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The bHLH gene *Hes6*, an inhibitor of *Hes1*, promotes neuronal differentiation

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SUMMARY

We have isolated the basic helix-loop-helix (bHLH) gene *Hes6*, a novel member of the family of mammalian homologues of *Drosophila hairy* and *Enhancer of split*. *Hes6* is expressed by both undifferentiated and differentiated cells, unlike *Hes1*, which is expressed only by the former cells. Hes6 alone does not bind to the DNA but suppresses Hes1 from repressing transcription. In addition, Hes6 suppresses Hes1 from inhibiting Mash1-E47 heterodimer and thereby enables Mash1 and E47 to upregulate transcription in the presence of Hes1. Furthermore, misexpression of *Hes6* with retrovirus in the developing retina promotes rod photoreceptor differentiation, like *Mash1*, in sharp contrast to *Hes1*, which inhibits cell differentiation. These results suggest that *Hes6* is an inhibitor of *Hes1*, supports *Mash1* activity and promotes cell differentiation. Mutation analysis revealed that Hes1- and Hes6-specific functions are, at least in part, interchangeable by alteration of the loop region, suggesting that the loop is not simply a nonfunctional spacer but plays an important role in the specific functions.

Key words: bHLH, Cell differentiation, Hes1, Hes6, Mash1, Mouse, Human

INTRODUCTION

Developmental processes of most tissues can be divided into two phases, the initial growth phase of dividing precursor cells and the subsequent differentiation phase of postmitotic cells. Proper timing from the growth phase to the differentiation phase is essential to generate the normal number of cells during development. Recent studies demonstrated that these growth and differentiation phases are controlled by positively and negatively acting transcription factors of the basic helix-loop-helix (bHLH) class (for reviews, see Weintraub et al., 1991; Anderson and Jan, 1997; Kageyama and Nakanishi, 1997; Lee, 1997). The bHLH factors such as Mash1 and MyoD form a heterodimer with the ubiquitously expressed bHLH factor E47, activate gene expression by binding to the E box (CANNTG), and promote determination and/or differentiation of neurons and muscle cells, respectively (Davis et al., 1987; Johnson et al., 1992; Guillemot et al., 1993). In contrast, the bHLH factor Hes1 inhibits the Mash1-E47 and MyoD-E47 heterodimers from binding to the E box and negatively regulates neuronal and muscle differentiation (Sasai et al., 1992; Ishibashi et al., 1994; Tomita et al., 1996). In addition, Hes1 represses *Mash1* expression by directly binding to the *Mash1* promoter (Chen et al., 1997). These positive and negative bHLH factors antagonistically regulate the differentiation process, and the balance between these two groups of factors is essential for normal morphogenesis of various tissues. For example, *Hes1*-null mutation leads to upregulation of *Mash1* expression, premature neuronal differentiation, and severe defects of the neural tube formation and eye morphogenesis (Ishibashi et al., 1995; Tomita et al., 1996; Ohtsuka et al., 1999).

In the developing nervous system *Hes1* is initially expressed by neural precursor cells in the ventricular zone, and during the differentiation process, precursors transiently coexpress *Hes1* and *Mash1*. However, differentiating neurons, which migrate out of the ventricular zone, express *Mash1* but not any more *Hes1* (Lo et al., 1991; Sasai et al., 1992; Guillemot and Joyner, 1993). It has been speculated that during this differentiation process positive bHLH factors eventually overcome the inhibitory activity of negative bHLH factors such as Hes1 by increasing their expression levels. However, a low level of *Hes1* expression is sufficient to block cellular differentiation (Ishibashi et al., 1994; Ström et al., 1997; Castella et al., 1999), and therefore the precise mechanism for the transition is not well understood.

Here, we have isolated a novel bHLH gene, designated *Hes6*, which encodes a protein that exhibits structural homology to other Hes factors, such as the conserved proline residue in the basic region and the carboxy-terminal WRPW sequence (Akazawa et al., 1992; Sasai et al., 1992; Ishibashi et al., 1993). The loop region of Hes6 is four or five amino acid residues shorter than that of the other Hes factors, however, which is a unique feature within the Hes family. *Hes6* is expressed in various regions including the nervous system during embryogenesis, and expression continues until adulthood. Surprisingly, misexpression of *Hes6* in the developing retina promotes rod photoreceptor differentiation, unlike *Hes1*. We found that Hes6 can functionally antagonize Hes1 and relieve positive bHLH factors from inhibition by Hes1, thereby

upregulating gene expression. Thus, *Hes6* may be important to promote cell differentiation by inhibiting *Hes1*. Mutational analysis revealed that the loop region is critical for the specific functions of Hes1 and Hes6.

MATERIALS AND METHODS

cDNA library screening

To clone novel *Hes*-like genes, the GenBank database was searched for expressed sequence tags (ESTs) containing sequences homologous to those of mouse *Hes1*. One mouse entry (W66929) showed a significant homology to the bHLH region of Hes1. Two primers, 5′-AGTAGTTTGCACTAGGGCGC-3′ and 5′-AGAACCTCGGCGTT-CTCTAG-3′, were designed based on the entry and used in PCR. The PCR product was used as a probe to screen about 200,000 clones of a mouse embryo (E9) cDNA library. One clone was obtained, and the gene was named *Hes6*. By searching the GenBank database for human ESTs using mouse Hes6 as a template, two EST entries for human Hes6 were found (AI417114 and AA286969).

Plasmids

The following wild-type cDNA (Hes6 and Hes1) and their mutant cDNA (Hes6ins and Hes1del) sequences were engineered in the mammalian expression vectors pCI (Promega) and pBK-CMV (Stratagene), the retroviral expression vector pCLIG (Hojo et al., 2000) and the bacterial expression vector pMNT (Sasai et al., 1992). In the case of Hes6 constructs, three copies of Myc sequences (MEQKLISEEDLNE) were tagged at the N-terminal site of Hes6.

Northern blot analysis and in situ hybridization

15 μg of total RNAs were electrophoresed on a formaldehyde/1.2% agarose gel and transferred to a nylon membrane (NEN). Hybridization was carried out as previously described (Sasai et al., 1992).

In situ hybridization analysis was performed as previously described (Takebayashi et al., 1997; Shimizu et al., 1995). Digoxigenin-labeled antisense RNA corresponding to the full-length mouse Hes6 cDNA fragment (1.3 kb) was synthesized in vitro. This probe was hybridized to whole mouse embryos and 16 μm cryostat sections of embryos and postnatal retinae.

DNA binding analysis (EMSA)

Proteins were prepared from *E. coli* by subcloning cDNA fragments of Hes6 (amino acid residues 1-224) and Hes1 (3-281) into pMNT T7 expression plasmid and overexpressing in *E. coli* (JM109[DE3]) as previously described (Sasai et al., 1992).

For the N box and E box probes, the double stranded oligonucleotide fragments containing either N box (top strand, 5′-GAAGTTTCACACGAGCCGTTCGC-3′; bottom strand, 5′-GCAC-GCGAACGGCTCGTGTGAAA-3′) present in the promoter region of the mouse *Hes1* gene or E box (top strand, 5′-GATCCA-ACACCTGCTGCCTGAG-3′; bottom strand, 5′-GATCCTCAGGC-AGCAGGTGTTG-3′) derived from the MCK enhancer sequence, respectively, were labeled with Klenow enzyme and [α-^{32}P]dCTP. Protein-DNA complexes were examined by the electrophoretic mobility-shift assay (EMSA) as previously described (Sasai et al., 1992).

Coprecipitation assay

COS-7 cells transfected with pBK-CMV, pBK-CMV-Hes1 and/or pBK-CMV-Hes6 were harvested and dissolved in lysis buffer (40 mM Tris, pH 7.4, 10 mM EDTA, 120 mM NaCl, 0.1% NP-40, 0.2 mM PMSF and 0.02 μg/μl aprotonin). Lysates were then incubated with anti-Myc agarose beads (Santa Cruz) for 12 hours with rotation at 4°C. After washing, beads were suspended in sample buffer. After boiling and centrifuge, the supernatant was subjected to western blot analysis.

