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Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system

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In the developing mammalian central nervous system, neural precursor cells present in the ventricular zone determine their fate to become neurons or glial cells, migrate towards the outer layers and undergo terminal differentiation. The transcriptional repressor HES-1, a basic helix-loop-helix (bHLH) factor structurally related to the *Drosophila hairy* gene, is expressed at high levels throughout the ventricular zone, but the level decreases as neural differentiation proceeds. Because of this negative correlation, we tested whether continuous expression of HES-1 inhibits neural differentiation. A *HES-1*- and *lacZ*-transducing retrovirus (SG-HES1) and a control *lacZ*-transducing retrovirus (SG) were injected into the lateral ventricles of mouse embryos, and the fate of the infected neural precursor cells was examined by X-gal staining. The SG virus-infected cells migrated and differentiated into neurons and glial cells. In contrast, the cells infected with SG-HES1 virus remained in the ventricular/subventricular zone, decreased to ~10% in number as compared with that of the newborn during the postnatal 4-5 weeks and, when they survived, were present exclusively in the ependymal layer. Furthermore, whereas cultured neural precursor cells infected with SG virus became immunoreactive for neuronal and glial markers, the cells infected with SG-HES1 virus did not. These results show that persistent expression of HES-1 severely perturbs neuronal and glial differentiation.

Key words: exo utero manipulation/*lacZ*/neural precursor cell/neurogenesis/retrovirus

Introduction

In the developing central nervous system (CNS) of mammals, neural precursor cells dividing in the ventricular zone determine their fate to become neurons and glial cells. These cells migrate from the ventricular zone towards the outer layers and undergo terminal differentiation (Jacobson, 1991). The underlying mechanisms are not yet well understood; however, invertebrate studies suggest that basic helix-loop-helix (bHLH) factors play a crucial role in neurogenesis (Campos-Ortega and Jan, 1991; Jan and Jan, 1993). For example, the *Drosophila* bHLH factors encoded by the *achaete-scute* complex (AS-C) are positive regulators and the bHLH factors encoded by *hairy* and *Enhancer of split* [*E(spl)*] are negative regulators for sensory organ formation (Moscoso del Prado and Garcia-Bellido, 1984; Klämbt *et al.*, 1989; Rushlow *et al.*, 1989; Skeath and Carroll, 1991; Campuzano and Modolell, 1992).

Recently, mammalian homologues of the *Drosophila* bHLH factors have been characterized to obtain new insights into the molecular nature of mammalian neurogenesis. MASH-1, a mammalian homologue of AS-C, is specifically expressed in subsets of neuronal precursor cells (Johnson *et al.*, 1990; Lo *et al.*, 1991). Null mutation of MASH-1 results in severe loss of the olfactory epithelium and sympathetic, parasympathetic and enteric ganglia, but presents no obvious abnormalities in the brain and spinal cord (Guillemot *et al.*, 1993). Thus, MASH-1 acts as a determination factor for olfactory and autonomic neurons, but is not essential for development of most of the CNS.

Another example concerns HES-1, a mammalian homologue of *hairy*, which acts as a transcriptional repressor by two different mechanisms: (i) by inhibiting such bHLH activators as MASH-1 and MyoD from binding to the E box enhancer (CANNTG) and (ii) by repressing transcription through directly binding to the N box [CACNAG, which was originally identified as a binding site of the *E(spl)* proteins by Tietze *et al.*, 1992] (Sasai *et al.*, 1992; Takebayashi *et al.*, 1994). HES-1 is expressed at high levels in the undifferentiated mesodermal cells but only at a low level in the adult muscles, and inhibits MyoD-induced myogenesis (Sasai *et al.*, 1992).

In the developing CNS, HES-1 is expressed at high levels throughout the ventricular zone, which consists of neural precursor cells, but not in the outer layers where differentiated neurons and glial cells are present (Sasai *et al.*, 1992). This negative correlation of HES-1 expression to neural differentiation, together with the previous findings that *Drosophila hairy* inhibits neurogenesis, led to the proposal that HES-1 may negatively regulate the developmental process of the mammalian CNS. In the experiments described here, this hypothesis was tested by infecting *HES-1*-transducing retrovirus into the neural precursor cells of the developing CNS.

Recombinant retrovirus has been successfully used for cell lineage analysis of the mammalian nervous system (Price *et al.*, 1987; Turner and Cepko, 1987; Luskin *et al.*, 1988; Walsh and Cepko, 1988; Galileo *et al.*, 1990). These viruses offer the possibility of manipulating the expression of genes in specific subsets of differentiating cells, in contrast to transgenic and gene targeting methods that in general affect the entire embryo. Neural progenitor cells infected with replication-defective retrovirus that carries the *lacZ* gene migrate towards the outer layers and undergo neuronal and glial differentiation which can be traced by X-gal staining. In one application of this technique, we infected the neural

progenitor cells with a replication-defective retrovirus that carries the *HES-1* gene in addition to the *lacZ* gene and subsequently performed X-gal staining to investigate the cell fate when HES-1 is persistently expressed. We found that persistent expression of HES-1 severely perturbs differentiation of neural precursor cells in the CNS. Inhibition of neural differentiation by continuous expression of HES-1 was also shown by immunocytochemical analysis using the cultured neural precursor cells. These findings indicate a critical involvement of bHLH factors in the CNS development.

