of MNS-8, -57, -70, -71 and -92, *Isl-1* was expressed only in the MNS-71 cells (the relative level of expression was 0.77±0.39, where that of the control E11.5 forebrain/midbrain tissue was designated as 1.00, n=3), and *Nkx-2.2* was expressed only in MNS-71 cells (0.33±0.08, n=3; also see Fig. 9C). In all the other cases, expression levels of *Isl-1*, *Nkx-2.1* and *Nkx-2.2* were less than 5% of the control tissue. Furthermore, none of the cell lines expressed *HNF-3β*, *Lim-1*, or *SHH* at a detectable level (data not shown). When MNS cell lines were grown in contact with CV1SHH cells, they showed differential induction profiles of the above genes (Fig. 9C). Upon contact with the CV1SHH cells, MNS-70 cells exhibited clearly elevated (more than 50- to 100-fold) levels of expression of *Isl-1*, *Nkx-2.1* and *Nkx-2.2*, which were comparable to those detected in the E11.5 forebrain/midbrain neuroepithelium (0.39±0.06 for *Isl-1*, 0.58±0.14 for *Nkx-2.1* and 2.03±0.45 for *Nkx-2.2*, n=3), although the accurate values for fold-induction were uncertain because of the very low levels of their expression in the control (SHH-untreated) cells. MNS-71 cells showed a very weak but detectable induction of *Nkx-2.1* in response to SHH, but the expression of *Isl-1* and *Nkx-2.2*