Transient transfection assay

NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco/BRL). After 24 hours of subculture (1.2×10^5 cells/well), indicated reporter plasmids and/or expression vectors were mixed with an appropriate volume of FuGENE6 (Boehringer Mannheim) and added to cells in 6-well plates. Cells were harvested after 48 hours, extracts prepared, and firefly luciferase activity was measured and normalized for transfection efficiency by *Renilla luciferase* activity (Promega). The total amount of DNA added in each transfection was kept constant by addition of an empty control vector.

Retinal explant culture and retrovirus infection

Retroviral DNAs were transfected with LipofectAMINE (Gibco-BRL) into ψ2mp34, an ecotropic packaging cell line (a gift from Kazuhiro Ikenaka). The supernatant was collected 2 days later and concentrated with Centriprep 100 (Amicon), as described previously (Ishibashi et al., 1994; Tsuda et al., 1998). The retinal explant culture and retroviral infection were performed as described previously (Tomita et al., 1996). 2 weeks after infection, retinae were fixed and embedded in OCT compound (Miles), and cryosections (16 μm) were cut.

Histological analysis

For immunohistochemistry, sections were preincubated in PBS containing 5% goat serum and 0.1% Triton X-100 for 30 minutes and then incubated in 1% goat serum and 0.1% Triton X-100 with the following antibodies: rabbit anti-GFP (diluted 1:1000; Clontech), rabbit anti-Myc (1:300; MBL), mouse anti-Myc (1:400; Invitrogen), mouse anti-nestin (1:500; Pharmingen), mouse anti-HPC1 (1:200; Sigma), mouse anti-calbindin (1:200; Sigma), mouse anti-PKC (1:200; Amersham), rabbit anti-rhodopsin (1:2000; LSL) and mouse anti-glutamine synthetase (1:200; Chemicon). To detect these antibodies, biotinylated anti-rabbit antibody (1:200; Vector), FITC avidinD (1:1000; Vector), and Fluorolink Cy3-labeled goat anti-mouse antibody (1:400; Amersham) were used. Retinal cell types were determined by their morphology, location and the following antibodies: anti-HPC1 (amacrine cells), anti-PKC (bipolar cells), anti-calbindin (horizontal and amacrine cells), anti-glutamine synthetase (Müller glia), anti-rhodopsin (rods) and anti-nestin (precursor cells). Fluorescently labeled preparations were imaged using a Carl Zeiss confocal microscope.

RESULTS

Structural analysis of Hes6

To identify a new bHLH gene, we searched the EST databases and found mouse and human EST clones for a new *Hes*-related bHLH gene, designated *Hes6*. A full-length *Hes6* cDNA (1.3 kb) was then isolated from the mouse embryo cDNA library and the entire sequence was determined. Mouse and human Hes6 consist of 224 amino acid residues (Fig. 1A) and show 96% identity in the bHLH domain and 85% identity in the whole structure with each other. In addition, mouse Hes6 shows 47% identity in the bHLH domain to mouse Hes1 (Fig. 1B). Hes6 contains a proline residue in the basic region (amino acid residue 31), which is conserved among mammalian Hes (Fig. 1B) and Hairy and Enhancer of split proteins in *Drosophila*. In addition, Hes6 has a conserved WRPW sequence at the carboxyl terminus, which is known to interact with the corepressor Groucho or its mammalian homologues

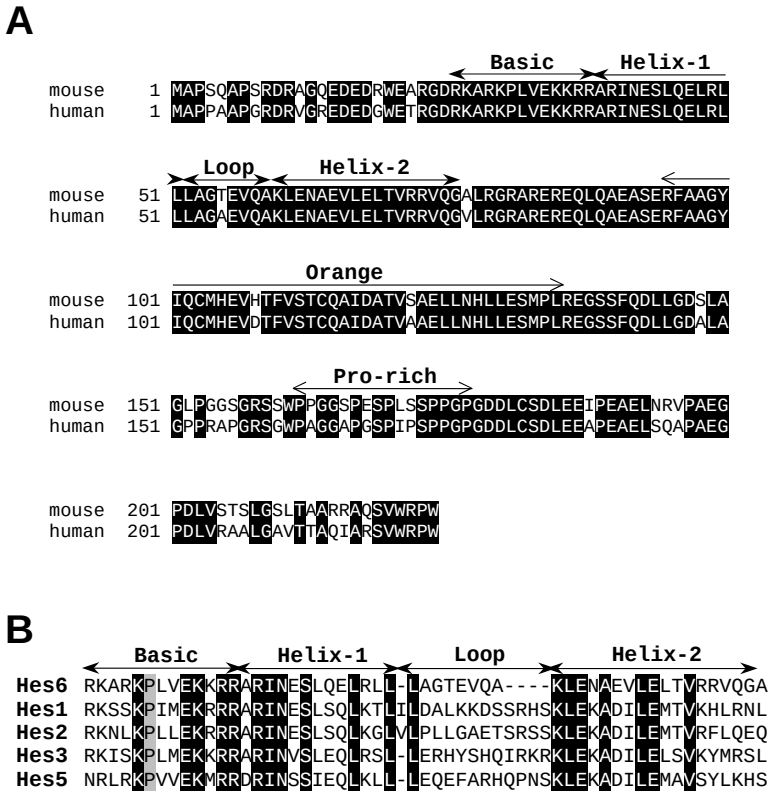

Fig. 1. Structural analysis of Hes6. (A) Deduced amino acid sequences of mouse and human Hes6. The basic region, helix-1, loop, helix-2, Orange domain and proline-rich region are indicated. Identical residues between mouse and human are boxed. Accession numbers of Hes6: AB035178 (mouse) and AB035179 (human). (B) Comparison of the bHLH domain of mouse Hes factors. Conserved residues, including the proline residue in the basic region, are boxed. The loop of Hes6 is four or five amino acid residues shorter than those of other Hes factors.

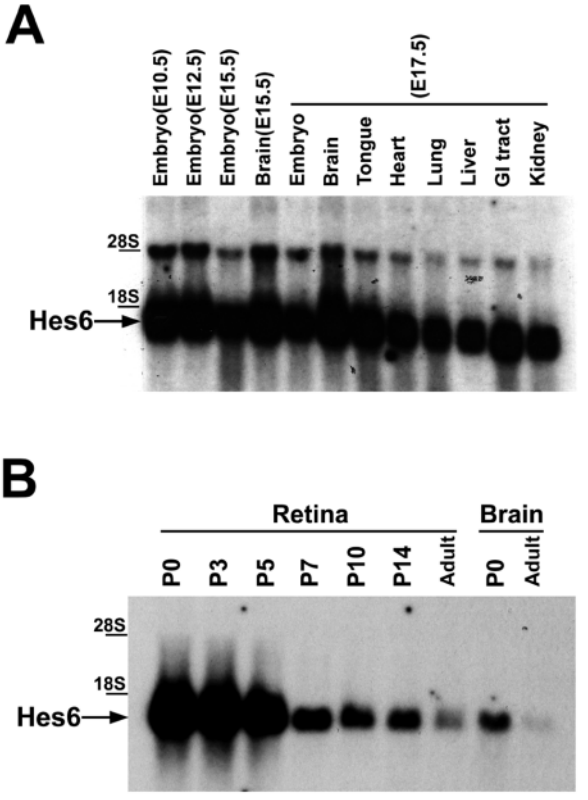

Fig. 2. Northern blot analysis. (A) *Hes6* expression in embryos. A high level of expression was observed from E10.5 onward. At E17.5, *Hes6* was expressed in all the tissues that we examined. (B) *Hes6* expression in the retina and brain. *Hes6* expression was reduced but continued postnatally until adulthood.

