Molecular Characterization of a Rat Negative Regulator with a Basic Helix-Loop-Helix Structure Predominantly Expressed in the Developing Nervous System*

Chihiro Akazawa, Yoshiki Sasai, Shigetada Nakanishi, and Ryoichiro Kageyama

From the Institute for Immunology, Kyoto University Faculty of Medicine, Yoshida, Sakyo-ku, Kyoto 606, Japan

We report here the cDNA cloning and characterization of a rat basic helix-loop-helix (HLH) factor, designated HES-5. This factor has a distant sequence homology to *Drosophila* hairy and Enhancer-of-split proteins, both of which are required for normal neurogenesis. DNase I footprinting analyses show that HES-5 binds to the sequence CACNAG (called N box), a recognition sequence of Enhancer-of-split proteins. Although HES-5 does not bind to the sequence CANTNG (called E box) recognized by other HLH factors, it attenuates the binding of E47, an HLH activator, to E box by forming a hetero-oligomer. In cotransfection analyses using NIH 3T3 cells, HES-5 significantly represses transcription originating from the promoter containing the N box sequences. Furthermore, HES-5 also partially inhibits the E47-induced expression from the promoter containing E boxes. Northern blot, RNase protection, and *in situ* hybridization analyses demonstrate that the HES-5 mRNA is specifically expressed in the nervous system. Prominent expression is observed in the ventricular zones of the embryonal brain vesicles and the outer nuclear layer of the neural retina. These results suggest that the negative regulator HES-5 may play an important role in neural development.

Mammalian neural development involves complex gene regulations and its mechanism remains to be fully elucidated. In *Drosophila*, various genes that play an important role in neural development have been identified and intensively characterized. For example, the proneural genes, *achaete* (ac) and *scute* (sc), positively regulate sensory neurogenesis, and two negative regulators, *hairy* (h) and *extramacrochaete* (emc), repress ac and sc gene expressions, respectively, thus controlling sensory organ formation (1-5). Therefore, the balance between positive and negative regulators is crucial for the normal neurogenesis. Because *Drosophila* and mammals share in part common mechanisms for tissue differentiation, isolation of mammalian homologues of *Drosophila* ac-sc proteins (called MASH1 and MASH2) (6). MASH1 is transiently expressed by subsets of neuroepithelial and neural crest cells, and is suggested to be involved in neural determination (7).

We have recently isolated four rat factors structurally related to the *Drosophila* h and Enhancer-of-split (E(spl)) proteins (designated HES-1, -2, -3, and -4), and further characterized two of them, HES-1 and HES-3. The *Drosophila* h gene encodes a helix-loop-helix (HLH) type transcription factor and is required not only for late development as a regulator of sensory neurogenesis but also for early development as a pair-rule segmentation gene (8, 9). The *Drosophila* E(spl) genes, classified as neurogenic genes, also encode HLH factors (10). Both HES-1 and HES-3 have an HLH domain, and they show contrasting tissue distribution: HES-1 is expressed in a variety of tissues, while HES-3 is present exclusively in cerebellar Purkinje neurons. These results suggest that HES factors may be involved in the functions of various cell types and prompted us to further examine other related factors. In this study, we have isolated another mammalian HLH factor, designated HES-5, which has a distant homology only in the basic HLH (B-HLH) domain to *Drosophila* h and E(spl) proteins, and found that this novel factor is expressed predominantly in embryonal neural lineage cells.

**EXPERIMENTAL PROCEDURES**

cDNA Library Screening—Construction of cDNA library was performed as described previously (11). cDNA was synthesized by oligot(dT) priming of poly(A)* RNA of rat embryos of embryonic day 17 (E17), and ligated into the *X*gt10 vector. The cDNA fragment of HES-4 was used as a probe. 36 positives were obtained by screening 2.6 × 10^6 plaques, and the longest insert was subcloned into the EcoRI site of pBluescript II SK− (Stratagene) (pHES-5).