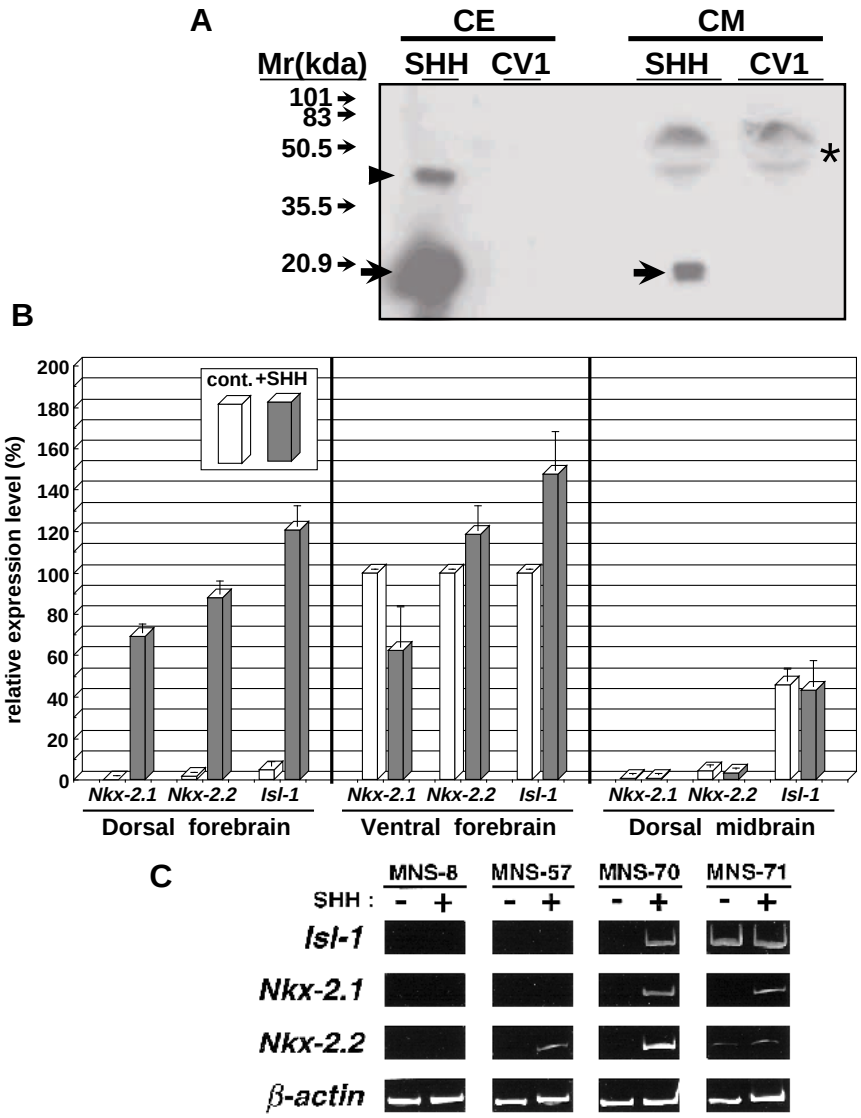

Fig. 9. SHH induced expression of ventral neuroepithelium-specific genes in MNS cell lines. (A) Western blot analysis of SHH expressed by CV1SHH cells. Whole cell extracts (CE) and conditioned media (CM) were prepared from 5×10^3 cells of the control and CV1SHH. These samples were subjected to 15% polyacrylamide gel electrophoresis and blotted with the anti-SHH antibody. The arrowhead and arrows with apparent relative molecular masses of 46×10^3 and 20×10^3 correspond to the full-length and its amino-terminal cleavage products of chicken SHH, respectively. The nonspecific bands in lanes of CM (marked by an asterisk) were due to the presence of large amounts of bovine serum albumin in the culture medium. (B) Primary culture of neuroepithelial cells was established as described in Materials and Methods, in which cells of three distinct regions, the dorsal forebrain, ventral forebrain, and dorsal midbrain were isolated separately. Note that in these experiments, the ventral two-thirds was removed from the caudal midbrain preparation used in Fig. 2. Subsequently, the cells were treated with the medium conditioned by the control CV1 (cont., shown by open bars) or CV1SHH (+SHH, shaded bars) cells for 3 days, and RNA was prepared for quantitative RT-PCR analysis of the *Isl-1*, *Nkx-2.1* and *Nkx-2.2* genes. For each gene, relative expression levels are shown as mean ±s.d. of three independent experiments, where the expression level in the ventral forebrain was designated as 100. (C) RNAs were prepared from the MNS cell lines cultured for 3 days in contact with control (–) or CV1SHH (+) cells. The induction of *Isl-1*, *Nkx-2.1* and *Nkx-2.2* was analyzed by quantitative RT-PCR. The cycle numbers of PCR reactions for Panels B and C were: 29 for *Isl-1*; 35 for *Nkx-2,1*; 32 for *Nkx-2.2*; and 20 for β-actin.

remained almost unchanged (0.69±0.15 for *Isl-1*, and 0.57±0.26 for *Nkx-2.2*, n=3, where the levels in the control cells were designated as 1.00). MNS-57 cells underwent significant induction of the *Nkx-2.2* expression (the level in the SHH-treated cells was 0.26±0.12 compared with 1.00 of the E11.5 sample, n=3) but not of *Isl-1* and *Nkx-2.1*, whereas none of these genes were induced in MNS-8 cells. In contrast, we did not observe detectable induction of *HNF-3β* or *Lim-1* in any of these cell lines (data not shown). Similar differential induction patterns were obtained by using the conditioned medium from CV1SHH cells but not with that from the parental CV1 cells (data not shown), indicating that the induction of the ventral genes directly resulted from exposure to SHH. These results demonstrate that distinct MNS cell lines, which maintain the expression of different sets of region-specific genes in vitro, show specific and differential responsiveness to the same inductive signal from the environment, i.e. SHH.

DISCUSSION

The generation of neural cell diversity in the developing central nervous system is thought to be regulated by both cell-intrinsic and -extrinsic mechanisms (see Jessell and Dodd, 1992; Ruiz i Altaba, 1994; Simon et al., 1995; Lumsden and Graham, 1995 for discussion). However, it is not fully understood how these two mechanisms contribute to the determination of a particular fate of each progenitor cell. Recently, a number of molecules have been identified that potentially regulate this complex process. These genes, collectively called region-specific genes, are expressed in the developing neuroepithelium in a spatially and temporally restricted manner, and have been implicated in the specification of particular domains or layers of the brain. Thus, studies on the regulation of these genes would provide crucial information to facilitate understanding of the molecular nature of the above two mechanisms. Recent studies have uncovered the important roles of inductive signals from the environment for the regulated expression of region-specific genes (Jessell and Dodd, 1992; Johnson and Tabin, 1995), but little is known about the involvement of the cell-autonomous mechanisms. In this study, we have established in vitro culture systems which enable us to study how cell-autonomous mechanisms and environmental signals contribute to the regulation of region-specific genes in neuroepithelial cells.

