Targeted disruption of mammalian *hairy* and *Enhancer of split* homolog-1 (*HES-1*) leads to up-regulation of neural helix–loop–helix factors, premature neurogenesis, and severe neural tube defects

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Mammalian *hairy* and *Enhancer of split* homolog-1 (*HES-1*) encodes a helix–loop–helix (HLH) factor that is thought to act as a negative regulator of neurogenesis. To directly investigate the functions of *HES-1* in mammalian embryogenesis, we performed a targeted disruption of the *HES-1* locus. Mice homozygous for the mutation exhibited severe neurulation defects and died during gestation or just after birth. In the developing brain of *HES-1*-null embryos, expression of the neural differentiation factor *Mash-1* and other neural HLH factors was up-regulated and postmitotic neurons appeared prematurely. These results suggest that *HES-1* normally controls the proper timing of neurogenesis and regulates neural tube morphogenesis.

[**Key Words:** Gene targeting; neurogenesis; neural tube defect; *HES* genes; *Mash-1*]

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During mouse neurulation, the neural folds elevate from the dorsal side of the embryo around embryonic day 7.5 (E7.5), approach each other medially, and fuse to form the neural tube (Jacobson 1991). The neural tube initially consists of a monolayer of dividing neural precursor cells. When neural precursor cells start to differentiate, they stop dividing, migrate toward the outer surface of the neural tube, and undergo terminal differentiation to become mature neurons or glial cells. To understand the molecular mechanisms underlying these complex developmental events, a very fruitful approach is to analyze mammalian genes homologous to *Drosophila* genes involved in similar processes.

HES-1 is a member of a family of mammalian basic helix–loop–helix (bHLH) factors homologous to the products of *Drosophila hairy* (*h*) and *Enhancer of split* [*E(spl)*] (Akazawa et al. 1992; Sasai et al. 1992; Ishibashi et al. 1993; Feder et al. 1993; Sakagami et al. 1994; Takebayashi et al. 1994, 1995), which negatively regulate neurogenesis (Moscoso del Prado and Garcia-Bellido 1984;

Campos-Ortega and Jan 1991; Campuzano and Modolell 1992; Jan and Jan 1993). *HES-1* is expressed in a variety of cells including embryonic neural and mesodermal cells (Sasai et al. 1992). In the developing nervous system, *HES-1* is expressed throughout the ventricular zone, where neural precursor cells are located. Its expression decreases as neurogenesis proceeds and is not detected in mature neurons or glial cells (Sasai et al. 1992). Neural precursor cells infected with *HES-1*-transducing retrovirus do not migrate out of the ventricular zone or differentiate into neurons or glial cells (Ishibashi et al. 1994). This suggests that *HES-1*, like *Drosophila h* and *E(spl)*, acts as a negative regulator of neurogenesis and that down-regulation of *HES-1* expression might be required for precursor cells to enter the differentiation processes.

HES-1 is a unique transcription factor with two different activities: (1) binding site-dependent repression and (2) dominant-negative activity (Sasai et al. 1992). Unlike most other bHLH factors that activate E-box (CANNTG)-dependent gene expression (Weintraub et al. 1991), *HES-1* represses transcription by binding to the N-box (CACNAG). For example, *HES-1* down-regulates

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its own expression by directly binding to the multiple N-box elements present in its own promoter (Takebayashi et al. 1994). In addition, like the dominant-negative HLH protein Id (Benezra et al. 1990), *HES-1* antagonizes the activity of bHLH-type transcriptional activators through nonfunctional heterodimer formation (Sasai et al. 1992). In particular, *HES-1* can antagonize the transcriptional activity of the muscle determination factor MyoD (Davis et al. 1987) by preventing the latter from binding to E boxes and thus can inhibit MyoD-induced myogenesis (Sasai et al. 1992). Thus, *HES-1* and Id may have overlapping functions in mammalian development. Furthermore, *HES-1* can also antagonize the activity of the neural bHLH activators Mash-1 and MATH-1, mammalian homologs of the products of the *Drosophila* proneural genes *achaete-scute* and *atonal*, respectively (Johnson et al. 1990; Sasai et al. 1992; Akazawa et al. 1995). This inhibitory activity on neural bHLH activators may account for the suppression of neural differentiation induced by the forced expression of *HES-1* (Ishibashi et al. 1994). Altogether, these results suggest a critical involvement of *HES-1* in mammalian embryogenesis.

To directly study the functions of *HES-1* during embryogenesis, we deleted the *HES-1* gene in mouse embryonic stem (ES) cells by homologous recombination. Most *HES-1*-null mutant mice exhibited severe defects in neurulation that subsequently resulted in exenceph-

aly and anencephaly. These mutant embryos died during embryogenesis or just after birth. In *HES-1*-deficient embryos, Mash-1 and other neural HLH factors were up-regulated and neural precursor cells of the telencephalon differentiated into mature neurons earlier than in wild-type embryos. This precocious neurogenesis may deter the process of closure of the cranial neural tube. Our findings suggest that *HES-1* normally regulates neural tube morphogenesis by controlling the proper timing of neurogenesis.