DNA-binding Analysis—The HES-5, E47, and CELF proteins were expressed in *Escherichia coli* and prepared as follows. The cDNA fragment of HES-5 (amino acid residues 1-166), E47 (473-end), and CELF (1-168) were ligated into either pGEMEX-1 (Promega) or pMNT, T7 expression plasmids. pGEMEX-1 was used for preparation of a fusion protein with a gene 10 product. pMNT was kindly provided by Dr. M. Nishizawa and Prof. S. Nagata (Osaka Bioscience Institute). JM109 (DE3) cells transformed by the expression vectors were treated with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h at 37 °C, and the extracted total cell proteins were applied to the SDS-polyacrylamide gel. The proteins were eluted from the gel, incubated in 6 M guanidine HC1 for 20 min, and dialyzed against 0.1 M KCl/HEPES (20 mM Hepes, pH 7.9, 1 mM MgCl2, 2 mM dithiothreitol, and 17% (v/v) glycerol) at 4 °C for 8 h, as described previously (11). The probe DNAs were prepared as follows. The double-stranded

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oligonucleotide fragments containing two N boxes (GGGCAACAGG and GCCACGAGTG) present in the promoter region of the E(spl)m8 gene, two repeats of a E2 sites (AGGGAGGTTGGCC), and two cAMP response elements were cloned into plBluescriptII KS— for the N box, E box, and cAMP response element probes, respectively. From each plasmid the Xhol-SaclII fragment labeled at the Xhol site was isolated and used as a probe.

The DNase I footprinting reactions were carried out in a total volume of 50 μl containing 5 ng of the probe, as described previously (11).

**Immunoprecipitation Analysis**—[35S]Methionine-labeled HES-5, MASH-1, ac, E47, and CELF were produced by a rabbit reticulocyte lysate (5 pl; Promega) and mixed with an excess amount of the gene 10-HES-5 fusion protein (200 ng) or the RNA was synthesized in the presence of [α-35S]CTP by T3 RNA polymerase and [35S]CTP according to the procedures described (12).

**Transient Transfection Analysis**—For the chloramphenicol acetyltransferase (CAT) reporter plasmids, the double-stranded oligonucleotides long and its molecular size 18.5 kDa. In the putative B-HLH domain (between amino acid residues 15 and 73), this factor showed 47, 36, 47, and 54% identity to h, E(spl), and HES-1, and HES-3, respectively, and thus we named this factor HES-5. The proline residue present in the basic region of HES-5 (amino acid residue 22), E(sp1) (lo), and hairy, and E(spl) proteins are indicated by an asterisk. Sources for sequences hairy (9), E(sp1) (10), N-myc (31), da (32), MyoD (33), MASH 1 (6), emc (34, 35), and Id (25), the carboxyl-terminal sequences are shown. The conserved residues are indicated by bold letters.

**RESULTS**

cDNA Cloning and Structural Analysis of HES-5—In the course of characterizing four HES factors, a cDNA clone for another HES-related protein was obtained from rat embryonal brain cDNA library. This clone had an insert of 1.3 kbp with the first methionine codon present at nucleotide residue 53 (Fig. 1A). Because there was no in-frame stop codon upstream of this methionine codon, we screened and examined 30 more independent cDNA clones. However, all of them started at similar positions (data not shown). Furthermore, Northern blot analysis showed that the mRNA was about 1.4 kb long (see Fig. 4A). This size agreed well with that of the cDNA we obtained, assuming that the poly(A) tail was approximately 100 bases. Therefore, the first ATG was assigned as an initiation codon, making this protein 166 amino acids long and its molecular size 18.5 kDa. In the putative B-HLH domain (between amino acid residues 15 and 73), this factor showed 47, 36, 47, and 54% identity to h, E(spl), MASH-1, and HES-3, respectively, and thus we named this protein HES-5. The probes were hybridized to the DNA-binding activity. The HES-5 protein was expressed in E. coli and subjected to the DNA-binding analysis. Although HLH factors such as MyoD have been shown to recognize a consensus sequence, CANNTG (called E box) (16), the introduction of a probe...
proline in the basic region inhibits the DNA-binding activity of E47. As shown in Fig. 2, the N box sequences were protected by HES-5 in the DNase I footprinting analysis. The addition of a 20-fold molar excess of other HLH factors, we next tested the possibility that the mutation of MyoD results in loss of the DNA-binding activity of HES-5 resulted in partial inhibition of the DNA-binding activity of E47 (lane 6). In contrast, a 20-fold and even a 100-fold molar excess of HES-5 did not alter the ability of CELF, which has a leucine zipper motif but not an HLH domain, to bind to the cAMP response element (lanes 10 and 11). These results suggested that the negative activity of HES-5 was specific for an HLH factor, although the interaction between HES-5 and E47 seemed rather weak.