(Paroush et al., 1994; Fisher et al., 1996; Grbavec and Stifani, 1996). Like other Hes factors, Hes6 has a proline-rich region (from amino acids 162-177) and an Orange domain (from amino acids 95-135), which confers specificity for protein interaction (Dawson et al., 1995). Thus, Hes6 has a structure that is generally conserved in the Hes family; however, the loop region of Hes6 is five amino acid residues shorter than Hes1 and Hes2 and four residues shorter than Hes3 and Hes5, which is a unique feature in the Hes family (Fig. 1B).

Spatiotemporal expression

Hes6 expression in mice was first determined by northern blot analysis. *Hes6* mRNA was detected in both embryos and adults (Fig. 2A,B). A high level of *Hes6* expression was detected from embryonic day (E) 10.5 onward. At E17.5, *Hes6* expression was observed in all the tissues that we examined (Fig. 2A), suggesting that *Hes6* may be involved in differentiation of almost all tissues. In the retina, *Hes6* was expressed at a high level at postnatal day (P) 0. The expression in the retina as well as in the brain decreased postnatally but was still detectable in the adult (Fig. 2B). The mRNA size was approximately 1.4 kb, which was close to the cDNA size that we had obtained (1.3 kb).

To analyze *Hes6* expression more precisely, in situ hybridization of mouse tissues was performed. *Hes6* expression was first detected at E8.5 (Fig. 3A). Expression was initially restricted to the forebrain and the optic vesicles (Fig. 3A), but soon extended to the midbrain during closure of the anterior neuropore (Fig. 3B). By E9.5, the expression further extended to the posterior part of the neural tube and was also observed in the cranial ganglia (Fig. 3C). At E10.5, *Hes6* was expressed mainly in the wall of brain vesicles (Fig. 3D). At this stage, *Hes6* was expressed in most of the nervous system including the dorsal root ganglia (Fig. 3E). By E11.5, *Hes6* expression occurred not only in the nervous system but also in the non-neural tissues (Fig. 3F), agreeing well with the results of the northern blot analysis (Fig. 2).

At E12.5, *Hes6* was expressed at a high level throughout the developing nervous system, including the telencephalon and diencephalon (Fig. 4A). In the forebrain, a high level of expression was observed in the ventricular zone, the innermost layer which contains dividing neural precursor cells (Fig. 4A,D). At E15.5, *Hes6* expression was now observed in the cortical plate as well as in the ventricular zone (Fig. 4B,E), suggesting that *Hes6* is expressed by both precursor cells and neurons, unlike *Hes1*, which is only expressed by precursor cells in the ventricular zone. At E17.5, *Hes6* was still expressed at a high level both in the ventricular zone and in the outer

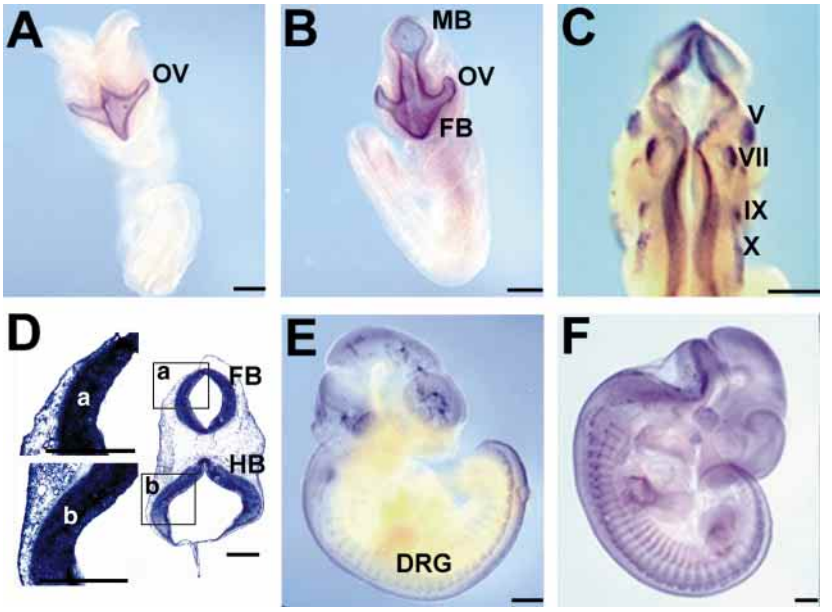

Fig. 3. In situ hybridization analysis of E8.5-E11.5 embryos. In situ hybridization was performed with a digoxigenin-labeled *Hes6* antisense riboprobe. (A) At E8.5, *Hes6* expression was restricted to the forebrain and optic vesicles (OV). (B) At E9.0, *Hes6* expression extended to the midbrain (MB) during closure of the anterior neuropore. (C) At E9.5, *Hes6* expression not only extended to the further posterior region of the neural tube but also occurred in the cranial ganglia: the trigeminal (V), geniculate (VII), petrosal (IX) and nodose ganglia (X). (D) At E10.5, *Hes6* was mainly expressed in the wall of brain vesicles. The left panels show a higher magnification of the boxed areas. (E) At E10.5, *Hes6* was expressed throughout the nervous system including the dorsal root ganglia (DRG). (F) At E11.5, *Hes6* expression occurred in the non-neural tissues as well as in the nervous system. FB, forebrain. HB, hindbrain. Bars, 100 μm (A,B); 200 μm (C); 500 μm (D-F).

layers (Fig. 4C,F). In the spinal cord and dorsal root ganglia, a high level of *Hes6* expression was observed at E12.5 and E15.5 (Fig. 4G,H) but the expression became weaker at E17.5 (Fig. 4I). *Hes6* expression in the olfactory epithelium was also detectable from E12.5 onward (Fig. 4J-L).

In the eye, *Hes6* expression started at E8.5 (Fig. 3A) and continued in the lens and neural retina during embryogenesis (Fig. 4M-O). At P0, the neural retina consists of two layers: the ganglion cell layer, which contains projection neurons, and the ventricular zone, which contains undifferentiated cells; *Hes6* was expressed in both layers (Fig. 4P). Cells in the ventricular zone differentiate postnatally and form two layers, the inner and outer nuclear layers. The outer nuclear layer contains rod and cone photoreceptors and the inner nuclear layer contains interneurons and glial cells. *Hes6* was expressed mainly in the ganglion and inner nuclear layers of the neural retina at P10 and P14 (Fig. 4Q,R). These results demonstrated that *Hes6* is expressed by both undifferentiated and differentiated cells in the retina, raising the possibility that *Hes6* has a function distinct from *Hes1* in development.

DNA-binding and coprecipitation analysis

The spatiotemporal expression patterns suggest that *Hes6* has a function distinct from *Hes1* in spite of their structural conservation. To compare the DNA-binding activity of Hes1 and Hes6, both proteins were expressed in *E. coli* and subjected to electrophoresis mobility-shift assay (EMSA). As previously described (Sasai et al., 1992), Hes1 bound to the N box (CACNAG) with a high affinity (Fig. 5A, lane 2) and to the E box (CANNTG) with a low affinity (Fig. 5B, lane 2), in contrast to most other bHLH factors, which bind only to the E box. The EMSA demonstrated that Hes6 did not bind to the N box or to the E box, unlike Hes1 (Fig. 5A, lanes 3-5 and B, lanes 3-5). These results suggest that Hes6 alone cannot bind to the DNA, although it is possible that Hes6 may bind to as-yet-unidentified sequences. Interestingly, when Hes1 and Hes6 were mixed together with the N box probe, an additional band migrated at a higher position (Fig. 5A, lanes 6-8, arrow) than the Hes1 homodimer (arrowhead). The appearance of the new band correlated with the increase of Hes6 input (Fig. 5A, lanes 6-8). Therefore, it is likely that this band was the result of complex formation of Hes1 and Hes6, although this interaction seemed to be weaker than Hes1 homodimer since the band was not detectable when equal amounts of Hes1 and Hes6 were mixed (lane 6). The N box-specific competitor DNA competed well with both bands (Fig. 5A, lane 9), indicating that the binding was specific to the N box. When Hes1 and Hes6 were mixed together with the E box probe, no new band was detected but the binding of Hes1 homodimer decreased (Fig. 5B, lanes 6-8), indicating that Hes6 suppressed the E box binding of Hes1. These results demonstrated that Hes6 influenced both the N box and E box binding of Hes1.