First, we showed that neuroepithelial cells from distinct regions of the brain express region-specific genes, including *Pax-3*, *Pax-5*, *Pax-6*, *Dlx-1*, *Dlx-2*, *Emx2* and *Dbx* at different levels. The overall pattern in each preparation was generally consistent with the results of previous in situ hybridization studies (Fig. 2). In addition, when cultured for 5 days free from the influences of cells in other regions, cell populations did not undergo significant changes in the overall gene expression pattern. These results support the notion that certain cell-autonomous mechanisms play an important role in maintaining the expression of particular sets of genes in neuroepithelial cells.

Next, we demonstrated that differential expression of region-specific transcription factors among different neuroepithelial cells can be reproduced in immortalized cell lines. MNS cell lines used in this study, which were established from E11.5 rat forebrain and midbrain (Nakafuku and Nakamura, 1995), shared properties of neural stem cells in that they expressed neuroepithelium-specific antigens and could generate neurons and glia under particular culture conditions (Figs 3, 4). Since MNS cell lines are derived from clonally distinct cells, they provide a useful model system in which to examine what types of region-specific genes are expressed in single neuroepithelial cells, and to study how they are regulated under conditions in which the influence of environmental signals and cell-cell interactions among heterogenous cell populations can be eliminated. We found that five distinct MNS cell lines expressed different combinations of transcription factors expressed in restricted regions of forebrain and midbrain. In contrast, region-specific genes that are expressed only in the caudal brain, including *Wnt*-3, *En-1*, *Hox-B1* and *Hox-B3*, were all below detectable levels in these cell lines. These results are consistent with the forebrain/midbrain origin of the MNS cell lines, and it is unlikely that the observed gene expression profiles of the MNS cell lines have resulted from some random events related to immortalization or in vitro culture. Furthermore, we demonstrated that these expression profiles remained essentially unchanged upon proliferation and differentiation (see Fig. 7). Immunocytochemical analysis demonstrated that when MNS-70 cells, which expressed the highest level of *Pax-6* among the cell lines examined, were induced to differentiate, *Pax-6*-positive neurons were indeed generated (Fig. 8). These results strongly suggest that at least in some cases, expression of region-specific genes in undifferentiated neural stem cells is directly inherited to their neuronal (and possibly also glial) progeny.

In relation to the above results, comparison of our data shown in Fig. 2 and Table 2 with the available information from a number of previous in situ hybridization studies (Bulfone et al., 1992; Puelles and Rubenstein, 1993; Stoykova and Gruss, 1994; Rubenstein et al., 1994 for details) demonstrated that the overall gene expression patterns found in some of the MNS cell lines closely matched those in particular regions of the developing brain. For example, MNS-70 cells expressed significant levels of *Pax-6*, *Dlx-1*, *Dlx-2* and *Dbx*, but not *Pax-3* or *Pax-5*. This profile is reminiscent of that in the septal region of the forebrain. However, MNS-8 cells shared similar expression patterns with the dorsal telencephalic region shown in Fig. 2 in that they expressed *Pax-6*, *Otx1* and *Emx2* at significant levels but not *Dlx-1*, *Dbx*, *Pax-3*, or *Pax-5*. MNS-57 cells expressed all the genes examined except *Pax-3*, but the relatively high levels of *Dlx-1*, *Dlx-2* and *Emx2* suggest its ventral forebrain origin, although our data shown in Fig. 2 did not clearly distinguished between the ventral and dorsal forebrain. We consider that these results may suggest the intriguing possibility that each MNS cell line inherited a particular regional identity from the neuroepithelial cell from which it originated. It should be noted, however, that although our data in Fig. 2 clearly showed differential expression profiles of various region-specific genes among distinct brain regions in vivo, we still do not know their exact expression patterns in particular single cells in a given region. For example, it is possible that even in the ventral forebrain where *Dlx-1* is strongly expressed, some cells express it at only very low levels. Thus, the comparison of the combinations of expressed genes alone is not enough at present to definitively

assign the position where each cell line was derived. Nevertheless, strict maintenance of gene expression profiles in MNS cell lines upon continuous cell growth and differentiation makes it likely that a particular set of genes expressed in individual cell lines reflect their distinct origins.