Results

Generation of mice lacking *HES-1*

The mouse *HES-1* gene is encoded by four exons, and the region encoding the bHLH domain of *HES-1* is located within the first three exons (Takebayashi et al. 1994). To mutate the *HES-1* locus in mouse ES cells, we constructed a targeting vector that deletes the protein-coding region located in the first three exons and replaces them with a neomycin (*neo*)-resistance expression cassette (Fig. 1A). R1 ES cells (Nagy et al. 1993) were electroporated with the linearized targeting vector and selected in the presence of G418 and gancyclovir. To isolate correctly targeted clones, we performed Southern blot analysis with a 5' probe and a 3' probe, both of which are external to the vector region, and with a *neo*

Figure 1. Targeted mutation at the *HES-1* locus. (A) Strategy for the targeted deletion of *HES-1*. (■) The protein-coding region; (□) the 5'- and 3'-noncoding regions of the locus. The first three exons are replaced with the PGK-*neo* cassette in the targeting vector, and a novel *Bam*HI site is introduced. The 5' and 3' probes that are external to the vector region are shown at the bottom. (B) Southern blot analysis of genomic DNA isolated from embryos. *Bam*HI-digested embryo DNA was hybridized with the 5' probe (lanes 1–3), 3' probe (lanes 4–6), and *neo* probe (lanes 7–9). The 5' probe detects a 12-kb wild-type and a 2.3-kb mutant band. The 3' probe detects a 12-kb wild-type and a 10.5-kb mutant band. The *neo* probe detects the 10.5-kb mutant band. (+/+) Wild-type; (+/-) heterozygous mutant; (-/-) homozygous mutant.

probe (Fig. 1B). Because a novel *Bam*HI site was introduced into the targeting vector, after digestion with *Bam*HI, the 5' and 3' probes should detect 2.3-kb and 10.5-kb mutant bands, respectively, in addition to the wild-type 12-kb band. The *neo* probe should also detect the 10.5-kb mutant band after digestion with *Bam*HI. Out of 185 ES colonies that grew in the selection medium, seven were correctly targeted (homologous recombination frequency of 3.8%) and two ES clones, #299 and #335, were aggregated with CD1 morula (Nagy et al. 1993). Chimeric mice were mated to CD1 females and transmitted the mutation to their progeny. The phenotypes of *HES-1*-null mice derived from the two independent ES clones were identical.

Mice heterozygous for the *HES-1* mutation showed no abnormalities after up to 1 year of life and were fertile. When heterozygous mice were intercrossed, no homozygous mutants were found alive 1 day after birth (Table 1). We therefore examined embryos from heterozygous intercrosses at various stages. Until E12.5, homozygous mutant embryos were present in the expected Mendelian distribution (~25%) (Table 1). However, after E12.5, we frequently found dead embryos. Some homozygous animals were found just after birth, but none of them survived for >1 day (Table 1), indicating that the *HES-1* null mutation is lethal (100% penetrance). Approximately 70% of *HES-1*-null embryos showed severe neural tube defects (see below). Although the remaining ~30% were morphologically normal, they also did not survive. The variance in phenotypes with respect to the time of death and the presence or absence of neural tube defects may be attributable to the different genetic backgrounds (129J×CD1) of the mice that were used. In the following sections, we present an analysis of the most frequently observed mutant phenotypes.

Defects of cranial neural tube closure in *HES-1*-null embryos

Most homozygous mutant embryos could not be distinguished morphologically from wild-type embryos at E8.5, when the anterior neuropore was still open (data

not shown). However, occasionally, *HES-1*-null embryos showed a kinked neural tube (Fig. 2A). At E8.5, the neural folds of wild-type embryos initiate fusion within the cranial region simultaneously at the anterior extremity of the forebrain, the forebrain–midbrain boundary, and the hindbrain–spinal cord boundary (Sakai 1989), and by E9.5, the anterior neuropore has completely closed (Fig. 2B). In *HES-1*-null embryos, the neural folds elevated and approached medially at E8.5; however, the mutant neural folds failed to fuse and the cranial region of mutant embryos was still partially or completely open at E9.5 (Fig. 2C). Furthermore, the head size of *HES-1*-null embryos was smaller than that of wild-type embryos (Fig. 2, c.f. B and C), suggesting that head growth was retarded without *HES-1*. Other regions of the body were apparently normal in *HES-1*-null embryos, except for the occasionally kinked neural tube (Fig. 2A). At E10.5, the neuroepithelium of *HES-1*-null embryos was everted (exencephaly) (Fig. 2E, arrow), the telencephalic vesicles were reduced and flattened, and a mid-facial cleft remained (Fig. 2E, arrowhead; Fig. 2G). By E18.5, *HES-1*-null embryos had become anencephalic (Fig. 2H,I). Thus, *HES-1* plays an important role in cranial neural-tube formation.

Cellular organization of the developing nervous system

To further investigate the morphology of mutant embryos, histological analysis was performed. Although the macroscopic structure of the neural tube was dramatically changed in *HES-1*-null embryos, the cellular organization of the walls of the neural tube was apparently normal at E10.5 (Fig. 3A–D). At this stage, the telencephalon of both *HES-1*-null and wild-type embryos consisted mostly of undifferentiated precursor cells (Fig. 3A,B). In the hindbrain of both mutant and wild-type embryos, brain walls consisted of two well-developed layers: the ventricular zone where neural precursor cells are dividing and the mantle layer where differentiating neural cells are present (Fig. 3C,D). In addition, both mutant and wild-type neural cells appeared aligned in radial arrays (Fig. 3C,D). At E12.5, although some regions began to undergo necrosis in the brain of mutant embryos (Fig. 3F, arrow and arrowhead), the cellular organization of the remaining parts of the nervous system seemed histologically normal. These results suggest that neural differentiation and migration are not blocked in *HES-1*-null mice.