Results

Construction and characterization of two recombinant retroviruses

To examine the function of HES-1 in mammalian neurogenesis, two replication-defective retroviruses, SG virus and SG-HES1 virus, were designed (Figure 1). SG virus, a control retrovirus, was constructed by replacing the *neo* gene of the retrovirus LXSN (Miller and Rosman, 1989) with the *lacZ−neo* fusion gene. This fusion gene was placed under the control of the internal SV40 promoter to allow visualization of infected cells and their progeny with blue precipitates by X-gal staining. This vector also conferred the *neo*-resistance so that virus-producing cells can be selected in medium containing G418. SG-HES1 virus was made by inserting rat HES-1 cDNA into the *Xho*I site located downstream of the long terminal repeat (LTR) to direct HES-1 expression from the LTR.

To show that SG-HES1 virus directs rat HES-1 expression, RNA was prepared from SG virus-infected, SG-HES1 virus-infected and non-infected C3H10T1/2 mouse cells, which produce the endogenous HES-1 RNA. Using the RNA, we performed reverse transcription PCR (RT-PCR), as shown in Figure 2a. Whereas the endogenous mouse HES-1 transcript was detected in all cells (lanes 1−3), the rat HES-1 transcript was found only in the SG-HES1 virus-infected cells (lane 5) but not in the SG virus-infected (lane 4) or non-infected cells (lane 6).

To confirm that SG-HES1 virus expresses a functional HES-1 protein after infection, we carried out a transient expression analysis. We showed previously that HES-1 negatively regulates transcription by binding to the N box sequences (CACNAG) (Sasai *et al.*, 1992). We thus transfected the reporter plasmid containing the luciferase gene under the control of the β-actin promoter or the synthetic promoter consisting of the β-actin promoter and six repeats of the N box elements (N6-β-actin), and subsequently infected with the retroviruses. As shown in Figure 2b, SG virus did not change the expression from the β-actin or the N6-β-actin promoter. In contrast, SG-HES1 virus efficiently repressed transcription only from the N6-β-actin promoter. These results confirmed that SG-HES1 virus directed the expression of the functional N box-dependent transcriptional repressor HES-1. We also observed good expression of β-galactosidase (lacZ) activity in the cells infected with SG-HES1 virus (data not shown, see below).

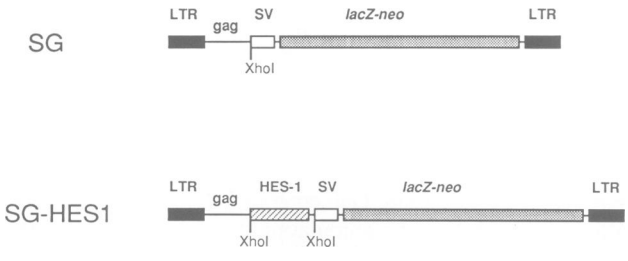

Fig. 1. Structures of the recombinant retroviruses used in this study. SG virus has Moloney sarcoma virus-LTR at the 5′-terminus, Moloney leukemia virus-LTR at the 3′-terminus and the *lacZ−neo* fusion gene which is under the control of the internal SV40 early promoter (SV). SG-HES1 virus was made by inserting the rat HES-1 cDNA (Sasai *et al.*, 1992) into the *Xho*I site of the SG virus vector so that rat HES-1 is expressed from the 5′-LTR.

Histochemical analysis of the control SG virus-infected brains

To examine the effects of HES-1 on neural differentiation, neural precursor cells were infected with the retroviruses. Virus was injected into the lateral ventricles of mouse embryos from day 12 or 13 (E12 or 13), which were allowed to develop *exo utero* (outside of the uterus but intraperitoneally) as described previously (Muneoka *et al.*, 1990). These embryos develop normally; thus the *exo utero* manipulation enables easy access to neural precursor cells