To determine whether HES-5 directly interacts with an HLH factor, we performed coimmunoprecipitation experiments using a gene 10-HES-5 fusion protein and an anti-gene 10 antibody. As shown in Fig. 3, when the gene 10-HES-5 fusion protein and the [35S]methionine-labeled HES-5 protein were mixed, the anti-gene 10 antibody efficiently immunoprecipitated the HES-5 protein (lane 1), indicating an interaction between the gene 10-HES-5 fusion protein and HES-5. As a negative control, when the gene 10 protein was mixed with the HES-5 protein, the anti-gene 10 antibody did not immunoprecipitate the HES-5 protein (lane 2), suggesting that there is no interaction between the gene 10 and HES-5 proteins. Other HLH factors such as E47, ac, and MASH1 were also coimmunoprecipitated with the gene 10-HES-5 fusion protein by the anti-gene 10 antibody (lanes 3, 5, and 7). The amount of the coimmunoprecipitated E47 was much lower than those of HES-5, ac, and MASH1 (less than 5% compared to HES-5), suggesting that the interaction between HES-5 and E47 was relatively weak.

In contrast to the HLH factors, the leucine zipper protein CELF was not coimmunoprecipitated with the gene 10-HES-5 fusion protein (lane 9), indicating that HES-5 specifically interacted with HLH factors but not with a leucine zipper type protein.

Transcriptional Analysis of HES-5—To characterize the interaction of HES-5 and E47, we performed the coimmunoprecipitation experiments using a gene 10-HES-5 fusion protein and an anti-gene 10 antibody. As shown in Fig. 3, when the gene 10-HES-5 fusion protein and the [35S]methionine-labeled HES-5 protein were mixed, the anti-gene 10 antibody efficiently immunoprecipitated the HES-5 protein (lane 1), indicating an interaction between the gene 10-HES-5 fusion protein and HES-5. As a negative control, when the gene 10 protein was mixed with the HES-5 protein, the anti-gene 10 antibody did not immunoprecipitate the HES-5 protein (lane 2), suggesting that there is no interaction between the gene 10 and HES-5 proteins. Other HLH factors such as E47, ac, and MASH1 were also coimmunoprecipitated with the gene 10-HES-5 fusion protein by the anti-gene 10 antibody (lanes 3, 5, and 7). The amount of the coimmunoprecipitated E47 was much lower than those of HES-5, ac, and MASH1 (less than 5% compared to HES-5), suggesting that the interaction between HES-5 and E47 was relatively weak.

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transcriptional activity of HES-5, we carried out a transient transfection analysis using NIH 3T3 cells (Fig. 4). The HES-5 and E47 cDNAs were cloned into the eukaryotic expression vector containing the cytomegalovirus enhancer and promoter. Reporter plasmids were comprised of the CAT gene under the control of the β-actin promoter linked to either six repeats of the N boxes (pN6-βA-CAT) or seven repeats of the E boxes (pE7-βA-CAT).

HES-5 significantly reduced the expression from the promoter containing the N boxes, while E47 did not affect the expression from the same promoter (Fig. 4, lanes 2–4). The negative activity of HES-5 depended upon the N boxes, because it did not repress transcription from the control β-actin promoter (lane 10). In contrast, HES-5 did not change the expression from the promoter containing the E boxes (lane 8), while E47 significantly activated the expression from the same promoter (lane 6). However, when the HES-5 expression vector was cotransfected with the E47 plasmid, the E47-induced CAT expression was partially inhibited (lane 7). These results demonstrated that HES-5 acted as a transcriptional negative regulator both by directly interacting with the N boxes and by attenuating the E47 activity of binding to the E boxes.

Spatial and Temporal Distribution of the HES-5 mRNA—
To analyze the tissue distribution and the ontogenetic expression pattern of HES-5, we performed Northern blot experiments. As shown in Fig. 5A, a single 1.4-kb band was detected

Fig. 4. Transcriptional analysis of HES-5. Full length cDNAs of HES-5 and E47 were cloned into the eukaryotic expression vector pSV-CMV. The CAT reporter plasmids contained the β-actin promoter linked to either six repeats of the N boxes (pN6-βA-CAT), seven repeats of the E boxes (pE7-βA-CAT), or no additional elements (pβA-CAT). 10 µg of pN6-βA-CAT were transfected into NIH 3T3 cells with either 8 µg of the control vector pSV-CMV alone (lane 1), 1 µg (lane 2), or 5 µg of the HES-5 plasmid (lane 3), or 5 µg of the E47 plasmid (lane 4). 2 µg of pE7-βA-CAT were transfected with either 16 µg of the control vector pSV-CMV alone (lane 5), 5 µg of the E47 plasmid (lane 6), 5 µg each of the E47 and HES-5 plasmids (lane 7), or 5 µg of the HES-5 plasmid (lane 8). As a control experiment, 2 µg of pβA-CAT were transfected with either 16 µg of the control vector pSV-CMV alone (lane 9), 5 µg of the HES-5 plasmid (lane 10), or 5 µg of the E47 plasmid (lane 11). The total DNA amounts were adjusted with the control vector pSV-CMV. Cells were harvested 48 h later, and CAT activities were measured. Each value of relative CAT activities is the average of at least four independent experiments.