HES-1 is also expressed in the peripheral nervous system, particularly in the cranial ganglia and dorsal root ganglia. However, no histological abnormalities were detected in those regions of *HES-1*-null embryos (Fig. 3A,B; data not shown).

Premature neurogenesis in *HES-1*-null embryos

To further investigate how neurogenesis is affected by the *HES-1* null mutation, we next conducted immunohistochemical studies with neural molecular markers.

Table 1. Genotypes in litters from heterozygous intercrosses

Age	Number of pups	Genotype (%)		
		+ / +	+ / -	- / -
E8.5	15	3 (20)	7 (47)	5 (33)
E9.5	89	25 (28)	47 (53)	17 (19)
E10.5	111	28 (25)	53 (48)	30 (27)
E11.5	15	2 (13)	10 (67)	3 (20)
E12.5	28	6 (21)	15 (54)	7 (25)
E18.5	71	25 (35)	40 (56)	6 (8)
$P \geq 1$	76	24 (32)	36 (68)	0 (0)

Embryos or neonates were harvested at the time indicated. Genomic DNA was extracted from the yolk sac or the tail of each pup, and subjected to Southern blot analysis or PCR to determine the genotype.

Figure 2. Morphological analysis of wild-type (*B,D,F*) and *HES-1*-null embryos (*A,C,E,G-I*). (*A*) Dorsal view of an E9.5 embryo. Most *HES-1*^{-/-} embryos show a normal trunk region. However, a few *HES-1*-null embryos showed a kinked neural tube (arrow). (*B,C*) Lateral view of E9.5 embryos. The cranial neural tube of (+/+) and (+/-) embryos is already closed, whereas that of (-/-) embryos is not (arrow). (*D,E*) Frontolateral view of E10.5 embryos. (*E*) The cranial neural tube of (-/-) embryos remains open at the position of the anterior neuropore and a mid-facial cleft persists (arrowhead). The face of this embryo is poorly formed, and the neuroepithelium is everted (arrow). (*F,G*) Dorsolateral view of E10.5 embryos. (*G*) The cranial neural tube of (-/-) embryos is open from the forebrain to a variable position ranging from the midbrain to the hindbrain-spinal cord boundary (arrow). (*H*) Frontal view of an E18.5 *HES-1*-null embryo. (*I*) Lateral view of an E18.5 *HES-1*-null embryo. (-/-) embryos exhibit anencephaly at this stage. Scale bars, (*B-G*) 500 μ m; (*H,I*) 2 mm.

Nestin is an intermediate filament specifically expressed in neural precursor cells in the developing nervous system (Lendahl et al. 1990). Nestin-positive cells were present in the ventricular zone of both wild-type (Fig. 4A) and mutant brains (Fig. 4B). To confirm that these nestin-positive cells are in a mitotic phase, bromodeoxyuridine (BrdU) was administered. Most cells in the ventricular zone of wild-type and mutant embryos were labeled with BrdU, and no detectable difference was observed between these embryos (data not shown). These results indicate that neural precursor cells proliferate apparently normally without *HES-1*.

To assess the differentiation status of neural cells in *HES-1*-null embryos, we then examined the expression of the postmitotic neuron-specific markers, neurofila-

ment (Lazarides 1982) and L1 (Rathjan and Schachner 1984). In wild-type embryos at E10.5, neurofilament-positive cells are aligned in the outer layer of the neural tube, caudally to the forebrain-midbrain boundary, and are thus excluded from the telencephalon (Fig. 4C,E). Neurofilament-positive cells, as well as L1-positive cells, first appear at E11.5 in the telencephalon of wild-type embryos (data not shown), indicating that terminal differentiation does not occur normally in this division of the brain until E11.5. In contrast, in the mutant telencephalon, neurofilament-positive cells and L1-positive cells were already present at E10.5 (Fig. 4D,F,H). Neurofilament-positive cells were aligned in the outermost layer of the telencephalon as far as its rostral extremity (Fig. 4F, arrowhead). These cells extended neurite-like

Figure 3. Histological analysis of wild-type (A,C,E) and *HES-1*-null embryos (B,D,F). (A,B) Horizontal section of the head of E10.5 embryos. In B, the neuroepithelium is everted (arrow). The trigeminal ganglia (tg) and Rathke's pouch (rp) are apparently normal. (C,D) Horizontal section of the hindbrain of E10.5 embryos. The cytoarchitecture of the neuroepithelium is not disturbed in *HES-1*-null embryos; the brain walls consist of two well-developed layers, the ventricular zone (v) and the mantle layer (m). (E,F) Sagittal section of E12.5 embryos. In F, the roof of the cephalic vesicles is lacking and part of the neuroepithelium is necrotic (arrow). A cystic lesion is also observed in the telencephalon (arrowhead). Other organs are normal in appearance. Abbreviations: (d) diencephalon; (gi) gastrointestinal tract; (h) hindbrain; (l) lung; (m) mantle layer; (rp) Rathke's pouch; (sc) spinal cord; (t) telencephalon; (tg) trigeminal ganglion; (v) ventricular zone. Scale bars, (A) 500 μ m; (B) 250 μ m; (C,D) 50 μ m; (E,F) 1 mm.

complex processes (Fig. 4F, arrows), suggesting that neuronal differentiation per se has already started in the telencephalon of *HES-1*-null embryos at E10.5. These results indicate that, in the absence of *HES-1*, postmitotic neurons appear prematurely in the telencephalon.