Based on the above results, we can conclude that certain cell-autonomous mechanisms play important roles in maintaining the expression of a specific set of region-specific genes. Several previous studies have also implicated similar mechanisms for the establishment of regional identity in the developing brain. For example, the limbic system-associated membrane protein (LAMP) is specifically expressed in the limbic cortex but not in other cortical areas (Horton and Levitt, 1988). Transplantation and in vitro culture experiments have shown that its specific expression in the limbic cortex is maintained after isolation from the original environment (Barbe and Levitt, 1991; Ferri and Levitt, 1993). Differential expression of the PC3.1 antigen (latexin), which also revealed regional heterogeneity of the developing cerebral cortex in vivo, was conserved in cultured neuroepithelial cells (Arimatsu et al., 1992). Likewise, chick/quail heterotopic transplantation demonstrated that midbrain and hindbrain neuroepithelia maintained their predetermined fates even after being placed into other brain regions (Nakamura, 1990). These studies collectively support our conclusion that cell-intrinsic mechanisms indeed play important roles in determination of the regional fate of neuroepithelial cells.

Finally, we examined the possible cooperative actions of the cell-autonomous and non-cell autonomous mechanisms in the regulation of region-specific gene expression. We first showed that SHH, which is one of the best characterized inductive signals from the environment (Martí et al., 1995; Roelink et al., 1995; Hynes et al., 1995; Ericson et al., 1995; Barth and Wilson, 1995), induced the expression of various ventral-specific genes, including *Nkx-2.1*, *Nkx-2.2* and *Isl-1* in primary cultures of distinct regions of the embryonic neuroepithelium (Fig. 9B). The dorsal forebrain expressed high levels of all these genes in response to SHH, and its pattern resembled that in the ventral forebrain. This result supports the notion that SHH acts as a major ventralizing signal in the forebrain region. A clear difference between the forebrain and midbrain was evident in that the dorsal midbrain did not express *Nkx-2.1* with or without exogenous SHH. These results were consistent with previous studies showing that neuroepithelial explants, derived from distinct regions, generated different cell types in response to SHH. In spinal cord explants SHH induced *Isl-1*-positive cells with the identity of motoneurons (Martí et al., 1995; Roelink et al., 1995), whereas it generated dopaminergic neurons in midbrain-derived explants (Hynes et al., 1995). On the other hand, SHH specifically induced *Nkx-2.1* and *Lim-1* in a population of forebrain-derived neuroepithelial cells in chick embryos (Ericson et al., 1995). However, as in the case of our study using primary culture of neuroepithelial cells, these studies utilized explant cultures which contained heterogeneous cell populations, and hence the contribution of cell-cell interactions among these populations in each explant has remained unclarified. Furthermore, distinct responsiveness among neuroepithelial cells has not yet been fully characterized at the single-cell level. To address this point, we extended the above observations by examining the properties of MNS cell lines. We demonstrated that the cell lines had the ability to respond to SHH and expressed these ventral cell-specific genes (Fig. 9C). Furthermore, we found that the combinations of the induced genes differed among different cell lines. These results strongly suggest that cell-intrinsic properties of neuroepithelial cells indeed define the responsiveness to environmental signals, as well as the repertoires of genes expressed in their absence. It is notable that SHH induced *Nkx-2.1* expression in MNS-70 and MNS-71 cells, which is consistent with their forebrain origin as suggested by the expression patterns of other region-specific genes (discussed above), since *Nkx-2.1* was specifically induced in the forebrain as shown in Fig. 9B. In addition, the use of homogeneous cell populations enabled us to clearly conclude that intrinsic properties of the cells themselves but not cell-cell interactions among heterogenous populations are responsible for specifying how neuroepithelial cells respond to SHH.