Fig. 5. Temporal and spatial distribution of the HES-5 mRNA. A, Northern blot analysis. 5 µg of poly(A)+ RNAs were analyzed by using the EcoRI fragment of pHES-5 as a probe. Tissue names are indicated above each lane. E, embryonic day; P, postnatal day; CNS, central nervous system. As a control experiment, the γ-actin probe was used. B, RNase protection analysis. 30 µg of total RNA of rat adult brain (lane 2) or embryonal tissues (lanes 3–11) were hybridized with the antisense cRNA of HES-5. The synthesized probe containing 131 nucleotides of the vector and 926 nucleotides of the anti-sense cRNA (total of 1057 nucleotides) and the protected band (926 nucleotides) are shown on the left. Human RNA prepared from HeLa cells (lane 12) was used as a negative control. The control experiments using the HES-1 probe are shown below. GI, gastrointestinal tract.
in the brain tissues (lanes 2 and 3), but not in the liver or kidney (lanes 4 and 5). The HES-5 mRNA was produced at a high level in the embryonal tissues (lanes 1 and 2), but significantly decreased in the adult brain (lane 3). In the eye, two species of 1.4 and 1 kb were expressed at a high level until postnatal day 6 (lanes 6 and 7), but sharply decreased to an almost undetectable level by postnatal day 14 (lane 8).

To examine the HES-5 mRNA expression pattern more precisely, we next conducted RNase protection assays. As shown in Fig. 5B, the HES-5 mRNA was produced only in the neural tissues of adults (lane 2) and embryos (lanes 3 and 4), but not in the peripheral tissues of embryos (lanes 5–11) or adults (data not shown). These results suggested that the HES-5 mRNA was predominantly expressed by neural tissues.

To determine the cell types expressing the HES-5 mRNA, we next carried out in situ hybridization experiments. On embryonic day 15.5, the HES-5 mRNA was detected throughout the central nervous system and was expressed at a high level in the wall of the brain vesicles (Fig. 6A). The areas of prominent expression included the telencephalon, diencephalon, mesencephalon, metencephalon, myelencephalon, and spinal cord (A). On embryonic day 18.5, the HES-5 mRNA was still produced at a high level in the developing nervous system (C). Higher magnification indicated that strong signals were present in the ventricular zones adjacent to the cerebral ventricles (E). The less intense signals were also detected in the immature neurons present in the outer layer (E). The HES-5 mRNA was also produced at a high level in the outer nuclear layer of the neural retina (F), and at a moderate level in the embryonal dorsal root ganglia (data not shown). In contrast, only weak and diffuse expressions of the HES-5 mRNA were observed in the adult brain (G), including cerebellar Purkinje cells (H). In the adult brain, the olfactory bulb produced a relatively high amount of the HES-5 mRNA. These results suggested that the HES-5 mRNA was expressed predominantly in the developing nervous system and that the mRNA level sharply decreased as neural differentiation proceeded.

**DISCUSSION**

**HES Family, HLH Factors of the Mammalian Nervous System**—In this study, we have described the molecular characterization of the neurospecific HLH factor HES-5 that represses transcription. HES-5 has a related but distinct B-HLH domain when compared to HES-1 and HES-3. Furthermore, these three HES factors exhibit different patterns of spatial and temporal tissue distribution. HES-1 is a ubiquitous factor produced by both embryos and adults and is also expressed by neural progenitor cells in the embryonal brain. In contrast, HES-3 is produced specifically by cerebellar Purkinje cells after birth. HES-5, on the other hand, is expressed at a high level in neural progenitor cells as well as at a low level in mature neurons such as Purkinje cells, thus overlapping with the HES-1 and HES-3 expressions. These results show that the expressions of HES factors are regulated at various stages of the mammalian neural differentiation, suggesting critical involvement of HES factors in neural development. Although the *Drosophila* h and E(spl) factors have been shown to regulate normal sensory neurogenesis, further studies will be necessary to provide direct evidence regarding the roles of HES factors in mammalian neural development.