We also examined the expressions of nestin and neurofilament in cranial and dorsal root ganglia but detected no significant changes in *HES-1*-null embryos (data not shown).

Altered gene expression in HES-1-null embryos

To understand the molecular basis for the premature neurogenesis observed in *HES-1*-null embryos, we then examined expression of the HLH factor genes *Mash-1* (Johnson et al. 1990), *HES-5* (Akazawa et al. 1992), and *Id-1* (Benezra et al. 1990). *Mash-1* and *HES-5* are expressed in neural precursor cells as well as in differentiating neural cells and are thus good markers for early neural differentiation. Furthermore, *Mash-1* has been shown to act as a positive regulator of neurogenesis (Guillemot et al. 1993). In contrast, *Id-1* is a dominant-negative HLH factor without a basic region, homologous to the *Drosophila* negative regulator *extramacrochaetae* (*emc*), and it is only expressed in undifferentiated precursor cells (Duncan et al. 1992; Evans and O'Brien 1993). We first examined the expressions of these genes by Northern blot experiments (Fig. 5).

At E9.5, both *Mash-1* (lane 6) and *HES-5* expression (lane 9) were clearly up-regulated in *HES-1*-null embryos, whereas *Id-1* and *elongation factor 1 α* (*EF1 α*) expressions were not changed (lanes 12,15). Thus, in the absence of *HES-1* (lane 3), expression of *Mash-1* and *HES-5* is specifically enhanced. Our previous studies showed that *HES-1* can antagonize the transcriptional activity of *Mash-1* by forming a nonfunctional complex (Sasai et al. 1992). Thus, these results suggest that, in the absence of *HES-1*, the neural differentiation factor *Mash-1* is not only relieved from this inhibitory protein-protein interaction, but also transcriptionally activated, raising the possibility that, in *HES-1* mutant embryos, the activity of this positive neural regulator becomes dominant over the negative control of neurogenesis, normally prevalent in neural precursors.

To determine how the expression patterns of these HLH factors are affected in the nervous system of *HES-1* mutant embryos, we then performed in situ hybridization analysis. In normal embryos at E8.5, *Mash-1* expression starts in the neural folds of the prospective midbrain and is not observed in other regions of the central nervous system (CNS) (Fig. 6A, arrowheads). At E10.5, *Mash-1* expression is observed at high levels in the developing nervous system in a patchy manner and is still low in the telencephalon (Fig. 6C, left embryo; Fig. 6D, arrows). In contrast, in *HES-1*-null embryos, *Mash-1* expression had already started in the neural folds of the prospective forebrain as well as in the midbrain at E8.5 (Fig. 6B, arrow and arrowheads), indicating that *Mash-1* expression starts prematurely in the forebrain of *HES-1*-null embryos. Furthermore, at E10.5 (Fig. 6C, right em-

Figure 4. Immunohistochemical analysis of wild-type (A,C,E,G) and *HES-1*-null embryos (B,D,F,H). Sagittal sections of E10.5 embryos immunostained with an anti-nestin antibody (A,B), an anti-neurofilament antibody (C–F), and an anti-L1 antibody (G,H). Approximate position of each section is indicated in I and J. In B, nestin is expressed in the head neuroepithelium and no apparent abnormalities are found. (C,E) In wild-type embryos, neurofilament-positive cells are present in the midbrain (m) but not in the forebrain (f). The forebrain–midbrain boundary is indicated by an arrowhead in C. (D,F) Neurofilament-positive cells are already present in the mutant forebrain (f, arrows). The region containing neurofilament-positive cells extends to the anterior extremity (arrowhead in F). These neurofilament-positive cells extend neurite-like processes (arrows in F). (G) In wild-type embryos, L1-positive cells are not found in the forebrain. (H) L1-positive cells appear in the mutant forebrain. Scale bars, (A–C,E,G,H) 100 μ m; (D,F) 50 μ m.

bryo; 6E), *Mash-1* was expressed at higher levels in the forebrain (Fig. 6C, arrowheads; Fig. 6E, arrows) as well as in the optic vesicles (ov) and spinal cord (Fig. 6C, arrows) in *HES-1*-null embryos than in wild-type embryos (Fig. 6C, left embryo). At this stage, *Mash-1* expression in mutant embryos appeared more uniform in other regions of the nervous system as well, suggesting that *Mash-1* expression is up-regulated throughout the CNS.

In normal embryos at E10.5, *HES-5* is generally expressed in a patchy manner in the developing nervous system (Fig. 7A). It is expressed in the ventral half and the most dorsal part of the spinal cord (Fig. 7C) and only at low levels in the telencephalic region (Fig. 7A, arrowheads). In contrast, in *HES-1*-null embryos, *HES-5* was uniformly expressed throughout the developing CNS, including the telencephalic region and the spinal cord (Fig. 7B,D). Thus, like *Mash-1*, *HES-5* expression is up-regulated in the absence of *HES-1*.

