NOTE TO USERS

Mechanisms of Hairy/Enhancer of Split 6 Function During Mammalian Neuronal Development

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CONTRIBUTIONS OF AUTHORS

Chapter 2. Gratton et al. (2003). Hes6 promotes cortical neurogenesis and inhibits Hes1 transcription repression activity by multiple mechanisms. *Mol. Cell. Biol.* 23, 6922-6935. I performed experiments shown in Figs. 2-6, 7b, 8, 9, 10a, b. Elena Torban performed studies shown in Figs 1 and 7, Stéphanie Bélanger-Jasmin performed studies shown in Figs. 10c and 10d, Franscesca M. Thériault contributed to the studies shown in Figure 1, and Dr. Michel S. German provided the constructs pFOX-Luc1, pFOX-ngn3p-Luc1 and pFOX-ΔN-box-ngn3p-Luc1. Dr. Stifani wrote the paper.

ABSTRACT

Hes1 is a mammalian basic helix loop helix (bHLH) factor that inhibits neuronal differentiation by mediating transcription repression mechanisms together with corepressors of the Gro/TLE family. The interaction of Hes1 with Gro/TLE is mediated by a WRPW tetrapeptide present at the carboxy-terminus of all Hes proteins. Another Hes protein, Hes6, also interacts with Gro/TLE through its WRPW motif. Contrary to Hes1, Hes6 promotes neuronal differentiation. It is shown here that Hes6 negatively regulates Hes1 activity by at least two mechanisms. Hes6 promotes a proteolytic degradation of Hes1. Moreover, Hes6 inhibits the interaction of Hes1 with its transcriptional corepressor, Gro/TLE. Hes6 inhibits Hes1-mediated transcriptional repression in cortical neural progenitor cells. Consistent with these observations, Hes6 promotes the differentiation of cortical neurons, a process normally inhibited by Hes1. Taken together, these results clarify molecular mechanisms underlying the neurogenic activity of Hes6.

RÉSUMÉ

Hes1 est une protéine chez les mammifères possédant une structure basique hélice boucle hélice qui inhibe la différentiation neuronale par des mécanismes de répression de la transcription par les co-répresseurs Gro/TLE. L'interaction entre Hes1 et Gro/TLE implique un tétrapeptide WRPW présent à l'extrémité carboxylique des protéines Hes. Hes6 interagit également avec Gro/TLE par le motif WRPW. Contrairement à Hes1, Hes6 induit la différentiation neuronale. Il est présenté ici que Hes6 contrôle négativement l'activité de Hes1 en utilisant au moins deux mécanismes. Hes6 fait la promotion d'un processus de dégradation de Hes1. De plus, Hes6 inhibe l'interaction entre Hes1 et Gro/TLE. Hes6 inhibe la répression transcriptionnelle médiée par Hes1 dans des cellules progénitrices neuronales corticales. En relation avec ces observations, Hes6 induit la différentiation de neurones corticaux, un processus normalement inhibé par Hes1. Ainsi, ces résultats clarifient les mécanismes moléculaires impliqués dans l'activité neurogenique de Hes6.

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INTRODUCTION

Neurogenesis involves the initial proliferation of neural progenitor cells, followed by their differentiation into post-mitotic neurons. Neurogenesis is regulated through the antagonistic activities of positive and negative regulators. These include transcription factors that contain the basic helix-loop-helix (bHLH) DNA-binding and dimerization motif (reviewed in Kageyama and Nakanishi, 1997; Anderson, 2001; Bertrand et al., 2002). Evolutionarily conserved bHLH factors that promote neurogenesis ("neurogenic proteins") include the Mash and Neurogenin families of proteins, which are homologs of the *Drosophila* proneural proteins Achaete-Scute and Atonal (Guillemot et al., 1993; Guillemot, 1995; Fode et al., 1998; Ma et al., 1998; Bertrand et al., 2002; Lo et al., 2002).