To show decisively that Hes6 and Hes1 associate with each other, we next performed coprecipitation assays. COS-7 cells were transfected with plasmids for Hes1 and Myc-Hes6, which had three repeats of Myc epitope tag at the amino terminus. 2 days after transfection, cell lysates were prepared. Western blot analysis of the cell lysates using anti-Hes1 and anti-Myc antibodies revealed that both Hes1 and Myc-Hes6 were expressed at similar levels (Fig. 4C, lanes 2-4). The lysates were next precipitated by the beads conjugated with anti-Myc antibody and subjected to western blot analysis with anti-Hes1 antibody. This analysis clearly showed that Hes1 was coprecipitated with Hes6 (lane 7), indicating that Hes1 and Hes6 physically associate with each other.

Transcriptional analysis

To analyze the transcriptional activity of Hes6, transient transfection analysis was performed. The luciferase reporter gene under the control of the N box-containing promoter was coexpressed with Hes6 and Hes1. As previously reported (Sasai et al., 1992), Hes1 repressed transcription (approx. threefold) from the N box-containing promoter (Fig. 6A, lane 2). In contrast, Hes6 did not repress or activate transcription from the N box-containing promoter (lane 4), in agreement with the above observation that Hes6 alone did not interact

with the N box. However, when Hes1 and Hes6 were coexpressed, Hes1-induced transcriptional repression was abolished (lane 3). Thus, Hes6 suppressed the N box-dependent transcriptional repression by Hes1.

When the luciferase reporter gene under the control of the E box-containing promoter was used, coexpression of Mash1 and E47 upregulated the transcription (approx. fivefold), as previously described (Johnson et al., 1992) (Fig. 6B, lane 2). Although Hes1 inhibited Mash1-E47-induced transcription (lane 3), Hes6 did not (lane 5). Or rather, Hes6 activated the Hes1-repressed Mash1-E47-dependent transcription (lane 4). Thus, Hes6 suppressed Hes1 from inhibiting Mash1-E47 heterodimer and enabled Mash1 and E47 to activate transcription. These results demonstrated that Hes6 functions as an inhibitor of Hes1 in transcription.

Hes6 in neural development

The transcriptional analysis suggested that Hes6 reverses Hes1 activity and supports Mash1-induced transcriptional activation. To more clearly define the Hes6 function in neural development, *Hes6*, *Mash1* and *Hes1* were misexpressed with retrovirus in the developing mouse retina and the fate of the virus-infected cells was determined. For the misexpression study, we used the replication-incompetent retrovirus CLIG, which directs expression of a test gene and green fluorescent protein (GFP) as a marker (Fig. 7A) (Hojo et al., 2000). Retinal explants, which mimic well the in vivo retinal development (Tomita et al., 1996), were prepared from mouse embryos at E17.5 and virus was infected on the same day (approx. E18.0). 2 weeks later, by which time most retinal cells had finished differentiation, the fate of the virus-infected cells was examined by monitoring GFP^+ cells. In the retina there are six types of neurons and one type of glial cells, which all differentiate from common precursors but with a different time

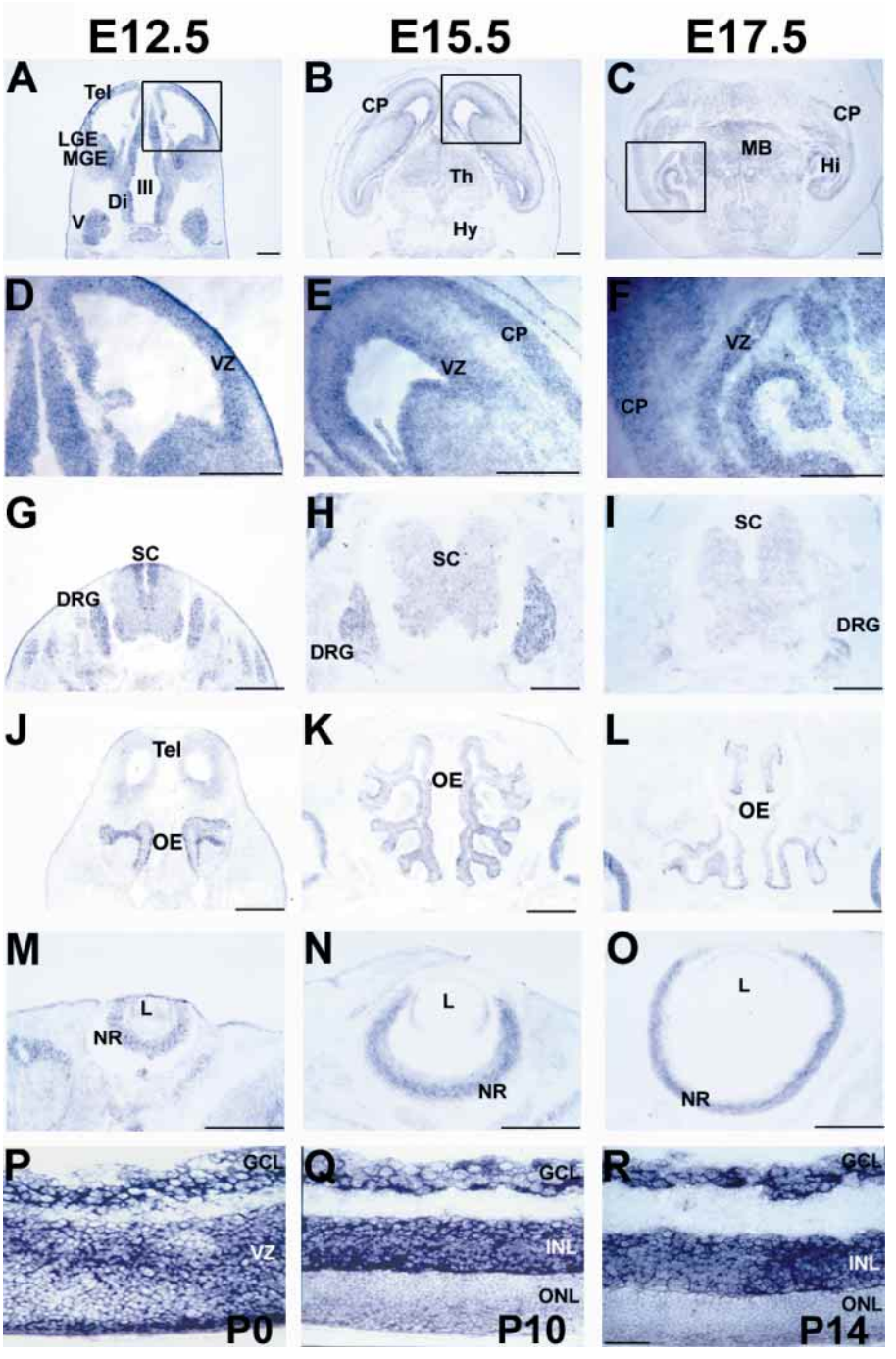

Fig. 4. In situ hybridization analysis of E12.5 (A,D,G,J,M), E15.5 (B,E,H,K,N) and E17.5 (C,F,I,L,O). (A-C) Brain sections. (A) *Hes6* was expressed in the ventricular zone, facing the ventricles of the telencephalon (Tel) and diencephalon (Di). It was also expressed in the outer layers. (B) *Hes6* was expressed in the cortical plate (CP) as well as in the ventricular zone. Strong expression was also observed in the thalamus (Th) and hypothalamus (Hy). (C) *Hes6* was still expressed at a high level in the brain. (D-F) A higher magnification of the boxed regions shown in A-C. Both precursor cells in the ventricular zone (VZ) and differentiating cells in the outer layers expressed *Hes6*. (G-I) Sections of the spinal cord (SC) and dorsal root ganglia (DRG). *Hes6* expression in the spinal cord and dorsal root ganglia was observed at a high level at E12.5 and E15.5 but became weaker at E17.5. (J-L) Sections of the olfactory epithelium. *Hes6* expression occurred from E12.5 onward. At E17.5, *Hes6* was expressed in a stripe pattern. (M-O) Eye sections. *Hes6* was expressed in both the lens (L) and neural retina (NR). (P) P0 retina. *Hes6* was expressed in the ventricular zone (VZ) and the ganglion cell layer (GCL). (Q,R) P10 and P14 retina. *Hes6* was expressed mainly in the GCL and inner nuclear layer (INL). Hi, hippocampus; LGE, lateral ganglionic eminence; MB, midbrain; MGE, medial ganglionic eminence; ONL, outer nuclear layer; III, the third ventricle; V, trigeminal ganglion. Bars, 500 μm (A-O); 25 μm (P-R).