In the nervous system, *Id-1* expression was restricted

to the ventricular zone of both wild-type and mutant embryos at E10.5 (Fig. 7E,F). Although no significant changes were observed in most regions, *Id-1* expression was up-regulated in the telencephalon of *HES-1*-null embryos (Fig. 7F, arrowheads). This up-regulation could compensate for *HES-1* deficiency to some extent (see Discussion).

We then examined another neural bHLH gene that is expressed at later stages of neurogenesis, *NSCL1* (Begley et al. 1992), by in situ hybridization analysis. At E10.5, *NSCL1*, which is structurally homologous to the hematopoietic bHLH factor SCL (TAL), was expressed only in the subependymal layer of the developing nervous system of both wild-type and *HES-1*-null embryos (Fig. 7G,H). However, its expression was quite low in the nasal side of the wild-type telencephalon (Fig. 7G, arrowheads), whereas it was expressed throughout the telencephalon of *HES-1*-null embryos (Fig. 7H, arrowheads), in good agreement with the above observation that neu-

Figure 5. Northern blot analysis of E9.5 embryos. Ten micrograms of total RNA from wild-type embryos (lanes 1,4,7,10,13) and heterozygous (lanes 2,5,8,11,14) and homozygous mutant embryos (lanes 3,6,9,12,15) were electrophoresed, transferred to a nylon membrane, and hybridized with 32 P-labeled *HES-1* (lanes 1–3), *Mash-1* (lanes 4–6), *HES-5* (lanes 7–9), *Id-1* (lanes 10–12), and *EF1α* (lanes 13–15) probes. The relative intensity of each band measured by densitometer is as follows: *HES-1* (+/+ : +/- : -/- = 1:0.8:0); *Mash-1* (1:2.1:4.8); *HES-5* (1:1.2:5); *Id-1* (1:1:0.8); *EF1α* (1:1:0.9). Expression of *Mash-1* and *HES-5* is clearly increased in (-/-), whereas that of *Id-1* and *EF1α* is not.

ral differentiation proceeds precociously in the telencephalon without *HES-1*.

To exclude the possibility that the premature neural differentiation observed in *HES-1*-null embryos results from an alteration in neural tube patterning, we also examined the expression of markers for cell populations along the dorsoventral and anteroposterior axis of the neural tube. The genes *Msx-1* (roof plate of the neural tube; Mackenzie et al. 1991), *MATH-1* (alar plate; Akazawa et al. 1995), *CRABP I* (various neuronal populations including cells in the dorsal mesencephalon and metencephalon; Ruberte et al. 1993), *Islet-1* (ventral neurons in spinal cord and brain; Ericson et al. 1992, 1995), and *netrin-1* (floor plate; Kennedy et al. 1994) showed similar expression patterns in the spinal cord and brain of wild-type and *HES-1*-null embryos (data not shown). Markers normally expressed in the dorsal part of the neural tube (*CRABP I*, *Msx-1*) were expressed along the everted edge of the open brain of *HES-1*-null embryos. Thus, lack of *HES-1* does not alter dorsoventral pattern-

ing in the neural tube. Finally, *BF-1*, which is specifically expressed in the telencephalon (Tao and Lai 1992), was

Figure 6. In situ hybridization analysis of wild-type (A,C, left embryo; D) and *HES-1*-null embryos (B;C, right embryo; E) with a *Mash-1* cRNA probe. (A,B) Whole-mount in situ hybridization of E8.5 embryos with a DIG-labeled probe. (A) In wild-type embryos, *Mash-1* is expressed in the neural folds of the prospective midbrain (arrowheads) but not in the forebrain. (B) *Mash-1* is expressed in the mutant forebrain (arrow) as well as in the midbrain (arrowheads). The expression extends to the anterior extremity of the forebrain. (C) Whole-mount in situ hybridization of E10.5 embryos with a DIG-labeled probe. *Mash-1* expression is increased in the forebrain (arrowheads) as well as in the optic vesicles (ov) and spinal cord (arrows) of the *HES-1*-null embryo (right embryo). (D,E) Sagittal sections of E10.5 embryos hybridized with a 35 S-labeled probe. In D, *Mash-1* is expressed in the wild-type developing nervous system in a patchy manner and the expression is low in the telencephalon (arrows). In E, strong signals are observed in the mutant telencephalon (arrows). In the rest of the developing CNS, *Mash-1* is expressed more uniformly than in wild-type embryos. Scale bar, (A,B) 250 μ m; (D,E) 500 μ m.

Figure 7. In situ hybridization analysis of wild-type (A,C,E,G) and *HES-1*-null embryos (B,D,F,H) with HLH factor cRNA probes. Sections of E10.5 embryos were hybridized with a DIG-labeled *HES-5* cRNA probe (A–D) and 35 S-labeled *Id-1* (E,F) and *NSCL1* (G,H) cRNA probes. (A) *HES-5* is expressed in the wild-type developing nervous system in a patchy manner. The expression is quite low in the forebrain (arrowheads). (B) *HES-5* is expressed in the mutant nervous system more uniformly, and the expression is high in the forebrain. (C,D) Higher magnification of the spinal cord. In C, *HES-5* is expressed in the ventral half and the most dorsal region of the wild-type spinal cord. The expression is low in other regions of the spinal cord (arrowheads). In D, *HES-5* is more uniformly expressed in the mutant spinal cord.