bHLH factors that inhibit neurogenesis ("antineurogenic proteins") include members of the Hairy/Enhancer of split (Hes) family of proteins, mammalian homologues of *Drosophila* Hairy and Enhancer of split (Akazawa et al., 1992; Sasai et al., 1992; Nuthall et al., 2002). In both invertebrates and vertebrates, most Hes proteins are DNA-binding, long range transcriptional repressors; in mammals, in particular, Hes1 and Hes5 are well-characterized negative regulators of neuronal differentiation (Akazawa et al., 1992; Sasai et al., 1992; Ishibashi et al., 1994; Kageyama and Nakanishi, 1997; Nakamura et al., 2000). *Hes* genes encode transcriptional regulators that all share a WRPW tetrapeptide motif at the extreme C-terminus. This motif has been shown to mediate interaction with the general transcriptional corepressors Groucho/Transducin-like Enhancer of split (Gro/TLE) (Paroush et al., 1994; Fisher et al., 1996; Grbavec and Stifani, 1996; McLarren et al., 2001). The ability of Hes proteins to recruit Gro/TLE factors is an important event in their repressive functions (Fisher et al., 1996; McLarren et al., 2001; Nuthall et al., 2002). Removal of the WRPW motif reduces Hes-1 mediated transcriptional repression while overexpression of Gro/TLE potentiates transcriptional repression mediated by Hes1 (Fisher et al., 1996;

Ohtsuka et al., 1999; McLarren et al., 2001; Nuthall et al., 2002). Gro/TLE are therefore transcriptional co-repressors of Hes1, through the formation of Hes1:Gro/TLE complexes that have the ability to repress transcription of target genes.

Another protein that has recently been implicated in the regulation of Hes1 activity is the related Hes family member, Hes6. Recent studies suggest that Hes6 could be involved in inhibitory mechanisms that antagonize the transcription repression ability of Hes:Gro/TLE complexes (Bae et al., 2000). Hes6 shares most of the characteristic features of Hes proteins but it has a shortened loop region and lacks the ability to bind to canonical Hes DNA binding sites, the E- or N- boxes, on its own (Bae et al., 2000; Koyano-Nakagawa et al., 2000). In addition, evidence suggests that Hes6 might function as a positive regulator of neuronal differentiation, in contrast to Hes1 and Hes5 (Bae et al., 2000; Koyano-Nakagawa et al., 2000). Hes6 is highly expressed in the developing nervous system in both neural precursor cells and post-mitotic neurons (Bae et al., 2000; Koyano-Nakagawa et al., 2000; Pissarra et al., 2000; Vasiliauskas and Stern, 2000), and in the developing mouse retina, Hes6 induces neuronal differentiation (Bae et al., 2000). Moreover, when ectopically expressed in the *Xenopus* neural plate, Hes6 promotes neuronal differentiation (Koyano-Nakagawa et al., 2000). The mechanisms underlying the neurogenic function of Hes6 are, however, poorly understood.

Taking into consideration that Hes6 1) forms heterodimers with Hes1 in transfected non-neural cells (Bae et al., 2000), 2) it cannot bind canonical Hes DNA-binding sites and that 3) it does not interfere with the ability of Hes1 to bind to N-boxes, but reduces Hes-1 mediated repression in transfected non-neural cells (Bae et al., 2000), we formulated the following hypothesis: *Hes6 promotes neuronal differentiation by acting as a negative regulator of Hes1.* We have therefore investigated this possibility, as well as the underlying molecular mechanisms.

Here we describe experiments showing that Hes6 negatively regulates Hes1 activity by a combination of at least two mechanisms. Hes6 promotes proteolytic degradation of Hes1. In addition, Hes6 inhibits the interaction of Hes1 with Gro/TLE. In agreement with these findings, both Hes6 and a truncated form of Hes6 lacking the WRPW motif inhibit Hes1-mediated transcriptional repression in cortical neural progenitor cells. Moreover, Hes6 promotes cortical neuronal differrentiation, a process normally inhibited by Hes1, and removal of the WRPW motif has a hypomorphic effect on this function, suggesting that Hes6 may promote neurogenesis through both WRPW motif-dependent and independent mechanisms. Taken together, these findings identify novel mechanisms through which Hes6 may act as a negative regulator of Hes1 activity and a positive regulator of neuronal differentiation.