In summary, it is highly likely that both cell-autonomous mechanisms and environmental factors contribute cooperatively to the differential and regulated expression of the genes specifying the identities of neuroepithelial cells. Many questions, however, still remain to be answered. At present, we do not know exactly how particular region-specific genes are activated in each cell line, how they are maintained, or how various environmental signals contribute to the initial activation and subsequent modulation of region-specific genes. We propose that the MNS cell lines described here will serve as a useful in vitro model to clarify the above questions at the molecular level. Such studies would provide further insight into the molecular basis of regional specification in the developing brain.

We are grateful to Drs H. Fujisawa and A. Kawakami for anti-Pax-6 antibody, J. C. Izpisua Belmonte for the cDNA for chicken SHH, A. McMahon and D. Bumcrot for anti-SHH antibody and Y. Ihara for anti-MAP2 antibody. We also thank Drs Y. Kaziro and Y. Nabeshima for encouragement and support, Drs F. Matsuzaki, N. Ohsumi-Yamashita, A. Wanaka and S. Yoshida for helpful discussion, Dr Keiko Ogura for preparing CV1SHH cells and Molecular Dynamics Japan for help in preparation of photographs. This work was supported in part by grants-in-aid from the Ministry of Education, Science and Culture, and from the Science and Technology Agency of Japan. Y. N. is supported by JSPS Research Fellowships for Young Scientists.

REFERENCES

Arimatsu, Y., Miyamoto, M., Nihonmatsu, I., Hirata, K., Uratani, Y., Hatanaka, Y. and Takiguchi-Hayashi, K. (1992). Early regional specification for a molecular neuronal phenotype in the rat neocortex. *Proc. Natl. Acad. Sci. USA* **89**, 8879-8883.

Asano, M. and Gruss, P. (1992). *Pax-5* is expressed at the midbrain-hindbrain boundary during mouse development. *Mech. Dev.* **39**, 29-39.

Barbe, M. F. and Levitt, P. (1991). The early commitment of fetal neurons to the limbic cortex. *J. Neurosci.* **11**, 519-533.

Barth, K. A. and Wilson, S. W. (1995). Expression of zebrafish *nk2.2* is influenced by *sonic hedgehog/vertebrate hedgehog-1* and demarcate a zone of neuronal differentiation in the embryonic forebrain. *Development* **121**, 1755-1768.

Basler, K., Edlund, T., Jessell, T. M. and Yamada, T. (1993). Control of cell pattern in the neural tube: regulation of cell differentiation by *dorsalin-1*, a novel TGFβ family member. *Cell* **73**, 687-702.

Birgbauer, E. and Fraser, S. E. (1994). Violation of cell lineage restriction compartments in the chick hindbrain. *Development* **120**, 1347-1356.

Boncinelli, E., Gulisano, M. and Broccoli, V. (1993). Emx and Otx homeobox genes in the developing mouse brain. *J. Neurobiol.* **24**, 1356-1366.

Boncinelli, E. (1994). Early CNS development: *Distal-less* related genes and forebrain development. *Curr. Opin. Neurobiol.* **4**, 29-36.

Bulfone, A., Puelles, L., Porteus, M. H., Frohman, M. A., Martin, G. R. and Rubenstein, J. L. (1993). Spatially restricted expression of Dlx-1, Dlx-2 (Tes-1), Gbx-2, and Wnt-3 in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries. *J. Neurosci.* **13**, 3155-3172.

Bumcrot, D. A., Takada, R. and McMahon, A. P. (1995). Proteolytic processing yields two secreted forms of Sonic hedgehog. *Mol. Cell. Biol.* **15**, 2294-2303.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analyt. Biochem.* **162**, 156-159.

Davis, C. A., Holmyard, D. P., Millen, K. J. and Joyner, A. L. (1991). Examining pattern formation in mouse, chicken and frog embryos with an *En*-specific antiserum. *Development* **111**, 287-298.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Edwards, M. A., Yamamoto, M. and Caviness, V., Jr. (1990). Organization of radial glia and related cells in the developing murine CNS. An analysis based upon a new monoclonal antibody marker. *Neuroscience* **36**, 121-144.

Eilers, M., Picard, D., Yamamoto, K. R. and Bishop, J. M. (1989). Chimaeras of myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. *Nature* **340**, 66-68.