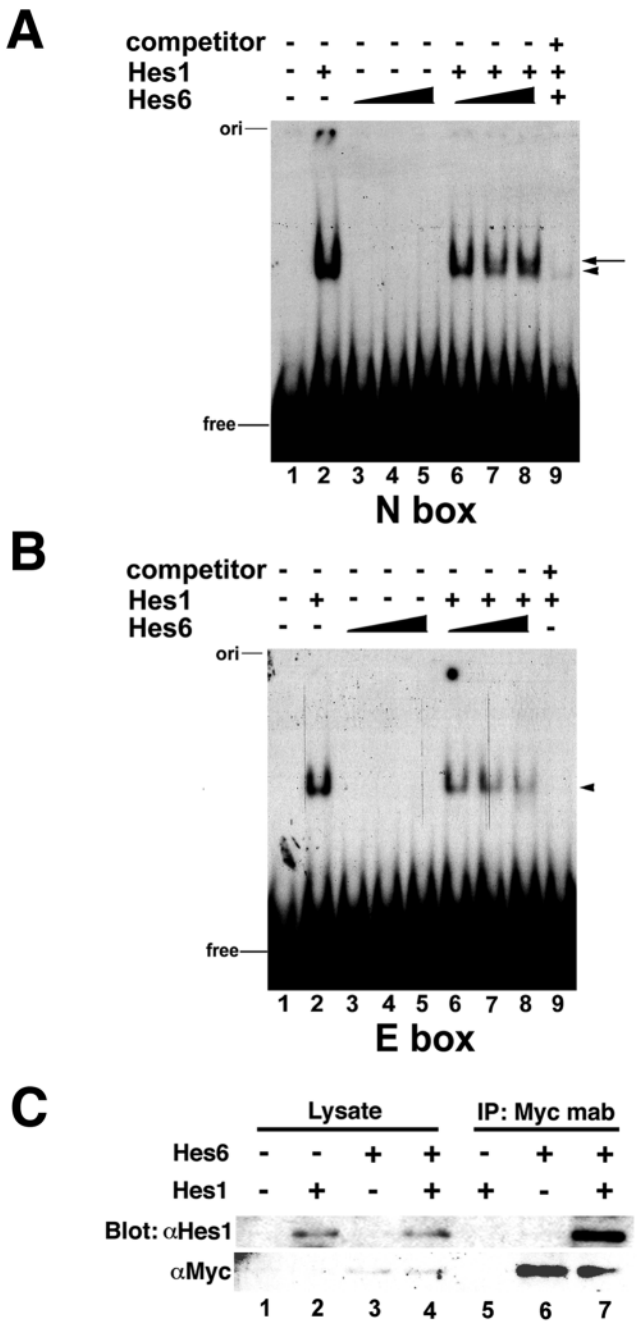

Fig. 5. DNA-binding and protein interaction. (A,B) Hes6 and Hes1 proteins were expressed in *E. coli* and subjected to EMSA. (A) The N box oligonucleotide probe (20 fmol) was used. The amounts of the proteins used are summarised above and were as follows: Hes1, 50 ng (lanes 2, 6-9) and Hes6, 50 ng (lanes 3 and 6), 150 ng (lanes 4 and 7) and 300 ng (lanes 5, 8 and 9), in a total volume of 20 μl. When Hes1 and Hes6 were mixed, a new band (arrow) was detectable, in addition to the Hes1 homodimer band (arrowhead). The competition experiment was performed with 10 pmol of the cold N box oligonucleotide probe (lane 9). (B) The E box oligonucleotide probe (20 fmol) was used. The amounts of the proteins used were the same as in A. Hes1 binding (arrowhead) was reduced in the presence of Hes6. The competition experiment was performed with 10 pmol of the cold E box oligonucleotide probe (lane 9). (C) Coprecipitation assay. Lysates were prepared from COS-7 cells transfected with expression plasmids for Hes1 and/or Myc-Hes6, as indicated above each lane. The lysates were, directly (lanes 1-4) or after immunoprecipitation with anti-Myc antibody (lanes 5-7), subjected to western blot analysis using anti-Hes1 (top) and anti-Myc (bottom) antibodies. Hes1 was coprecipitated with Hes6 (lane 7), indicating that Hes1 and Hes6 associate with each other.

course. The birthdate is crucial for the choice of cell fate and the most preferred cell fate at E18, when virus was applied, is the rod photoreceptor (Cepko et al., 1996). When cells were infected with the control virus CLIG, approximately 80% of the virus-infected cells became rods in the outer nuclear layer (Fig. 7B,M), and more than 10% of the virus-infected cells became bipolar cells or Müller glial cells in the inner nuclear layer (Fig. 7B,M). When CLIG-Mash1 virus was used, almost all of the virus-infected cells became rods (Fig. 7C,M). Thus, misexpression of *Mash1* at E18 exclusively promoted differentiation of rods, the most preferred cell fate at this stage. In contrast, when CLIG-Hes1 virus was used, nearly 50% of the virus-infected cells remained at the outermost layer of the neural retina (Fig. 7J). Furthermore, more than 85% of the virus-infected cells, including those migrated, expressed the progenitor-specific intermediate filament nestin (Fig. 7J-L), indicating that the majority of *Hes1*-expressing cells were inhibited from differentiation (Fig. 7M). However, the average clonal size of CLIG-Hes1-infected cells was approximately 2.0, which was almost the same as that of CLIG- or CLIG-Mash1-infected cells (average: 1.5-2.0), suggesting that *Hes1*-expressing cells did not proliferate although they were inhibited from differentiation. When CLIG-Hes6 virus was used, almost all of the virus-infected cells became rods in the outer nuclear layer (Fig. 7D,M). To verify the coexpression of *Hes6*, the Myc epitope was fused with Hes6, and we found that more than 90% of the GFP^+ cells were positive for Myc expression (Fig. 7D-F). None of the *Hes6*-expressing cells (Myc^+) remained as nestin-positive precursors (Fig. 7G-I). The intact Hes6 (without Myc tag) was also misexpressed and the same results were obtained (data not shown). The average clonal size was approximately 2.0, suggesting that *Hes6* did not induce cell proliferation. Thus, *Hes6* did not inhibit but promoted rod differentiation in the retina, like *Mash1*.