CHAPTER I: LITERATURE REVIEW

BASIC HELIX LOOP HELIX PROTEINS AS POSITIVE AND NEGATIVE REGULATORS OF NEUROGENESIS

The neocortex of the mammalian central nervous system (CNS) derives from mitotic neuroepithelial cells in the ventricular zone of the telencephalon. It is from this region that neural precursor cells arise, proliferate, and then exit the cell cycle at defined time points to generate the complex repertoire of neurons that constitute the neocortex. The commitments of neural progenitor cells to the neuronal fate and the subsequent progression to mature neurons are controlled by both extrinsic and intrinsic factors. The proper timing between the growth phase of neural progenitor cells and the differentiation phase of post-mitotic neurons is essential to generate the normal number of cells during nervous system development. The underlying mechanisms controlling this transition are not well understood but invertebrate and vertebrate studies suggest that the antagonistic activities of transcription factors containing a bHLH motif play crucial roles.

1 NEUROGENIC BHLH PROTEINS

A family of conserved bHLH proteins promotes neurogenesis by acting as transcriptional activators of genes that promote the acquisition of the neuronal phenotype (reviewed in Lee, 1997). These bHLH proteins are collectively referred to as proneural proteins. In vertebrates, proneural bHLH proteins include factors that are structurally and functionally related to the product of the Drosophila *atonal* gene, such as Neurogenins, NeuroD, Math1 and Math3, and other proteins related to the Drosophila achaete (ac) and scute (sc) proteins, such as Mash1, Xash3 or Cash4 (reviewed in Campos-Ortega, 1993; Jan and Jan, 1994). Similar to their Drosophila counterpart, the targeted disruption of proneural genes such as *Mash1*, *Ngn1*, *Ngn2* and *Math1* in mice lead to the loss of specific subsets of

neurons due to inhibition of neuronal differentiation (Guillemot et al., 1993; Ben-Arie et al., 1997; Fode et al., 1998; Ma et al., 1998; Miyata et al., 1999). As an example, mice carrying a null mutation of *Mash1* have severe defects in neurogenesis in the ventral telencephalon and olfactory sensory epithelium (Guillemot et al., 1993; Casarosa et al., 1999; Horton et al., 1999; Cau et al., 2002). On the other hand, the ectopic expression of these vertebrate proneural genes results in the differentiation of supernumerary neurons, thus promoting ectopic neurogenesis. This has been observed when Neurogenins are ectopically expressed in chick or Xenopus embryos, or mammalian cells (Lee, 1995; Takebayashi et al., 1997; Perez et al., 1999; Farah et al., 2000). Proneural bHLH proteins form heterodimeric complexes with the ubiquitously expressed bHLH E proteins. In Drosophila, such E proteins are encoded by the gene *daughterless*. In mammals, they include the products of the gene *E2A* (which produces two alternatively spliced products termed E12 and E47), and the factors HEB and E2-2 (Cabrera and Alonso, 1991; Johnson et al., 1992; Massari and Murre, 2000). Heterodimeric complexes of proneural proteins and E proteins bind specifically to DNA sequences that contain an E-box (CANNTG), a process that results in an activation of transcription of the target genes (Johnson et al., 1992; Sasai et al., 1992). Therefore, factors that interfere with proneural E-protein complex formation or with the ability of these complexes to bind to the E-box act as repressors of proneural protein transcriptional activity. Thus, proneural proteins are evolutionary conserved bHLH factors, which form complexes with E proteins to act as transcriptional activators of target genes that promote neuronal differentiation.