Ekker, S. C., Ungar, A. R., Greenstein, P., von Kessler, D. P., Porter, J. A., Moon, R. T. and Beachy, P. A. (1995). Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. *Curr. Biol.* **5**, 944-955.

Ericson, J., Muhr, J., Placzek, M., Lints, T., Jessell, T. M. and Edlund, T. (1995). Sonic hedgehog induces the differentiation of the ventral forebrain neurons: A common signal for ventral patterning within the neural tube. *Cell* **81**, 747-756.

Ferri, R. T. and Levitt, P. (1993). Cerebral cortical progenitors are fated to produce region-specific neuronal populations. *Cerebr. Cortex* **3**, 187-198.

Figdor, M. C. and Stern, C. D. (1993). Segmental organization of embryonic diencephalon. *Nature* **363**, 630-634.

Gard, A. L. and Pfeiffer, S. E. (1990). Two proliferative stages of the oligodendrocyte lineage (A2B5+O4- and O4+GalC-) under different mitogenic control. *Neuron* **5**, 615-625.

Goulding, M. D., Chalepakis, G., Deutsch, U., Erselius, J. R. and Gruss, P. (1991). Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. *EMBO J.* **10**, 1135-1147.

Goulding, M. D., Lumsden, A. and Gruss, P. (1993). Signals from the notochord and floor plate regulate the region-specific expression of two Pax genes in the developing spinal cord. *Development* **117**, 1001-1016.

Gravel, M., DeAngelis, D. and Braun, P. E. (1994). Molecular cloning and characterization of rat brain 2′,3′-cyclic nucleotide 3′-phosphodiesterase isoform 2. *J. Neurosci. Res.* **38**, 243-247.

Guazzi, S., Price, M., De Felice, M., Damante, G., Mattei, M.-G. and Di Lauro, R. (1990). Thyroid nuclear factor 1 (TTF1) contains a homeodomain and displays a novel DNA binding specificity. *EMBO J.* **9**, 3631-3639.

Guthrie, S., Muchamore, I., Kuroiwa, A., Marshall, H., Krumlauf, R. and Lumsden, A. (1992). Neuroectodermal autonomy of *Hox-2.9* expression revealed by rhombomere transplantation. *Nature* **356**, 157-159.

Guthrie, S. (1995). The status of neural segment. *Trends Neurosci.* **18**, 74-79.

Horton, H. L. and Levitt, P. (1988). A unique membrane protein is expressed on early developing limbic system axons and cortical targets. *J. Neurosci.* **8**, 4653-4661.

Hynes, M., Porter, J. A., Chiang, C., Chang, D., Tessier-Lavigne, M., Beachy, P. A. and Rosenthal, A. (1995). Induction of midbrain dopaminergic neurons by Sonic hedgehog. *Neuron* **15**, 35-44.

Jessell, T. M. and Dodd, J. (1992). Floor plate-derived signals and the control of neural cell pattern in vertebrates. *Harvey Lect.* **86**, 87-128.

Johnson, R. and Tabin, C. (1995). The long and short of *hedgehog* signaling. *Cell* **81**, 313-316

Joyner, A. L. and Martin, G. R. (1987). *En-1* and *En-2*, two mouse genes with sequence homology to the *Drosophila engrailed* gene: expression during embryogenesis. *Genes Dev.* **1**, 29-38.

Kindler, S., Sshwanke, B., Schulz, B. and Garner, C. C. (1990). Complete cDNA sequence encoding rat high and low molecular weight MAP2. *Nucl. Acids. Res.* **18**, 2822.

Lendahl, U., Zimmerman, L. B. and McKay, R. D. (1990). CNS stem cells express a new class of intermediate filament protein. *Cell* **60**, 585-595.

LeVine, S. M. and Goldman, J. E. (1988). Embryonic divergence of oligodendrocyte and astrocyte lineages in developing rat cerebrum. *J. Neurosci.* **8**, 3992-4006.

Liem, K. F., Tremml, J. G., Roelink, H. and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969-979.

Lewis, S. A., Balcarek, J. M., Krek, V., Shelanski, M. and Cowan, N. J. (1984). Sequence of a cDNA encoding mouse glial fibrillary acidic protein: structural conservation of intermediate filaments. *Proc. Natl. Acad. Sci. USA* **81,** 2743-2746.