Alteration of the loop region

Whereas Hes6 and Hes1 have a conserved structure, their activities were totally different. We next explored the region responsible for the functional difference between Hes6 and Hes1. Among the conserved and functionally important regions (the bHLH, Orange and WRPW domains), one of the most prominent structural differences between the two Hes factors is their loop region, which is five amino acid residues shorter in Hes6 (Fig. 1B). We therefore generated two constructs by changing the loop region of Hes6 and Hes1: Hes6ins, which has a five amino acid insertion in the loop of Hes6, and Hes1del, which has a five amino acid deletion in the loop of Hes1 (Fig. 8A). The transcriptional activities of these mutant constructs were examined by a transient transfection assay. When the luciferase gene under the control of the N box-containing promoter was used, Hes6ins repressed transcription, as did Hes1 (Fig. 8B, lane 2). Thus, insertion of five amino acid residues in the loop conferred the N box-dependent repression activity on Hes6. Conversely, Hes1del did not repress

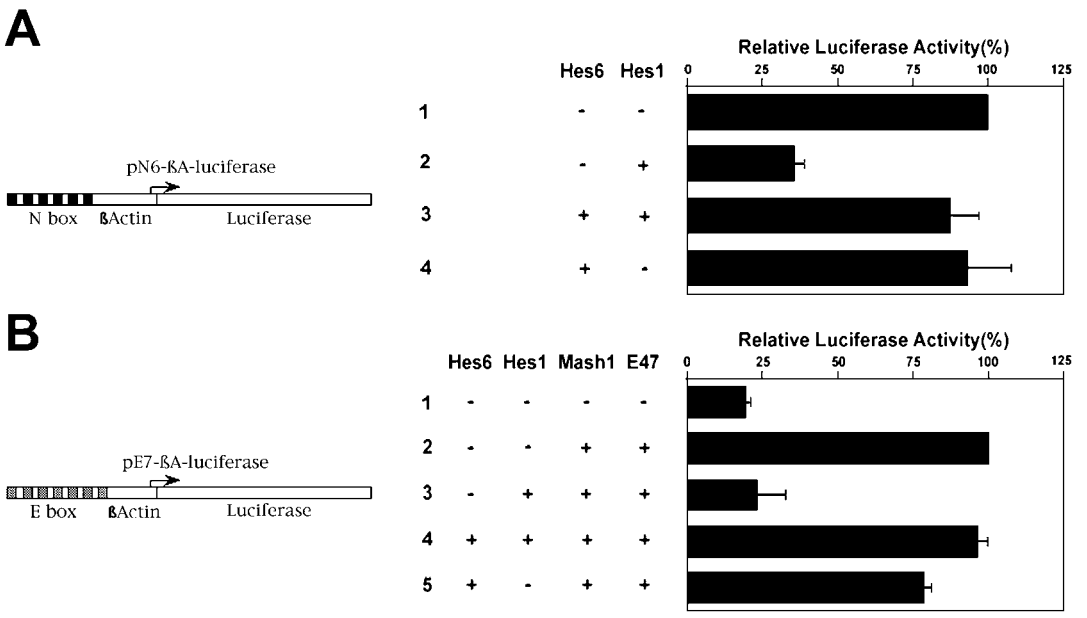

Fig. 6. Transcriptional analysis. The luciferase reporter and expression vectors were transiently transfected into NIH3T3 cells and after 48 hours the luciferase activities were determined. Values are mean + s.d. of the relative activities in at least three independent experiments performed in duplicate. (A) The luciferase reporter under the control of the N box-containing promoter (0.1 μg) was cotransfected with Hes1 (0.6 μg) and/or Hes6 expression vectors (1.8 μg), as indicated on the left. While Hes1 repressed transcription (lane 2), Hes6 did not (lane 4). When Hes1 and Hes6 were coexpressed, Hes6 suppressed Hes1-induced repression (lane 3). (B) The luciferase reporter under the control of the E box-containing promoter (0.05 μg) was cotransfected with the expression vectors (E47, 0.2 μg; Mash1, 0.2 μg; Hes1, 0.8 μg; Hes6, 2.4 μg), as indicated on the left. Hes6 suppressed Hes1 from inhibiting Mash1 and E47 (lane 4).

transcription, as did Hes6 (Fig. 8B, lane 3). Thus, deletion of five amino acid residues in the loop completely abolished the N box-dependent repression activity of Hes1. These results indicated that the loop region was critical for specific activities of Hes6 and Hes1 and that some of these specific activities were interchangeable by alteration of the loop region. The transcriptional activities of these Hes mutants against the Mash1-E47 complex were also examined. In spite of acquisition of the N box-dependent repression activity, Hes6ins only weakly inhibited Mash1-E47-induced transcription (Fig. 8C, lane 3). Thus, insertion of just five amino acid residues in the loop was not sufficient to confer full activities, indicating that additional changes are required for the full inhibitory activity to Mash1 and E47. In contrast, Hes1del did not inhibit but rather augmented Mash1-E47 activity (lane 4). In addition, this mutant suppressed Hes1 and enabled Mash1-E47 complex to activate transcription, like Hes6 (compare lanes 5 and 7). Thus, deletion of just five amino acids was sufficient for functional conversion of Hes1 into Hes6.

The effect of the loop alteration was further examined by using retinal explant cultures. Misexpression of Hes6ins with retrovirus did not inhibit differentiation but generated many rods (Fig. 9A,J). In addition, none of the virus-infected cells expressed nestin (Fig. 9A-C). Thus, Hes6ins failed to exhibit the Hes1-like inhibitory activity for neuronal differentiation. This failure may reflect the insufficient activity of Hes6ins to inhibit Mash1-E47 (Fig. 8C, lane 3). In contrast, misexpression of Hes1del generated many rods (nearly 80%) and did not inhibit neuronal differentiation (Fig. 9D,J). None of the virus-infected cells expressed nestin (Fig. 9D-F). Thus, a five-amino-acid deletion from the loop region completely abolished Hes1-specific inhibitory activity and conferred Hes6-like activity. Interestingly, about 20% of the virus-infected cells became Müller glial cells, which expressed glutamine synthetase (GS) (Fig. 9G-I, arrowheads, and J), suggesting that Hes1del was functionally similar to but still different from Hes6. These results demonstrated that, although the alteration of just five amino acid residues is not sufficient to interchange all activities, the loop region is not a nonfunctional spacer but is critical for specific Hes activities.

DISCUSSION

Hes6 suppresses Hes1 and promotes cell differentiation

The differentiation process is antagonistically controlled by positive and negative bHLH factors. During this process the positive regulator Mash1 and the negative regulator Hes1 are transiently coexpressed in neural precursor cells, but it is not well understood how Mash1 eventually overcomes inhibition by Hes1, since a low level of Hes1 expression is sufficient to block neuronal differentiation. In this study, we showed that Hes6 suppresses Hes1, supports Mash1 activity and promotes cell differentiation. It has been shown that Hes1 represses transcription by two different mechanisms: repression by directly binding to the N box and dominant-negative effects on bHLH activators such as Mash1 (Sasai et al., 1992). We here show that Hes6 can suppress both activities of Hes1 and enable Mash1 and E47 to activate transcription in the presence of Hes1. Thus, it is likely that Hes6 may promote cell differentiation by suppressing Hes1 and supporting positive