2 ANTI-NEUROGENIC bHLH PROTEINS

2.a Structure of the bHLH transcriptional repressors of the Hes family

The second group of bHLH proteins involves factors that antagonize neuronal differentiation in both invertebrates and vertebrates by inhibiting the function of genes that promotes the neuronal fate such as the proneural genes. These factors include members of the Hairy/Enhancer of split (Hes) family of proteins (Akazawa et al., 1992; Sasai et al., 1992; Nuthall et al., 2002). These phylogenetically conserved bHLH transcriptional regulators all share two crucial structural similarities: a bHLH domain that contains a proline residue at a conserved position in the basic region, and a conserved WRPW tetrapeptide motif located at the extreme C-terminus. Mutation analysis in *Drosophila* originally suggested that both of these structural features are important for Hes protein activity. (Wainwright and Ish-Horowicz, 1992). Hence, Hes are antineurogenic bHLH factors containing a WRPW motif at their C-termini and an invariant Pro residue within the basic arm of their bHLH motif.

2.a.1 The bHLH domain as a dimerization motif and a DNA binding motif

The HLH portion of the bHLH motif acts as a protein dimerization region (Ferre-D'Amare et al., 1993; Ellenberger et al., 1994; Ma et al., 1994). The crystal structure of bHLH proteins such as the myogenic bHLH protein MyoD shows that the interaction between the two helices of each bHLH partners leads to the formation of dimers (Ma et al., 1994). The ability of theses bHLH proteins to form dimers is an essential functional event since it is a prerequisite for DNA binding (Ferre-D'Amare et al., 1993; Ellenberger et al., 1994). In agreement with this, members of the Hes family, including mammalian Hes1 and Hes5, have the ability *in vitro* to form homodimers or heterodimers via their HLH domain (Akazawa et al., 1992; Sasai et al., 1992; Van Doren et al., 1994). This ability was also demonstrated using yeast two-hybrid assays (Alifragis et al., 1997). Whereas the HLH portion of the bHLH

motif acts as a dimerization motif, the basic arm mediates DNA binding (Tietze et al., 1992). In fact, *Drosophila* extramacrochaetae and the vertebrate Id proteins have a HLH domain, but lack an adjacent basic arm. As a result, these proteins have the ability to form heterodimers with other bHLH factors but are unable to bind DNA (Massari and Murre, 2000; Campuzano, 2001; Davis et al., 2001). Moreover, recent studies have shown that the basic region of the bHLH domain is not the only feature important for DNA-binding. The length of the loop region in the helix-loop-helix domain is another critical feature for DNA binding activity and a sole loop region residue is crucial for high affinity DNA binding as shown with *Drosophila* bHLH transcription factor Deadpan by Winston et al. (2000). In sum, the HLH portion of the bHLH motif acts as a protein dimerization region while the basic arm and the loop region mediate DNA binding.

2.a.2 Hes proteins contain a particular type of bHLH domain

A unique feature of the Hes factors is the presence of a proline residue in the basic arm of the bHLH domain, which is absent in other bHLH factors that bind to the E-box. The introduction of a proline residue into the basic region of other bHLH factors can lead to the loss of the E-box-binding activity (Davis et al., 1990). Indeed, Hes proteins bind more poorly to the E box than to the N-box (CACNAG). Thus, homodimers of Hes1 and Hes5 preferentially bind to the N-box (Akazawa et al., 1992; Sasai et al., 1992), although they can also bind to an E-box (Hirata et al., 2000). Hes1 homodimers also have the ability to bind to class C DNA binding sites (CACGCA) as shown by previous studies in cultured cells done by Chen et al. (1997), which suggested that Hes1 can repress transcription of *Hash1* (Human achaete-scute homologue-1) gene by directly binding to its promoter region. Therefore, these studies indicate that the proline residue in the basic arm of the bHLH domain plays a key role in the affinity of Hes protein homodimers with specific DNA binding sequences.