Lu, S., Bogarad, L., Murtha, M. T. and Ruddle, F. H. (1992). Expression pattern of a murine homeobox gene, *Dbx*, displays extreme spatial restriction in embryonic forebrain and spinal cord. *Proc. Natl. Acad. Sci. USA* **89**, 8053-8057.

Lumsden, A. (1990). The cellular basis of segmentation in the developing hindbrain. *Trends Neurosci.* **13**, 329-335.

Lumsden, A., Clarke, J. D. W., Keynes, R. and Fraser, S. (1994). Early phenotypic choices by neuronal precursors, revealed by clonal analysis of the chick embryo hindbrain. *Development* **120**, 1581-1589.

Lumsden, A. and Graham, A. (1995). A forward role for hedgehog. *Curr. Biol.* **5**, 1347-1350.

Macdonald, R., Xu, Q., Barth, K. A., Mikkola, I., Holder, N., Fjose, A., Krauss, S. and Wilson, S. W. (1994). Regulatory gene expression boundaries demarcate sites of neuronal differentiation in the embryonic zebrafish forebrain. *Neuron* **13**, 1039-1053.

Martí, E., Bumcrot, D. A., Takada, R. and McMahon, A. P. (1995). Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. *Nature* **375**, 322-325.

Martinez, S., Geijo, E., Sanchez-Vives, M. V., Puelles, L. and Gallego, R. (1992). Reduced junctional permeability at interrhombomeric boundaries. *Development* **116**, 1069-1076.

Murphy, M., Drago, J. and Bartlett, P. F. (1990). Fibroblast growth factor stimulates the proliferation and differentiation of neural precursor cells in vitro. *J. Neurosci. Res.* **25**, 463-475.

Nakafuku, M. and Nakamura, S. (1995). Establishment and characterization of a multipotent neural stem cell line that can conditionally generate neurons, astrocytes, and oligodendrocytes in vitro. *J. Neurosci. Res.* **41**, 153-168.

Nakamura, H., Takagi, S., Tsuji, T., Matsui, K. A. and Fujisawa, H. (1988). The prosencephalon has the capacity to differentiate into the optic tectum: analysis by chick-specific monoclonal antibodies in quail-chick-chimeric brains. *Dev. Growth Differ.* **30**, 717-725.

Nakamura, H. (1990). Do CNS anlagen have plasticity in differentiation? Analysis in quail-chick chimera. *Brain Res.* **511**, 122-128

Nudel, U., Zakut, R., Shani, M., Neuman, S., Levy, Z. and Yaffe, D. (1983). The nucleotide sequence of the rat cytoplasmic beta-actin gene. *Nucl. Acids Res.* **11**, 1759-1771.

Ogura, T., Alvarez, I. S., Vogel, A., Rodriguez, C., Evans, R. M. and Izpisua Belmonte, J. C. (1996). Evidence that *Shh* cooperates with a retinoic acid inducible co-factor to establish ZPA-like activity. *Development* **122**, 537-542.

Price, M., Lazzaro, D., Pohl, T., Mattei, M. G., Ruther, U., Olivo, J. C., Duboule, D. and Di Lauro, R. (1992). Regional expression of the homeobox gene Nkx-2.2 in the developing mammalian forebrain. *Neuron* **8**, 241-255.

Price, M. (1993). Members of the Dlx- and Nkx2-gene families are regionally expressed in the developing forebrain. *J. Neurobiol.* **24**, 1385-1399.

Puelles, L., Amat, J. A. and Martinez-de-la-Torre, M. (1987). Segment-related, mosaic neurogenetic pattern in the forebrain and mesencephalon of early chick embryos: I. Topography of AChE-positive neuroblasts up to stage HH18. *J. Comp. Neurol.* **266**, 247-268.

Puelles, L. and Rubenstein, J. L. (1993). Expression patterns of homeobox and other putative regulatory genes in the embryonic mouse forebrain suggest a neuromeric organization. *Trends Neurosci.* **16**, 472-479.