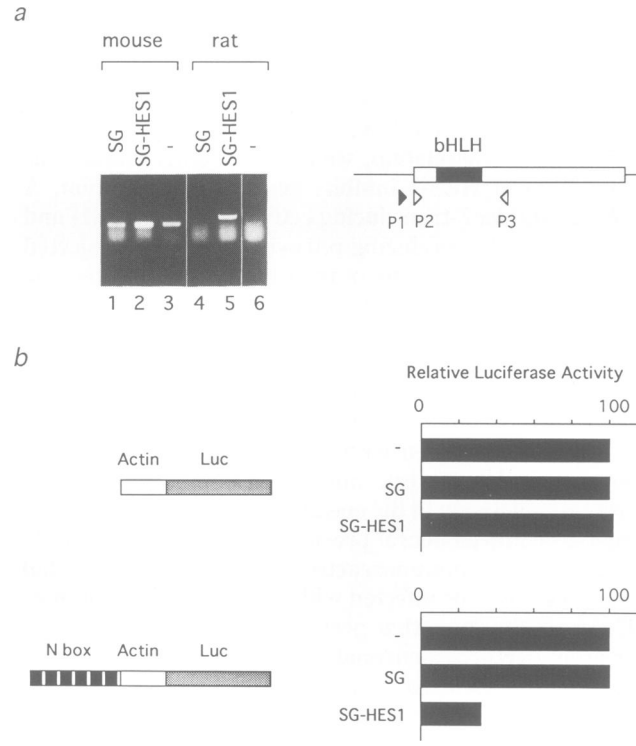

Fig. 2. Characterization of the recombinant retroviruses by RT-PCR and luciferase assays. (**a**) Detection of HES-1 transcript by RT-PCR. RNA was recovered from SG virus-infected (lanes 1 and 4), SG-HES1 virus-infected (lanes 2 and 5) and non-infected C3H10T1/2 mouse cells (lanes 3 and 6). The endogenous mouse HES-1 mRNA was detected by primers P2 and P3 (lanes 1−3) and the rat HES-1 transcript was detected by the rat HES-1-specific primers P1 and P3 (lanes 4−6). Schematic presentation of the PCR primers is shown on the right. (**b**) Detection of HES-1 activity by luciferase assays. C3H10T1/2 cells were transfected with the luciferase reporter plasmid under the control of the β-actin promoter or the N box-containing β-actin promoter, as schematized on the left. Cells were subsequently mixed with either the control medium (−), SG virus or SG-HES1 virus. Relative luciferase activities determined by at least four independent experiments are shown.

which differentiate in the physiological condition. Only mitotic cells can stably integrate the viral DNA into their genome and transmit it into their progeny. Brains (cerebra) were removed on postnatal days 0−4 (P0−4; P0 is defined as the first day after birth by Caesarean section on E19), the period in which neurogenesis is complete but glial cell production still continues, and on P14 and 33, and were subjected to X-gal staining.

In the control SG virus-infected brains, X-gal-stained cells were distributed throughout the ventricular/subventricular zone, white matter and cortex on P0−4 (Figure 3b−f).

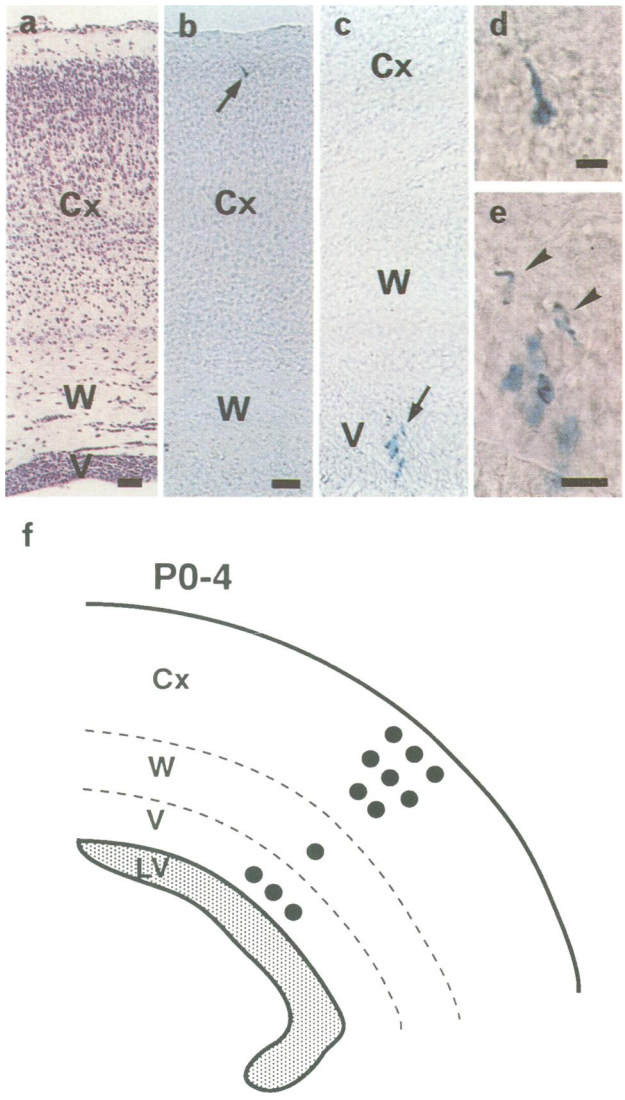

Fig. 3. Distribution of X-gal-stained cells in P0−4 brains infected with SG virus. (**a**) Hematoxylin-eosin staining of P4 cerebrum. (**b**) and (**c**) X-gal-stained cells (arrows) were present in various regions including the cortex (b) and the ventricular/subventricular zone (c) of P4 cerebrum. (**d**) Higher magnification of the labelled cell seen in (b). This clone consisted of one labelled cell which had a neuron-like morphology. (**e**) Higher magnification of the labelled cells seen in (c). This clone consisted of eight labelled cells. Some of them had processes (arrowheads). (**f**) Diagrammatic summary showing the distribution of X-gal-stained clones in P0−4 brains infected with SG virus. Each closed circle represents one X-gal-stained clone per brain in an average of four examined brains. Each clone consists of one to 10 labelled cells, as described in the text. Cx, cortex; W, white matter; V, ventricular/subventricular zone; LV, lateral ventricle. Bar (μm): (a−c) 50; (d) 10; and (e) 20.