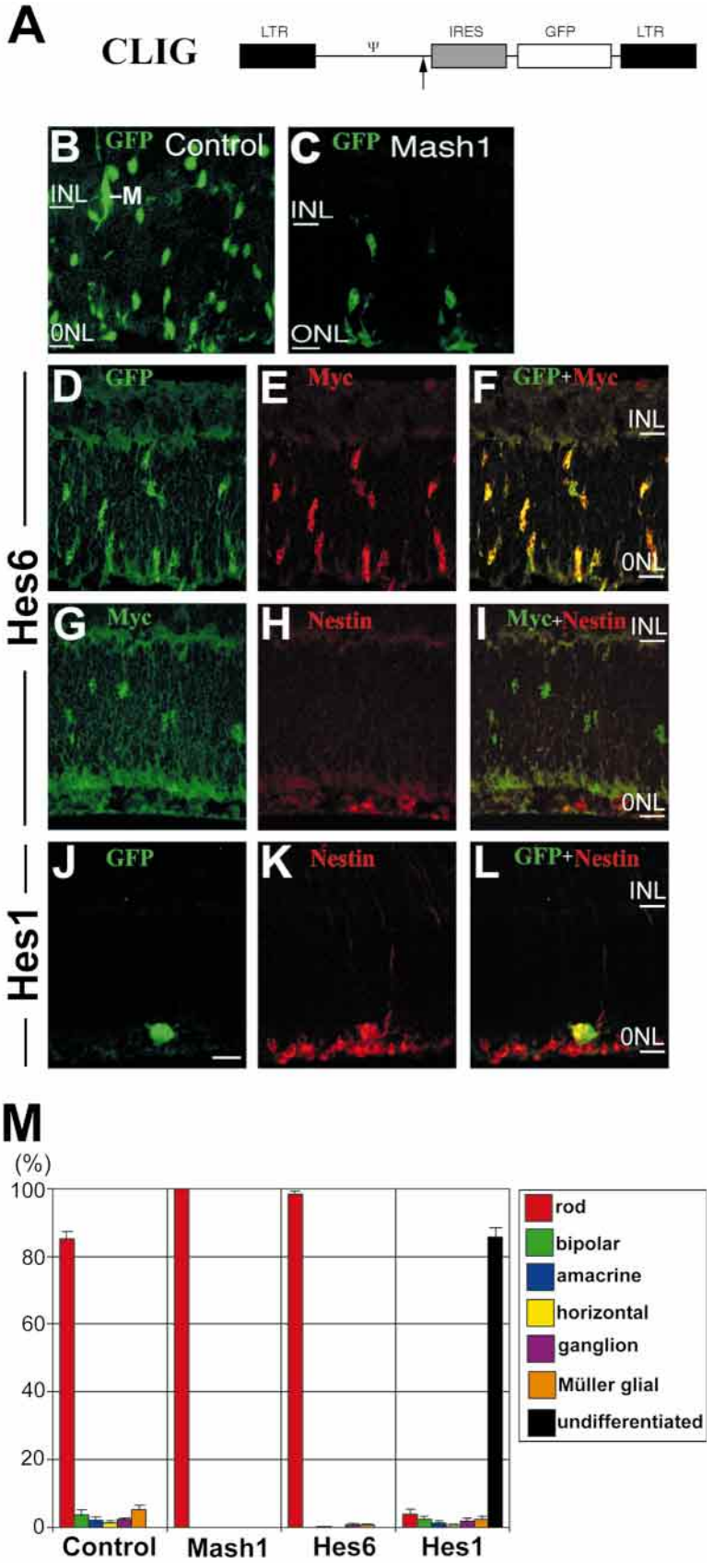

Fig. 7. Retinal explant cultures infected with *Hes6*-transducing retrovirus. Retinal explants were prepared from E17.5 embryos and recombinant retrovirus was infected on the same day (approx. E18). After 2 weeks of culture, the explants were subjected to immunohistochemistry. (A) Schematic structures of the recombinant retrovirus CLIG. The position of the test gene is indicated by an arrow. The internal ribosomal entry sequence (IRES) allows bicistronic expression. LTR, long terminal repeat; GFP, green fluorescent protein. (B) Infection with the control virus CLIG. About 80% of the virus-infected cells (GFP^+) became rods in the outer nuclear layer (ONL). Some of them migrated into the inner nuclear layer (INL). M, Müller glial cell. (C) Infection with CLIG-Mash1. Almost all of the virus-infected cells became rods in the ONL. (D-I) Infection with CLIG-Hes6. Here, Hes6 fused with the Myc epitope tag was expressed. This virus directed coexpression of GFP (D,F, green) and Myc (E,F, red). Almost all of the virus-infected cells became rods. None of the Hes6-expressing cells (G,I, green) expressed the precursor-specific intermediate filament nestin (H,I, red). (J-L) Infection with CLIG-Hes1. A cluster of three virus-infected cells remained at the outermost layer of the neural retina. These cells expressed nestin. Thus, these Hes1-expressing cells were inhibited from differentiation. Bar, 20 μm. (M) Percentages of types of cells produced in cultures of the virus-infected cells (means + s.e.m.). More than 200 cells in at least three independent retinal explants were examined.

bHLH factors. In agreement with this hypothesis, misexpression of *Hes6* in the developing retina promoted differentiation of rods, which were the most preferred cell type at the stage examined, and this activity is very similar to that of *Mash1* but totally different from that of *Hes1*. Nevertheless, it is still possible that *Hes6* and *Mash1* independently promote cell differentiation, and therefore it remains to be determined whether *Mash1*-induced cell differentiation really depends upon *Hes6*. Analysis of *Hes6* knock-out mice is required to formally answer this question.

While Hes6 promotes cell differentiation, it is not yet clear how the timing of differentiation is regulated. One possibility is that, when expression of *Hes6* is induced in *Hes1*-expressing precursor cells, then Hes6 may suppress the Hes1 activity and thereby enable *Mash1* expression to start, and this induces the differentiation program. In this case, the onset of *Mash1* expression is the initiation of differentiation. Alternatively, precursor cells may initially express both *Hes1* and *Mash1*, but they cannot differentiate since Mash1 is inhibited by Hes1. When *Hes6* expression is induced, then Hes1 is suppressed and Mash1 may start cell differentiation. In this case, the onset of *Hes6* expression is the initiation of differentiation. From the present expression study, it is difficult to tell which model is likely. Analysis at the single cell level may be required to understand this problem.

Since Hes1 is an important Notch effector (Ohtsuka et al., 1999), our results raise the possibility that Hes6 may be involved in regulation of the Notch pathway. Notch is a transmembrane protein and plays an essential role in the differentiation of a variety of cell types (Dorsky et al., 1997). Upon activation by Notch ligands,

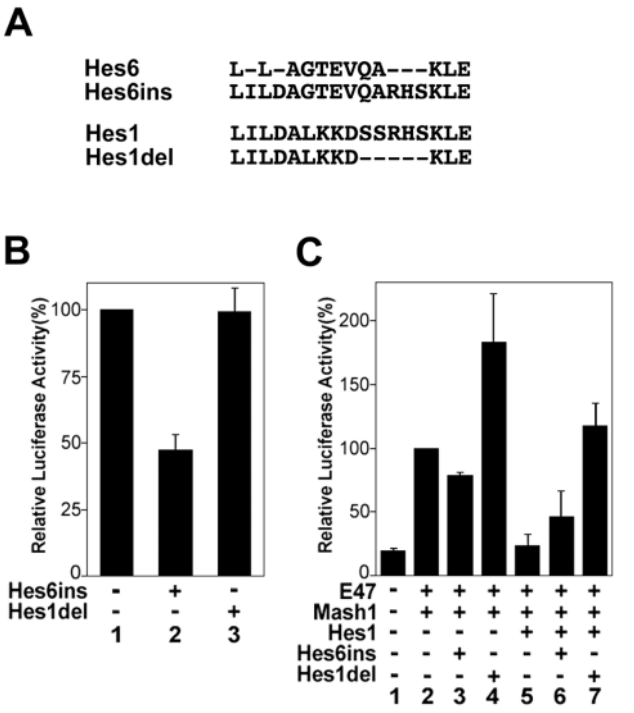

Fig. 8. Transcriptional analysis of Hes mutants. (A) The loop structure of Hes6, Hes1 and their mutants, Hes6ins and Hes1del. (B) The luciferase reporter under the control of the N box-containing promoter (0.1 μg) was cotransfected with Hes6ins (0.6 μg) or Hes1del expression vector (1.8 μg). (C) The luciferase reporter under the control of the E box-containing promoter (0.05 μg) was cotransfected with various expression vectors (E47, 0.2 μg; Mash1, 0.2 μg; Hes1, 0.8 μg; Hes1del, 2.4 μg; Hes6ins, 0.8 μg), as indicated below. Values are means + s.d. of the relative activities in at least three independent experiments performed in duplicate.