**HES-5, a Negative Regulator of Neural Progenitor Cells**—The HES-5 mRNA is most abundant in the ventricular zones of the embryonal brain, where neuroepithelial cells proliferate (19, 20). In contrast, the level of the HES-5 mRNA is low in the adult brain, where neurogenesis mostly ceases. These results indicate that the HES-5 mRNA decreases as neural differentiation proceeds. A similar change of the HES-5 mRNA level is also observed in the neural retina. In the retina, a high level of the HES-5 mRNA expression continues until postnatal day 6, by which time retinal progenitor cells undergo their final division in rodents (21–23). At or after this division, these cells differentiate into various types of neurons and glial cells (24), coinciding with a decrease of the HES-5 mRNA, the level of which is quite low on postnatal day 14. These features of a high expression in progenitor cells and a low expression in mature cells, together with the repressor activity, indicate the similarity between HES-5 and Id, a negative regulator of the muscle determination factor MyoD (25). In undifferentiated myoblasts, Id is expressed at a high level and inhibits the expression of the muscle-specific genes, but decreases upon terminal differentiation into muscles. Thus, it is possible that HES-5 may prevent transcription of the genes that are programmed to express in differentiated neurons or glial cells. As neural differentiation proceeds, HES-5 sharply decreases, but remains at a low level in mature neurons such as Purkinje cells. Whether the decrease of HES-5 to this low level by itself results in gene activation, or whether another HLH activator that antagonizes HES-5 is necessary to activate gene expression should be further tested.

Identification of the target genes regulated by HES-5 is a very important problem. Our preliminary results of cotransfection assays indicate that HES-5 negatively regulates the transcription of the *Pcp-2/L7* gene, which is specifically expressed postnatally in Purkinje cells and retinal bipolar neurons (26, 27), thus raising the possibility that HES-5 may be involved in repression of the *Pcp-2/L7* gene expression in the precursor cells. Interestingly, the presence of a transcriptional repressor interacting with the *Pcp-2/L7* gene promoter has been implicated from the transgene analysis (28), and thus the relationship between HES-5 and the repressor indicated by the transgene analysis should be further studied.

**Intercommunication between HES-5 and Other Factors**—HES-5 negatively regulates transcription by directly interacting with N boxes as well as by inhibiting other HLH activators from binding to E boxes. The mechanism of how HES-5 represses transcription by binding to N boxes is an intriguing problem. Other HLH negative regulators such as Id and HLH462 do not bind to the DNA template by themselves but inhibit other activators from binding by forming nonfunctional heterodimers (25, 29). Because HES-5 binds to the DNA template by itself, its repressor activity seems unique compared to Id and HLH462. HES-5 may simply compete for binding to E boxes. The mechanism of how HES-5 inhibits the E47 activity is another interesting question. HES-5 does not alter the DNA-binding activity of CELF, a leucine zipper type transcription factor.
FIG. 6. In situ hybridization analysis of HES-5 with embryo (E15.5 and E18.5) and adult tissue sections. The HES-5 probe was hybridized to the sagittal sections of embryos of E15.5 (A) and E18.5 (C–F) and adult brains (G and H). A, strong signals are detected in the wall of the embryonal brain vesicles including the telencephalon, diencephalon, and mesencephalon as well as in the spinal cord, whose positions are indicated in B, C, high expression is observed in the central nervous system of E18.5. LV, lateral ventricle; SC, spinal cord. D, the control experiment was carried out with excess cold cRNA to evaluate specific signals in C. E, higher magnification of the regions surrounding the lateral ventricle in C is shown. Strong and less intense signals are seen in the ventricular cells adjacent to the cerebral ventricle and the immature neurons in the outer layers, respectively. F, the upper part of the embryo is shown. The outer nuclear layer of the embryonal retina expresses a high level of the HES-5 mRNA. G, weak and diffuse expression is observed in the adult brain. The olfactory bulb (OB) produces a relatively high level of the HES-5 mRNA. Cb, cerebellum. H, higher magnification of the adult cerebellum is shown. The HES-5 transcript is detected in Purkinje cells (indicated by arrowheads). Photos were taken in a dark field (A and C–G) or a bright field (H). Bars represent 10 mm (A, C, F, G), 0.75 mm (E), or 50 μm (H).

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factor, and thus its negative activity seems specific for an HLH factor. Therefore, it is likely that heterodimer formation through HLH domains may be responsible for the negative activity of HES-5. However, the negative effect of HES-5 against E47 is only partial in the DNase I footprinting experiment as well as in the transient transfection assay, and it is possible that HES-5 more strongly inhibits different HLH factors that recognize E boxes. Those HLH factors, on the other hand, could regulate the HES-5 activity, and this reciprocal interaction may help to produce the diversity in tran-
spatiotemporal control. Further studies will reveal more detailed relationships between HES-5 and other HLH factors and help understand the molecular mechanisms of complex mammalian neural development.

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