These labelled cells appeared alone or as discrete clusters of two to 10 cells. As reported previously, cells of each group were most likely clonal in origin (Price *et al.*, 1987; Turner and Cepko, 1987; Luskin *et al.*, 1988; Walsh and Cepko, 1988; Galileo *et al.*, 1990). The average number of labelled clones per brain was 12.0 (n = 4), and 25% (average three clones per brain) remained in the ventricular/subventricular zone, 8% (average one clone) in the white matter and 67% (average eight clones) reached the cortex (Figure 3f). Some of the labelled cells found in the cortex appeared to extend neurites, and thus had a neuron-like morphology (Figure 3d).

As shown in Figure 4, many labelled cells (average 19 clones per brain) were also found in the ventricular/subventricular zone, white matter and cortex of the SG virus-

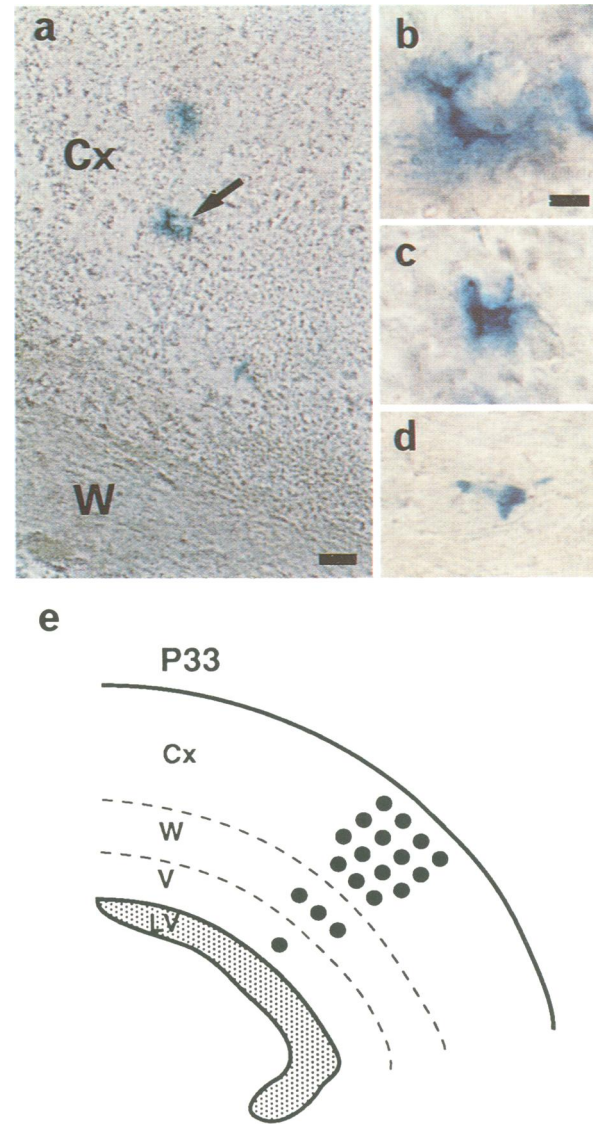

Fig. 4. Distribution of X-gal-stained cells in P33 brains infected with SG virus. (**a**) A cluster of three X-gal-stained cells was present in the cortex. A neuron-like labelled cell is indicated by an arrow. The other labelled cells are also probably neurons. (**b**) Higher magnification of the neuron-like labelled cell, shown in (a) by an arrow, with well-developed processes. (**c**) An astrocyte-like labelled cell. (**d**) An oligodendrocyte-like labelled cell. (**e**) Diagrammatic summary showing the distribution of X-gal-stained clones in P33 brains infected with SG virus. Two brains were examined. See Figure 3 for abbreviations. Bar (μm): (a) 50; (b−d) 10.

infected brains on P33. Nearly 80% of the labelled cells were present in the cortex (Figure 4e). Furthermore, some of them showed the neuron-like morphology and others the astrocyte- or oligodendrocyte-like morphology (Figure 4b–d). These results indicated that neural precursor cells infected with the control SG virus reached the cortex or white matter and differentiated into neurons or glial cells.

Histochemical analysis of the SG-HES1 virus-infected brains

In contrast to the cells infected with SG virus, all labelled cells infected with SG-HES1 virus were found in the ventricular/subventricular zone (71 clones examined) and none were found in the outer layers on P0–4 (Figure 5). All of the labelled cells were round in appearance and had no mature processes (Figure 5b). These results suggest that neural precursor cells forced to persistently express HES-1 are inhibited from migration and that their differentiation process is severely perturbed. Those labelled cells occurred mostly alone (Figure 5a and b) and, out of 71 clones examined, only three clones consisted of more than one labelled cell. This may suggest that cell proliferation was also somewhat affected by HES-1 expression.