(E) In the nervous system, *Id-1* expression is restricted to the ventricular zone. The expression is quite low in the wild-type forebrain (arrowheads). (F) *Id-1* expression is increased in the mutant forebrain (arrowheads). In the other regions of the embryo, *Id-1* expression is not changed. (G,H) *NSCL1* is expressed in the subependymal layer. In G, the expression is quite low in the nasal side of the wild-type forebrain (arrowheads). In contrast, in H, the expression is observed throughout the mutant forebrain (arrowheads). (E',F',G',H') Cresyl violet staining. Scale bar, (A,B) 500 μ m; (C,D) 100 μ m; (E–H,E'–H') 500 μ m.

present in the rostral brain of *HES-1*-null embryos, although in a smaller domain than in wild-type embryos, owing to a reduced development of the telencephalic vesicles (data not shown). Thus, identity of telencephalic cells seems unaffected in *HES-1*-null embryos, and alteration in neural tube patterning does not appear to account for the premature neuronal differentiation observed in the mutant forebrain.

Discussion

Premature neurogenesis and neural tube defects in HES-1-null mice

In this study, we generated *HES-1*-null mutant mice to investigate the role of *HES-1* in mammalian development. One of the most prominent phenotypes of *HES-1*-null embryos is their severe neurulation defects. In most

cases, the cranial neural tube remains partially or completely open, the telencephalic vesicles are reduced in size, and a mid-facial cleft persists. Furthermore, growth of the head appears retarded in these embryos. Histological analyses indicated no significant abnormalities in the lamination and radial arrangement in the CNS of *HES-1*-null embryos. However, at the molecular level, neurogenesis is apparently accelerated in the telencephalon of mutant embryos; neurofilament-positive and L1-positive neurons appear earlier in mutants than in normal embryos. In addition, expression of the neural bHLH factors *Mash-1* and *HES-5* is up-regulated in *HES-1*-null embryos. These results, together with previous studies showing that the transcriptional activity of *Mash-1* can be antagonized by *HES-1* through an inhibitory protein-protein interaction (Sasai et al. 1992), demonstrate that neural development is negatively regulated by *HES-1*. In addition, our results raise the possibility that *Mash-1* derepression is a direct cause of premature neuronal differentiation in *HES-1*-null embryos. We also propose that accelerated neurogenesis could lead to neural tube closure defects in the cranial region, if it results in a significant reduction of proliferation by depletion of the population of dividing neuronal progenitors. To test the role that *Mash-1* up-regulation plays in the *HES-1* mutant phenotype, we are presently analyzing *HES-1*-null embryos on a *Mash-1* mutant background.

It is believed that abnormalities in both the neuroepithelium and the surrounding tissues can result in neural tube defects (Copp et al. 1990; Schoenwolf and Smith 1990), and the recent generation of a mutation in the mouse *twist* gene demonstrates that mesodermal cells underlying the neuroepithelium play an important role in neural tube formation. Mouse *twist* encodes a bHLH factor expressed in mesoderm and neural crest-derived cranial mesenchyme (Wolf et al. 1991). In mice without *twist*, the shape of mesenchymal cells underlying the neural tube is changed and intercellular contacts are reduced, thereby affecting the process of neural tube closure (Chen and Behringer 1995). Because *HES-1* is expressed in undifferentiated mesenchymal cells in the craniofacial region, it is possible that these cells are also involved in the neural tube defects of *HES-1* mutant embryos. However, facial mesenchymal cells in *HES-1*-deficient embryos are normal in appearance and in expression of specific molecular markers [*Msx-1* (Mackenzie et al. 1991), *CRABPI* (Ruberte et al. 1993), *Cart-1* (Zhao et al. 1994); data not shown]. Furthermore, when BrdU was administered to *HES-1*-null embryos, mesenchymal cells incorporated BrdU without any significant changes (data not shown), suggesting that mesenchymal cells proliferate and develop without *HES-1*. Thus, it seems likely that neural cells, rather than mesenchymal cells, are the primary cause for the neural tube defects in *HES-1*-null embryos, although the possibility that undetectable abnormalities of mesenchymal cells may contribute to the neural tube defects still remains.

It is not yet known whether rates of cell differentiation are affected in other regions of the embryo, particularly in the spinal cord. As in the cranial region, *HES-5* and

Mash-1 are up-regulated in the spinal cord of *HES-1*-null embryos. Furthermore, we occasionally observed a kinked neural tube in *HES-1*-null embryos, although this phenotype appears associated with defective somites and lack of elongation of the embryo, which could be the primary cause of the spinal cord defect (this aspect of the phenotype will be reported in a separate article). Thus, if the lack of *HES-1* may in some cases affect spinal cord development, it appears to be mostly compensated for, as in *HES-1*-null embryos without neurulation defect, presumably by other HLH factors. It is not yet known which HLH factors substitute for *HES-1*, but it is possible that other members of the *HES* family such as *HES-2* and *HES-5*, which are both expressed during embryogenesis, are involved in this compensation. However, unlike *HES-1*, *HES-5* has only a weak dominant-negative activity (Akazawa et al. 1992) and does not antagonize *Mash-1* activity (C. Akazawa, Y. Sasai, and R. Kageyama, unpubl.). Furthermore, *HES-2* has no dominant-negative activity (Ishibashi et al. 1993). Thus, it is unlikely that these factors substitute for *HES-1*. In this regard, *Id* factors may be more likely candidates for compensation for *HES-1* deficiency because, like *HES-1*, they have a strong dominant-negative activity (Benezra et al. 1990). Furthermore, *HES-1*, *Id-1*, and *Id-2* are expressed in the same or largely overlapping regions. The hypothesis that *HES-1* and *Id* factors can compensate for each other can be examined by generating *HES-1*-null; *Id*-null double mutant mice. *Id-1* expression is up-regulated in the telencephalic region of *HES-1*-null embryos, and this may explain why some *HES-1*-null embryos are normal in appearance; in some mutant embryos, *Id-1* and other related factors could be expressed at a high enough level to compensate for *HES-1* deficiency in the formation of the neural tube.