2.a.3 The WRPW motif of Hes proteins interacts with the transcriptional co-repressor Gro/TLE

Another typical feature of Hes family members is the presence of a conserved protein-protein interaction motif Trp-Arg-Pro-Trp (WRPW) at their carboxy-terminus. This motif has been shown to mediate interaction with the general transcriptional corepressors Groucho/Transducin-like Enhancer of split (Gro/TLE) (Parouch et al., 1994; Fisher et al., 1996; Grbavec and Stifani, 1996; Grbavec et al., 1998; McLarren et al., 2001). In *Drosophila*, genetic studies demonstrate that the loss of *groucho* function and the loss of *Hes* gene function result in the same phenotype, namely the production of supernumerary neurons (Delidakis et al., 1991; Schrons et al., 1992; Paroush et al., 1994; Heitzler et al., 1996). Mutation that consists of a deletion of the WRPW motif results in the inability of Hes proteins to interact with Groucho (Fisher et al., 1996). These findings indicate that in invertebrates, Hes proteins form transcription repression complexes with Groucho. This interaction is also observed between mammalian Hes proteins, such as Hes1, and homologues of Drosophila Groucho, the TLE1-4 proteins. The interaction of Hes proteins with Gro/TLE is a key event in the promotion of Hes transcription repression activities (see below "Gro/TLE interaction with Hes1 is required for transcriptional repression"). Thus, the WRPW motif of Hes proteins mediates an interaction with Gro/TLE transcriptional corepressors.

2.b Hes1 inhibits neuronal differentiation

2.b.1 Biological function of Hes1 and Hes5 during mammalian neurogenesis

Two members of the Hes family of proteins, Hes1 and Hes5, have key roles in the timing of differentiation in telencephalic development due to their antineurogenic function (Akazawa et al., 1992; Sasai et al., 1992; Ishibashi et al., 1994; Kageyama and Nakanishi, 1997; Nakamura et al., 2000). The role of Hes1 and Hes5 in neurogenesis is supported by expression studies that revealed that both are generally expressed in restricted regions of the developing mammalian nervous system containing undifferentiated neural precursors (Akazawa et al., 1992; Sasai et al., 1992; Ishibashi et al, 1995). As such, Hes1 and Hes5 are expressed at high levels throughout the ventricular zone of the developing telencephalon, which consists of neural precursor cells, but the level decreases as neural differentiation proceeds in the outer layers (Akazawa et al., 1992; Sasai et al., 1992). A similar pattern is also seen in the developing retina where Hes1 is expressed in retinal progenitor cells (Tomita et al., 1996). Furthermore, gain and loss of function analysis have clarified the role of Hes1 in neuronal development. The constitutive expression of Hes1 in neural precursors, using a retroviral vector, prevented neuronal differentiation in brain (Ishibashi et al., 1995), retina (Tomita et al., 1996), and primary rat hippocampal neural precursors (Castella et al., 1999). Conversely, targeted disruption of the *Hes1* gene in mice leads to premature differentiation of neurons in the telencephalon (Ishibashi et al., 1995), retina (Tomita et al., 1996) and causes severe defects during neural tube formation (Ishibashi et al., 1995; Tomita et al., 1996; Ohtsuka et al., 1999). On the contrary, *Hes5*-null mice are morphologically normal in spite of premature neuronal differentiation while transient misexpression of Hes5 keeps embryonic telencephalic cells undifferentiated, which suggests that Hes5 inhibits neurogenesis (Ohtsuka et al., 1999; Ohtsuka et al., 2001). Together, these findings demonstrate that Hes1 and Hes5 have the ability to inhibit neuronal differentiation.

2.b.2 Hes1 and Hes5 are essential Notch signaling effectors during neuronal differentiation