To see the final fate of the cells infected with SG-HES1 virus, we next examined brains of P14 and P33. Although the average number of labelled clones per brain was 5.1 on P0–4 (n = 14) (Figure 5c), the average number was only 1.2 on P14 (n = 6) and 0.6 on P33 (n = 8) (Figure 6c). Thus 4–5 weeks after birth, the cells infected with SG-HES1 virus decreased to the ~10% compared with the number on P0–4. This is in sharp contrast to the SG virus-infected brains which showed more labelled cells on P33 than on P0–4 (see Figures 3f and 4e). In addition to reduction of the cell number, the labelled cells infected with SG-HES1 virus still stayed in the ventricular/subventricular zone on P14, except for one clone which was present in the cortex (data not shown). Furthermore, on P33 the labelled cells were found only in the ependymal layer, the epithelial lining of the ventricles (Figure 6a–c). These results suggest that HES-1-expressing cells failed to differentiate, died during the postnatal 4–5 weeks and, when they survived, became exclusively ependymal cells.

Cytochemical and immunocytochemical analyses of cultured neural precursor cells infected with the retroviruses

To obtain more evidence that continuous expression of HES-1 inhibits neuronal and glial differentiation, we next performed immunocytochemical analysis in addition to X-gal staining. Because brain slices used for X-gal staining were relatively thick (60 μm), it was difficult to determine whether the X-gal staining and immunocytochemical staining occurred in the same cell. Thus, we used cultured neural precursor cells for immunochemical study. Cultured neural precursor cells have been shown to undergo neural differentiation in a similar time course to *in vivo* differentiation and become immunoreactive for neuronal and glial proteins (Kitani *et al.*, 1991). Thus, this cell culture provides a good system to analyse early events in neural differentiation.

Cultured neural precursor cells prepared from E10 mouse embryos were infected with the retroviruses (day 1) and subjected to X-gal staining and immunochemical analysis (Figure 7). Many clones consisting of one to 20 X-gal-stained cells were observed in both SG virus-infected and SG-HES1 virus-infected cultures on days 2, 10 and 14. During this period, the number of labelled clones did not reduce and some clones consisted of >10 labelled cells in the cultures infected with SG-HES1 virus (Figure 7c and d), suggesting that the cultured neural precursor cells forced to express HES-1 did not die but proliferated.

On day 10, many labelled cells infected with the control SG virus extended processes and >50% of the X-gal-stained cells were immunoreactive for neurofilament (Figure 7a) or glial fibrillary acidic protein (GFAP) (Figure 7b). These results suggest that cultured neural precursor cells infected with SG virus differentiated into neurons and glial cells. In contrast, the labelled cells infected with SG-HES1 virus always formed aggregates and did not extend any processes (Figure 7c and d). Furthermore, none of them were immunoreactive for neurofilament or GFAP (Figure 7c and d). These results thus demonstrated that neuronal and glial differentiation was severely perturbed by persistent expression of HES-1.

Discussion

HES-1 is a negative regulator of mammalian neurogenesis

HES-1 is expressed at high levels in the ventricular zone of the developing CNS but decreases as neural differentiation proceeds. We found that the differentiation process of the CNS is severely perturbed when HES-1 is persistently expressed. Neural precursor cells infected with SG-HES1 virus did not extend mature processes or become immunoreactive for neuronal or glial proteins. Furthermore, almost all cells infected with SG-HES1 virus (~99%) stayed in the ventricular/subventricular zone or the ependymal layer. Thus, most of the cells continuously expressing HES-1 were inhibited from migration and differentiation, suggesting that down-regulation of HES-1 expression is required for both neuronal and glial cell differentiation.

During the postnatal 4–5 weeks, the X-gal-stained cells infected with SG-HES1 virus decreased in number to ~10% that of P0–4. In addition, most of the labelled cells did not appear in clusters but occurred alone *in vivo*. These results suggest that *in vivo* many neural precursor cells continuously expressing HES-1 may die after birth. Occurrence of cell death was also reported in recent studies with null mutation of the positive neural regulator MASH-1, which shows that olfactory neural precursor cells prevented from differentiating due to a lack of MASH-1 result in death (Guillemot *et al.*, 1993). Thus, inhibition of differentiation by either the presence of the negative regulator HES-1 or the absence of the positive regulator MASH-1 seems to result in the death of many neural precursor cells. However, we did not observe such cell death in our primary culture experiment. *In vitro*, X-gal-stained cells persistently expressing HES-1 did not decrease in number, and many of them were found in clusters of >10 clonal cells. These results suggest that the cells infected with SG-HES1 virus are proliferating in the primary culture and that cell death does not occur at undifferentiated stages or early stages of differentiation *in vitro*, and therefore cell death may be a secondary event *in vivo*. Thus, HES-1