Structural and functional conservation between mammalian and Drosophila neural factors

The data shown above indicate that *HES-1* regulates neural tube formation by preventing premature neurogenesis. *Drosophila* HLH factors encoded by *h* (*HES-1* homolog) and *emc* (*Id* homolog) both act as negative regulators of peripheral nervous system development (Rushlow et al. 1989; Ellis et al. 1990; Garrell and Modolell 1990). Interestingly, accelerated neurogenesis is also observed during *Drosophila* eye development when clones of cells double null mutant for *h* and *emc* are generated (Brown et al. 1995). In these clones, morphogenetic furrow progression, neuronal development, and expression of the eye proneural gene *atonal* all occur prematurely, indicating that the two HLH proteins encoded by *h* and *emc* act together to negatively regulate the rate of neuronal development. Thus, structurally related mammalian and *Drosophila* HLH factors regulate neural development in a similar manner (negative regulation), suggesting that the underlying mechanisms in which they participate have also been conserved during evolution.

Recent studies have shown that the product of *h* represses transcription of the proneural gene *achaete* by

directly binding to its promoter (Ohsako et al. 1994; Van Doren et al. 1994). In *HES-1*-null embryos, expression of *Mash-1*, a mammalian homolog of *achaete*, is specifically up-regulated, suggesting that *HES-1* represses transcription of *Mash-1*. These results again show that the function as well as the structure of the neural HLH factors have been conserved during evolution. However, it is not yet known whether *HES-1* can interact directly with the promoter region of *Mash-1*. If *Mash-1*, like *MyoD* (Thayer et al. 1989), can interact with its own promoter and up-regulate its own expression (positive autoregulation), *HES-1* could repress *Mash-1* expression by inhibiting the transcriptional activity of *Mash-1* without interacting with its promoter. In the case of *HES-5*, there are several N-box sequences in its promoter, and *HES-1* most likely represses *HES-5* expression by binding to the *HES-5* promoter (Takebayashi et al. 1995).

In spite of the striking similarities described above, a functional difference between mammalian and *Drosophila* HLH factors is also noted. *Drosophila* *achaete* is involved in developmental decisions between neural and epidermal fates. Thus, by regulating *achaete* expression, *h* also functions in the neural–epidermal fate choice, in addition to controlling the rate at which neurogenesis proceeds in the eye. In contrast, *Mash-1* is expressed in the neural tube only after the neural fate is determined (Lo et al. 1991; Guillemot and Joyner 1993) and is therefore not involved in the neural–epidermal decision. Mammalian proneural genes that initially determine the neural fate have not yet been identified. From the phenotype of mutant embryos, *HES-1* does not appear to be involved in this process either.

By antagonizing bHLH-type differentiation factors, *HES-1* keeps cells undifferentiated. In the case of neurogenesis, persistent expression of *HES-1* inhibits both neuronal and glial differentiation (Ishibashi et al. 1994). Thus, *HES-1* may keep cells uncommitted to either neuronal or glial fates in the nervous system. This effect is very similar to that of the transmembrane protein Notch; activated Notch blocks cell fate commitment in both *Drosophila* and vertebrate (Coffman et al. 1993; Fortini et al. 1993; Kopan et al. 1994; Nye et al. 1994; for review, see Artavanis-Tsakonas et al. 1995). In addition, it was reported recently that activated *Xotch* (*Xenopus Notch*) inhibits neuronal and glial differentiation in the *Xenopus* retina (Dorsky et al. 1995), suggesting that the nuclear factor *HES-1* and the transmembrane protein Notch may function in either the same or a closely related regulatory pathway.

Interestingly, *E(spl)* (also homolog to *HES-1*) is a nuclear target of the *Notch* signaling pathway in *Drosophila* (Artavanis-Tsakonas et al. 1995). When Notch is activated by its ligand Delta, Suppressor of Hairless [Su(H)] and the cytoplasmic domain of Notch translocate into the nucleus, and activate *E(spl)* expression (Artavanis-Tsakonas et al. 1995). Although it is not yet known whether this signaling pathway is conserved in mammals, mammalian homologs to these *Drosophila* molecules have been identified (Ellisen et al. 1991; Weinmaster et al. 1991; Furukawa et al. 1992; Artavanis-

Tsakonas et al. 1995; Chitnis et al. 1995; Henrique et al. 1995; Lindsell et al. 1995). Furthermore, RBP-J κ , a mammalian homolog of *Drosophila* Su(H) (Furukawa et al. 1992), has been shown to interact with the promoter of the *HES-1* gene and activate its expression (Brou et al. 1994). These results thus raise the interesting possibility that, in addition to the structural and functional conservation between mammalian and *Drosophila* HLH factor networks, the *Drosophila* Notch–Su(H)–*E(spl)* signaling pathway is also conserved as the vertebrate Notch–RBP-J κ –*HES-1* pathway for regulating cellular differentiation.