Robel, L., Ding, M., James, A. J., Lin, X., Simeone, A., Leckman, J. F. and Vaccarino, F. M. (1995). Fibroblast growth factor 2 increases Otx2 expression in precursor cells from mammalian telencephalon. *J. Neurosci.* **15**, 7879-7891.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. M. and Dodd, J. (1994). Floor plate and motor neuron induction by *vhh-1*, a vertebrate homolog of *hedgehog* expressed by the notochord. *Cell* **76**, 716-775.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A. and Jessell, T. M. (1995). Floor plate and motor neuron induction by

different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81**, 445-455.

Rubenstein, J. L., Martinez, S., Shimamura, K. and Puelles, L. (1994). The embryonic vertebrate forebrain: the prosomeric model. *Science* **266**, 578-580.

Ruiz i Altaba, A. (1994). Pattern formation in the vertebrate neural plate. *Trends Neurosci.* **17**, 233-243.

Salinas, P. C. and Nusse, R. (1992). Regional expression of the Wnt-3 gene in the developing mouse forebrain in relationship to diencephalic neuromeres. *Mech. Dev.* **39**, 151-160.

Scherer, S. S., Braun, P. E., Grinspan, J., Collarini, E., Wang, D. Y. and Kamholz, J. (1994). Differential regulation of the 2′,3′-cyclic nucleotide 3′-phosphodiesterase gene during oligodendrocyte development. *Neuron* **12**, 1363-1375.

Schneeberger, C., Speiser, P., Kury, F. and Zeillinger, R. (1995). Quantitative detection of reverse transcriptase-PCR products by means of a novel and sensitive DNA stain. *PCR Methods Appl.* **4**, 234-238.

Shimamura, K., Hartigan, D. J., Martinez, S., Puelles, L., Rubenstein, J. L. R. (1995). Longitudinal organization of the anterior neural plate and neural tube. *Development* **121**, 3923-3933.

Simeone, A., Acampora, D., Gulisano, M., Stornaiuodo, A. and Boncinelli, E. (1992a). Nested expression domains of four homeobox genes in developing rostral brain. *Nature* **358**, 687-690.

Simeone, A., Gulisano, M., Acampora, D., Stornaiuodo, A., Rambaldi, M. and Boncinelli, E. (1992b). Two vertebrate homeobox genes related to the *Drosophila empty spiracles* gene are expressed in the embryonic cerebral cortex. *EMBO J.* **11**, 2541-2550.

Simon, H., Hornbruch, A. and Lumsden, A. (1995). Independent assignment of anterior-posterior and dorso-ventral positional values in the developing chick hindbrain. *Curr. Biol.* **5**, 205-214.

Stoykova, A. and Gruss, P. (1994). Roles of *Pax* genes in developing and adult brain as suggested by expression patterns. *J. Neurosci.* **14**, 1395-1412.

Stuart, E. T., Kioussi, C. and Gruss, P. (1993). Mammalian Pax genes. *Annu. Rev. Genet.* **27**, 219-236.

Vicario-Abejón, C., Cunningham, M. G. and McKay, R. D. G. (1995). Cerebellar precursors transplanted to the neonatal dentate gyrus express features characteristic of hippocampal neurons. *J. Neurosci.***15**, 6351-6363.

Walther, C. and Gruss, P. (1991). *Pax-6*, a murine paired box gene, is expressed in the developing CNS. *Development* **113**, 1435-1449.

Wilkinson, D. G., Bhatt, S., Cook, M., Boncinelli, E. and Krumlauf, R. (1989a). Segmental expression of Hox-2 homeobox-containing genes in the developing mouse hindbrain. *Nature* **341**, 405-409.

Wilkinson, D. G., Bhatt, S., Chavrier, P., Bravo, R. and Charnay, P. (1989b). Segment-specific expression of a zinc-finger gene in the developing nervous system of the mouse. *Nature* **337**, 461-464.

Williams, R. W. and Goldowitz, D. (1992). Lineage versus environment in embryonic retina: a revisionist perspective. *Trends Neurosci.* **15**, 368-373.

Wilson, S. W., Placzek, M. and Furley, A. J. (1993). Border disputes: do boundaries play a role in growth-cone guidance? *Trends Neurosci.* **16**, 316-323.

(Accepted 9 May 1996)