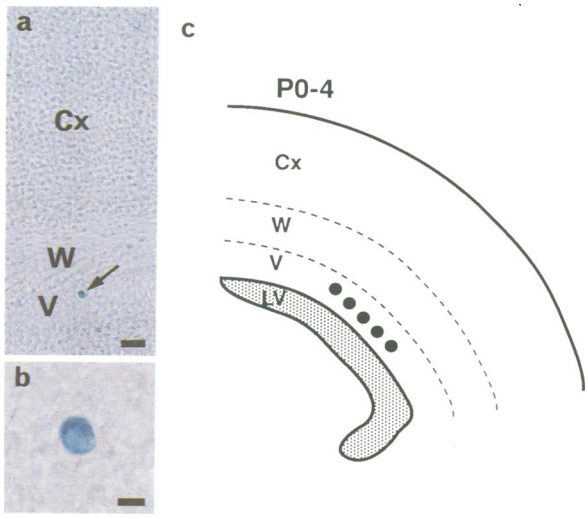

Fig. 5. Distribution of X-gal-stained cells in P0−4 brains infected with SG-HES1 virus. (**a**) A labelled cell (arrow) remained in the ventricular/subventricular zone of P4 cerebrum. (**b**) Higher magnification of the cell shown in (a), which was round in appearance. (**c**) Diagrammatic summary showing the distribution of X-gal-stained clones in P0−4 brains infected with SG-HES1 virus. 14 brains were examined. Each clone mostly consists of one labelled cell. See Figure 3 for abbreviations. Bar (μm): (a) 50; (b) 10.

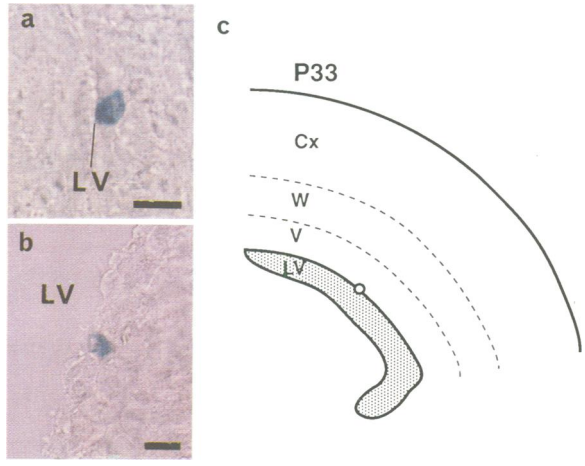

Fig. 6. Distribution of X-gal-stained cells in P33 brains infected with SG-HES1 virus. (**a** and **b**) X-gal-stained cells were found only in the ependymal layer, the epithelial lining of the lateral ventricles, of P33 brains. (**c**) Diagrammatic summary showing the distribution of X-gal-stained clones in P33 brains infected with SG-HES1 virus. The open circle represents 0.6 X-gal-stained cell per cerebrum in an average of eight examined brains. See Figure 3 for abbreviations. Bar: (a and b) 20 μm.

inhibits neural differentiation, but it is probably not the result of cell death, although the possibility that HES-1 may indirectly prevent neural differentiation by specifically killing the differentiating daughter cells is not totally excluded yet. It remains to be determined why most of the cells inhibited from neuronal and glial differentiation die *in vivo* rather than remain as undifferentiated cells. This discrepancy between the *in vivo* and *in vitro* experiments may reflect a difference in the environmental conditions, such as the level of growth factors or the difference in the stages of neural development.

When the HES-1-expressing cells survive *in vivo*, they seem to become exclusively the ependymal cells which form

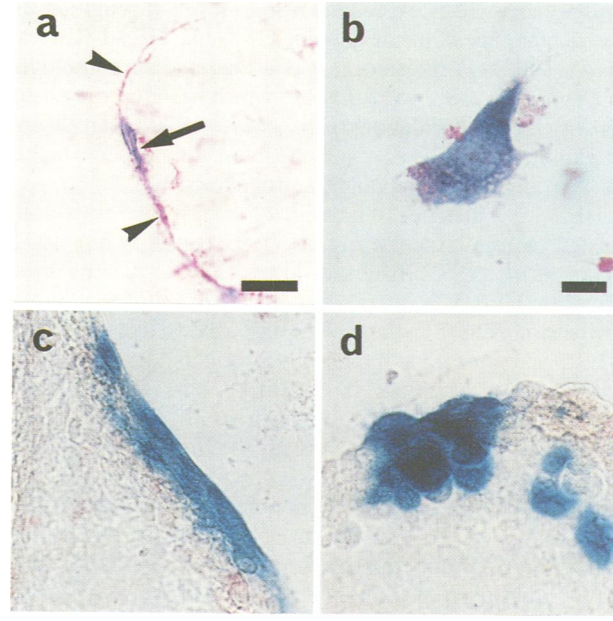

Fig. 7. Cytochemical and immunocytochemical analyses of cultured neural cells infected with SG virus (**a** and **b**) or SG-HES1 virus (**c** and **d**). (a) An X-gal-stained cell (blue-stained, arrow) with well-developed processes, which were immunoreactive for neurofilament (red-stained, arrowhead), was found in the SG virus-infected culture. (b) An X-gal-stained cell, which was immunoreactive for GFAP, was present in the SG virus-infected culture. (c and d) SG-HES1 virus-infected cells (blue-stained) were not immunoreactive for neurofilament (c) or GFAP (d). Bar (μm): (a, c and d) 20, (b) 10.