Materials and methods

Construction of a *HES-1* targeting vector

The *HES-1* genomic clone was obtained from a 129SVJ mouse library (Stratagene) and was described previously (Takebayashi et al. 1994). The targeting vector contains an HSV-TK expression cassette, 1.2 kb of 5'-flanking and 5'-untranslated regions, a *neo* expression cassette in place of the bHLH-coding region, and 6 kb of the fourth exon and 3'-flanking region (Fig. 1A).

Generation and genotyping of *HES-1* mutant mice

R1 ES cells (Nagy et al. 1993) were electroporated with the linearized targeting vector and cultured in the presence of G418 and gancyclovir. Genomic DNA was recovered from selected clones and subjected to Southern blot hybridization to detect homologous recombination events (Wurst and Joyner 1994). The 0.9-kb *Bam*HI–*Pvu*II fragment that is 5'-external to the vector region and the 0.4-kb *Acc*I–*Bam*HI fragment that is 3'-external to the vector region were used as 5' and 3' probes, respectively (Fig. 1A). DNA was digested with *Bam*HI and hybridized with the 5' probe. This probe detects a 12-kb wild-type genomic DNA fragment and a 2.3-kb mutant fragment. The 3' probe was also hybridized to the *Bam*HI-digested DNA to detect a 12-kb wild-type fragment and a 10.5-kb mutant fragment. The 0.6-kb *Pst*I–*Sac*I fragment of *neo* was used as an internal probe to ensure that no insertion is present at extra sites. Two ES cell clones positive for homologous recombination were aggregated with CD1 morula to generate chimeric mice.

Chimeric males were bred to CD1 females. To determine the genotypes of offspring, tail DNA was digested with *Bam*HI and hybridized with the 5', 3', and *neo* probes, as described above. To determine the genotypes of embryos from heterozygous intercrosses, we performed PCR with tail or yolk sac DNA with the following primers. The sense strand primer, 5'-CCCCTT-TGCAGTCATCAAAG-3', and the antisense strand primer, 5'-GCATTGCTCACTTACATCTTTC-3', produced a 122-bp wild-type band, which was detected only in wild-type and heterozygous mutant mice. The sense strand primer, 5'-ATGGAT-TGCACGCAGGTTCTC-3', and the antisense strand primer, 5'-CTGATGCTCTTCGTCCAGATC-3', produced a 476-bp *neo* band, which was detected only in heterozygous and homozygous mutant mice.

Histology and immunohistochemistry

For histological analysis, embryos were fixed overnight in 4% paraformaldehyde in phosphate buffer (pH 7.2) at 4°C, embedded in wax, and sectioned at 5 μ m. Sections were then dewaxed, rehydrated, and stained with hematoxylin and eosin. For immunohistochemistry, embryos were fixed as above, rinsed in phos-

phate-buffered saline (PBS), transferred to PBS plus 30% sucrose, and left until they sank. The samples were then embedded in O.C.T. compound, frozen, and sectioned at 10 μ m. The sections were preincubated in PBS containing 5% normal goat or donkey serum for 1 hr and incubated overnight in PBS containing 1% bovine serum albumin and primary antibodies. The following antibodies were used: rabbit antiserum to nestin (used at a 1:100 dilution) (Tomooka et al. 1993), which was a gift from Dr. Y. Tomooka; a mouse monoclonal antibody to 160 kD of neurofilament (Amersham RPN.1104; 1:100 dilution); a rat monoclonal antibody to neural cell adhesion molecule L1 (Boehringer Mannheim; 1:20 dilution). To visualize immunoreactivity, a fluorescein isothiocyanate-conjugated second antibody (Vector, Cappel) was used for nestin and neurofilament. L1 immunoreactivity was detected by the avidin-biotin method using a biotin-conjugated secondary antibody combined with fluorescein isothiocyanate-conjugated avidin (Vector). Coverslips were mounted in 90% glycerol in PBS.

Northern blot analysis

Total RNA was prepared from E9.5 whole embryos. Ten microgram of total RNA was electrophoresed on a formamide/1.2% agarose gel and transferred to a nylon membrane. The filter was hybridized with 32 P-labeled probes at 42°C in 50% formamide, 5 $\times$ SSC, 5 $\times$ Denhardt's solution, 50 mM sodium phosphate buffer (pH 6.8), 0.1% SDS, and 100 μ g/ml of heat-denatured salmon sperm DNA and washed at 65°C in 0.2 $\times$ SSC and 0.1% SDS.

RNA in situ hybridization

RNA in situ hybridization using radioisotope-labeled cRNA probes was performed as described previously (Akazawa et al. 1992). For nonradioactive in situ hybridization, sections or whole-mount samples were hybridized with digoxigenin-labeled cRNA probes as described previously (Ang and Rossant 1993; Akazawa et al. 1995; Shimizu et al. 1995). The *HES-5* and *Mash-1* cRNA probes were synthesized as described previously (Akazawa et al. 1992; Guillemot and Joyner 1993). The other probes contained the following sequences: *Id-1*, 79–535 of cDNA (Benezra et al. 1990); *NSCL-1*, 446–855 of cDNA (Begley et al. 1992). The probes showed the same hybridization pattern as described previously in wild-type mice [*Id-1* (Duncan et al. 1992; Evans and O'Brien 1993), *NSCL-1* (Begley et al. 1992)].

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