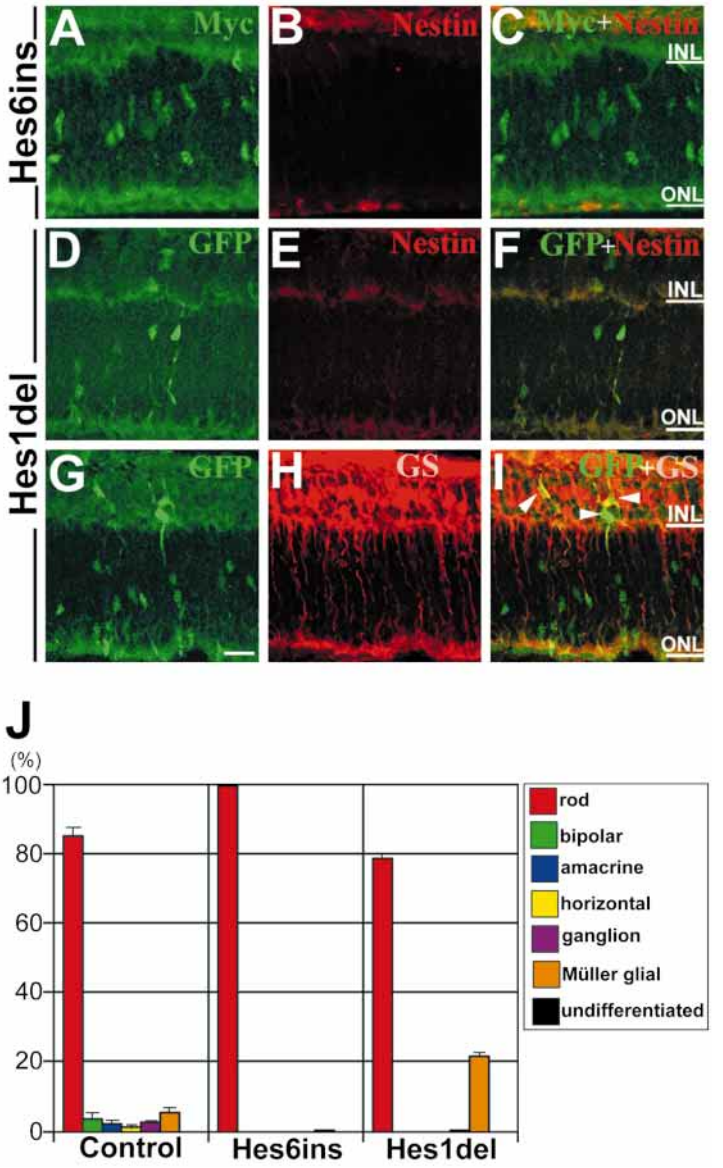

Fig. 9. Misexpression of Hes mutants in retinal explant cultures. Retinal explants were prepared from E17.5 embryos, and infected with recombinant retroviruses (CLIG-Hes6ins and CLIG-Hes1del) on the same day. After 2 weeks of culture, the explants were subjected to immunohistochemistry. (A-C) Infection with CLIG-Hes6ins. Since Hes6ins was fused with the Myc epitope tag, the virus-infected cells were identified as Myc^+ cells. Almost all of the virus-infected cells became rods. None of them expressed nestin. (D-I) Infection with CLIG-Hes1del. Nearly 80% of the virus-infected cells (GFP^+) became rods (D) and about 20% became Müller glial cells (G-I), which expressed the glial marker glutamine synthetase (GS), as indicated by arrowheads (I). None of the CLIG-Hes1del-infected cells expressed nestin (D-F). Bar, 20 μm. (J) Percentages of types of cells produced in cultures of the virus-infected cells (means + s.e.m.). More than 200 cells in at least two independent retinal explants were examined.

Notch is processed to release its intracellular domain (ICD), which then moves into the nucleus (Artavanis-Tsakonas et al., 1999; Honjo, 1996). In the nucleus, ICD forms a complex with RBP-J, a mammalian homologue of *Drosophila* Suppressor of Hairless [Su(H)], and this complex induces *Hes1* expression (Jarriault et al., 1995; Nishimura et al., 1998), thereby inhibiting cell differentiation. It has been shown that this pathway is regulated at multiple steps. For example, Hairless forms a complex with and inhibits Su(H) and dominant-negatively regulates the Notch pathway in *Drosophila* (Bang et al., 1994; Brou et al., 1994). Since Hes6 suppresses Hes1, it is possible that Hes6 may also dominant-negatively regulate the Notch pathway like Hairless.

Hes6 expression continues in differentiated cells, which do not express *Hes1*. Adult tissues, including the brain, still express *Hes6* although at a much reduced level than embryos. This expression pattern suggests that *Hes6* may also function in mature cells in addition to undifferentiated cells. Since some neuronal bHLH genes such as *NeuroD* and *Math2*/*NEX-1* are expressed by mature neurons (Bartholomä and Nave, 1994; Lee et al., 1995; Shimizu et al., 1995; Schwab et al., 1998), it is possible that Hes6 may support the functions of these bHLH factors.

The mechanism of suppression of Hes1 by Hes6

Hes6 alone does not bind to the N box or E box sequences but interferes with the E box binding of Hes1. This activity is similar to the helix-loop-helix (HLH) factor Id1, a dominant-negative regulator of bHLH factors (Benezra et al., 1990).

Since Id1 lacks the basic region, it cannot bind to the DNA by itself. However, it forms a heterodimer complex with other bHLH factors through the HLH domain and interferes with their DNA binding. It is possible that Hes6 interferes with the E box binding of Hes1 in a similar manner.

Regarding the N box-dependent suppression, the mechanism seems more complex. Whereas Hes6 does not abrogate the N box binding of Hes1, it suppresses Hes1 from N box-dependent transcriptional repression. Hes1 and Hes6 associate with each other and this complex may be transcriptionally inactive. However, even if this is the case, it still remains to be determined why the Hes1-Hes6 complex cannot repress transcription, since both have the repression domain (WRPW). One possible mechanism is that the carboxy-terminal WRPW regions are not properly arranged in the Hes1-Hes6 complex so that the corepressors (Groucho homologues) cannot interact. Another possibility is that the loop of Hes1 but not of Hes6 could interact with an as-yet-unidentified cofactor of transcriptional repression, since just a five-amino-acid insertion into the loop conferred the repressor activity on Hes6. It is also possible that Hes6 may inhibit the repression activity of Hes1 by sequestering Groucho homologues.

The loop region is essential for proper functions of Hes

In the Hes family, Hes6 is unique since it suppresses Hes1 and promotes cell differentiation. This uniqueness partly resides in the loop region; the loop of Hes6 is four or five amino acid residues shorter than that of other Hes factors. In addition, deletion of five amino acid residues from the loop of Hes1 completely abolishes the repression activity and confers Hes6-like activity. Conversely, insertion of five amino acid residues into the loop of Hes6 confers Hes1-like repressor activity on the N box. Thus, the loop region is critical for the proper functions of Hes6 and Hes1 and these functions are partially interchangeable by alteration of the loop. It remains to be determined whether just the length of the loop is important, or some specific amino acid residues are required, or both. It was reported that the loop region of Id1 is essential for its activity (Pesce and Benezra, 1993). In this case, specific amino acid residues are required for the proper Id1 activity. Sequence comparison between Hes6 and Id1 did not show any homology, but Id1 targets to E47 while Hes6 targets to Hes1, the difference in the loop region could be responsible for this specific protein interaction. While both Hes and Id1 studies revealed the importance of the loop region, alteration of the loop of Id1 simply resulted in loss of activity, whereas in contrast, alteration of the loop of Hes factors caused interchange of some specific activities of different Hes factors. Deletion and insertion of just five amino acid residues partially converted the negative regulator Hes1 into the positive regulator Hes6 and vice versa. This interchangeability of transcriptional activities simply by altering the loop region is unique to Hes, and therefore further mutational analysis of Hes proteins will provide more insight into the functional importance of the loop region.

Our data suggest that the transition from proliferation to differentiation is not a simple quantitative balance between Hes1 and Mash1, but that Hes6 may play a role to inhibit Hes1 and support Mash1-dependent cell differentiation. This hypothesis raises another important question of how Hes6 expression is controlled during embryogenesis. Further characterization of *Hes6* may help us to understand more precisely the mechanism of the differentiation process of many cell types.

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