the lining of the ventricles. We do not know yet whether persistent expression of HES-1 converts neuronal- or glial-lineage cells to the ependymal cells or whether only the cells destined to be ependymal cells survive. Because relatively few labelled cells were found in the ependymal layer of both the SG virus-infected and SG-HES1 virus-infected brains, it is more likely that only the cells destined to the ependymal cell fate survive when HES-1 is expressed continuously.

Genetic evidence indicates that *Drosophila hairy* negatively regulates sensory organ formation (Moscoso del Prado and Garcia-Bellido, 1984). Thus, our data show the remarkable functional conservation of the structurally related *Drosophila* and mammalian factors, and further support the notion of the critical involvement of bHLH factors in neural differentiation.

Multiple HLH factors may be involved in differentiation of the mammalian CNS

Our data shown above are the first indication that a bHLH factor is involved in neural differentiation in the mammalian CNS. The mechanism of how HES-1 prevents mammalian neurogenesis is an interesting problem. We showed previously that HES-1 inhibits myogenesis by antagonizing the muscle determination factor MyoD (Sasai *et al.*, 1992). Furthermore, HES-1 also represses MASH-1-induced transactivation by preventing it from binding to the E box (Sasai *et al.*, 1992). We thus speculate that HES-1 inhibits neurogenesis by antagonizing the determination factors of the CNS, which probably belong to the bHLH family, although such factors have not been identified yet.

Id, a negative regulator of MyoD (Benezra *et al.*, 1990), is another HLH factor which may be involved in mammalian

neurogenesis. Id is expressed at high levels throughout the ventricular zone of the developing CNS, like HES-1 (Duncan *et al.*, 1992). Furthermore, it is a mammalian homologue of *Drosophila extramacrochaetae*, which negatively regulates sensory organ formation (Ellis *et al.*, 1990; Garrell and Modolell, 1990). Thus, it may be interesting to see whether Id also inhibits mammalian neurogenesis.

HES-1 is a member of a family of at least five structurally related factors (Akazawa *et al.*, 1992; Sasai *et al.*, 1992; Feder *et al.*, 1993; Ishibashi *et al.*, 1993). Among these proteins, HES-5, a neural-specific factor, shows a similar expression pattern in the developing CNS to that of HES-1. HES-5 is expressed at high levels throughout the ventricular zone of the developing CNS, but the level decreases as neural differentiation proceeds (Akazawa *et al.*, 1992). It also represses transcription by directly binding to the N box. However, whereas HES-1 efficiently inhibits the activity of E12/E47, mammalian bHLH factors structurally related to the product of *Drosophila* proneural gene *daughterless*, HES-5 only partially inhibits the activity of E12/E47. Thus, HES-5 may also negatively regulate differentiation in the CNS but seems to target different factors from those of HES-1.

A model for down-regulation of HES-1 expression in the course of neurogenesis and myogenesis

The mechanism of how HES-1 is repressed in the course of neural and muscle differentiation is another interesting problem. Our recent studies show that the mouse *HES-1* gene contains multiple copies of the N box in the promoter region and that *HES-1* represses its own expression by interacting with the N boxes (Takebayashi *et al.*, 1994). Thus, it is possible that the negative autoregulation plays an important role in the down-regulation of HES-1 in the course of neurogenesis and myogenesis, although we do not have any definitive evidence yet. In undifferentiated myoblasts, HES-1, like Id (Jen *et al.*, 1992), may be complexed with E12/E47 because HES-1 efficiently heterodimerizes with E12/E47 (Sasai *et al.*, 1992). When determination factors such as MyoD are induced, they form heterodimers with their co-factors E12/E47, leaving HES-1 a free molecule. This may increase the HES-1 homodimer, which represses transcription by directly binding to the N box of various genes including the *HES-1* gene itself. This is in sharp contrast to the positive autoregulation of MyoD in the course of myogenesis. We speculate that a similar mechanism may also be responsible for mammalian neurogenesis, although it is not yet known whether E12/E47 and other bHLH activators are involved in differentiation of the CNS. Further analysis of these bHLH factors will help to understand the molecular mechanisms of mammalian neurogenesis.

Materials and methods

Construction of recombinant retroviruses

For construction of SG virus, the *lacZ–neo* fusion gene was made as described previously (Friedrich and Soriano, 1991), and was replaced with the *neo* gene of LXSN (Miller and Rosman, 1989). For construction of SG-HES1 virus, the 1038 bp *SspI–DraI* fragment containing the full coding region of rat HES-1 cDNA (Sasai *et al.*, 1992) was ligated into the *XhoI* site of SG vector which is located at the junction of the truncated *gag* and SV40 promoter regions. Both viral vector DNAs were linearized with *ScaI* and transfected into $\psi 2$ cells which produce ecotropic virus. After 2 days, medium was changed to selection medium containing 0.5 mg/ml G418, and 10 days later single colonies were isolated. Supernatant recovered from a large-scale culture of each colony was concentrated by Centriprep 100 (Amicon) to obtain a titre of $\sim 1 \times 10^6$ c.f.u./ml. Only viral stocks whose helper activity was <0.01 c.f.u./injected volume (1 μl) were used for subsequent analyses.

Characterization of the retroviruses by RT-PCR

Total RNA prepared from SG virus-infected, SG-HES1 virus-infected or non-infected C3H10T1/2 cells was subjected to reverse transcription using oligo(dT) as a primer. Primers used for PCR were as follows: P1, CGGAATTCCTCTGGGGATTGAGAAGAAAG; P2, GGCGAATTCATGCCAGCTGATATAATGGAG; P3, GCGGGATCCACGCTCGGGTCTGTGCTGAGAGC. P1, a rat HES-1-specific primer, and P3 were used to detect the rat HES-1 transcript expressed by SG-HES1 virus. P2 and P3 were used to detect the endogenous mouse HES-1 transcript.

Characterization of the retroviruses by luciferase assays

Reporter plasmids contained the luciferase gene under the control of the β-actin promoter or the synthetic promoter consisting of the β-actin promoter and six repeats of the N box (Sasai *et al.*, 1992). 1×10^5 cells (C3H10T1/2) were transfected with 10 μg luciferase reporter plasmid by using the calcium phosphate co-precipitation method. After 3 h, the control medium or $\sim 1 \times 10^5$ c.f.u. SG virus or SG-HES1 virus was mixed with the cells in the presence of 8 μg/ml polybrene and incubated for 3 h. Then, three volumes of medium were added to dilute polybrene. After 2 days, cells were harvested and luciferase activity was determined, as described previously (Ow *et al.*, 1986).

Injection of virus into embryos

Pregnant ICR mice were obtained from SLC (Shizuoka, Japan). The day when the vaginal plug was detected was designated E0. Animals were operated on on E12 or E13, as described previously (Muneoka *et al.*, 1990). Anaesthesia was induced by intraperitoneal injection of Nembutal (Abbott Laboratories, North Chicago, IL) at a dose of 1.2 μl/g body weight. After midline laparotomy, the uterine wall was incised to expose embryos with extraembryonic membranes. Using a heat-pulled glass micropipette, 1.0 μl of virus solution with 0.2 mg/ml polybrene and 0.05% trypan blue was injected into one lateral ventricle. Embryos were allowed to develop *exo utero* and fostered on E19 (=P0). Those embryos developed normally.

Histochemistry

Brains were harvested on P0–4, P14 or P33 and fixed in cold 2% paraformaldehyde solution in 0.1 M PIPES buffer (pH 6.9) with 2 mM $MgCl_2$ for 6 h. Brains were then rinsed in PBS and transferred to 30% sucrose and 2 mM $MgCl_2$ in PBS. After sinking, they were embedded in OCT compound and frozen. Serial 60 μm sections were cut using a cryostat and fixed in paraformaldehyde for 5 min. They were rinsed subsequently in PBS and incubated for 12 h at 37°C in 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl β-galactopyranoside) solution, including 35 mM $K_3Fe(CN)_6$, 35 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40 as described previously (Halliday and Cepko, 1992).

Neural precursor cell culture

Neural precursor cells were isolated from E10 fetal heads, as described previously (Kitani *et al.*, 1991). Cells were seeded at 5×10^5 cells/well in 24 multiwell dishes with poly-D-lysine-coated coverslips (Becton Dickinson Labware, Bedford, MA). The culture medium was a 1:1 mixture of DME/F12 supplemented with $NaHCO_3$ (2.4 g/l), streptomycin (100 μg/ml), penicillin G (100 U/ml), fetal bovine serum (1%), transferrin (10 μg/ml), insulin (10 μg/ml), cholera toxin (10 ng/ml) and sodium selenite (1×10^{-8} M). 2 μl virus solution was added with 8 μg/ml polybrene to each well 3 h after plating. Cells were incubated for 3 h, then three volumes of the medium were added to each well. The medium was changed every other day and neural precursor cells were allowed to differentiate.

Cytochemistry and immunocytochemistry

Cells were rinsed with PBS and fixed with paraformaldehyde as described above. After permeabilization with 0.01% sodium deoxycholate and 0.02% Nonidet P-40, cells were incubated with X-gal solution for 6 h at 37°C. They were then rinsed and refixed with 4% paraformaldehyde for 30 min at 4°C. The fixed samples were next treated with mouse mAb to 160 kDa neurofilament protein (1:50; Amersham, Arlington Heights, IL) and/or glial fibrillary acidic protein (1:50; Amersham), followed by biotinylated horse antiserum to mouse IgG (Vector, Burlingame, CA), and examined with a Vectastain ABC-alkaline phosphatase detection kit (Vector).

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