Notch signaling controls cell fate decisions and other developmental process in both invertebrates and vertebrates (reviewed in Artavanis-Tsakonas et al., 1999). Several lines of studies show that, similar to *Drosophila* Hes proteins, Hes1 and Hes5 act as nuclear effectors of the Notch signaling pathway during mammalian neurogenesis. The overexpression of Hes1, Hes5 and a constitutively active form of Notch (caNotch) results in the same phenotype, namely the inhibition of neuronal differentiation (Ishibashi et al., 1994; Chitnis et al., 1995; Ohtsuka et al., 2001). Translocation of caNotch into the nucleus leads to a complex formation with the mammalian homolog of *Drosophila* Suppressor of Hairless, the recombination signal-binding protein-Jκ (RBP-Jκ) (Furukawa et al. (1992). This complex has the ability to activate *Hes1* and *Hes5* promoters through the RBP-J-binding sites and can directly induce transcription of Hes1 and Hes5 by interacting with its promoter element (Jarriault et al. (1995), Hsieh et al. (1997), Nishimura et al. (1998)). Thus, a caNotch can induce the endogenous expression of Hes1 and Hes5. This is also supported by the fact that, in mice mutant for *Notch1* or *RBP-Jκ* the expression of Hes5 is altered (de la Pompa et al. (1997), Barrantes et al. (1999)), whereas treatment with the Notch ligand Delta induces the endogenous expression of Hes1 or Hes5 in neighbouring cells (Jarriault et al. (1998), Wang et al. (1998)). In addition, retrovirally misexpressing caNotch in neural precursor cells prepared from wt, *Hes1*-null, *Hes5*-null and *Hes1-Hes5* double-null mutant mouse embryos induces endogenous expression of Hes1 and Hes5 and inhibits neuronal differentiation in the wt, *Hes1*-null and *Hes5*-null background, but not in the *Hes1-Hes5* double-null background (Ohtsuka et al. (1999)). These findings demonstrate that Hes1 and Hes5 are essential Notch effectors in the regulation of mammalian neuronal differentiation even though they are not

functionally equivalent. Together these findings demonstrate that Hes1 is a crucial negative regulator of neuronal differentiation in mammals.

2.c Mechanisms involved in Hes1 function

2.c.1 Active transcriptional repression

2.c.1.1 Direct binding of Hes1 to target gene promoters leads to transcriptional repression

An active transcriptional repression mechanism consists of direct binding of a transcriptional regulator to DNA sequences, leading to the repression of a target gene. As described previously, Hes homodimers bind DNA through class C or N box consensus sites. A few possible Hes1 target genes have been identified by the presence of these target sequences in their promoters (Akazawa et al. (1992), Sasai et al. (1992), Ishibashi et al. (1993), Hirata et al. (2000)). One example is the *Hes1* gene itself. In reporter gene assays in cultured mammalian cells, mutations of the N-box sequences in the *Hes1* promoter suggests that Hes1 negatively autoregulates itself (Takebayashi et al. (1994)). Another potential target gene of Hes1 is *Neurogenin3* (*Ngn3*), which contains several N-boxes in its promoter (Lee (2001)). Ngn3 is a pro-endocrine factor in the developing pancreas and induces differentiation of pancreatic progenitors cells to become islet cells (Gradwohl et al. (2000)). Interestingly, Ngn3 is also expressed in limited regions of the developing spinal cord and hypothalamus (Sommer et al. (1996)). Hes1 has the ability to bind the *Ngn3* promoter's N-boxes and specifically negatively regulates *Ngn3* activity (Lee (2001)). As mentioned previously, mammalian homologues of *Drosophila ac-sc* such as *Hash1* and *Mash1* are other potential target genes of Hes1 (Chen et al. (1997)). Consistently, biological studies have revealed that targeted disruption of the *Hes1* gene in mice prematurely upregulates *Mash1* mRNA levels (Ishibashi et al. (1995); Cau et al. (2000)). Loss of *Hes1* function results in earlier

differentiation of neural precursor cells into mature neurons in the telencephalon and, in the olfactory placodes, in an increase in the density of Mash1-positive progenitors and an excess of neurons (Ishibashi et al. (1995); Cau et al. (2000)). The involvement of Hes1 in the regulation of the expression of mammalian homologues of *Drosophila ac-sc* genes resembles the transcriptional inhibition of the expression of the *achaete* gene by Hairy through binding to class C binding sites in its promoter (Ohsako et al. (1994), Van Doren et al. (1994)). Therefore, these data suggest that Hes1 functions as a negative regulator of neurogenesis by repressing proneural gene activity.

2.c.1.2 Gro/TLE interaction with Hes1 is required for transcriptional repression

The active transcriptional repression mediated by Hes1 requires the WRPW tetrapeptide at its extreme carboxy-terminus. This motif mediates interaction with the general transcriptional corepressors Gro/TLE. Hes1 and TLE1 are coexpressed in the mammalian developing nervous system (Sasai et al. (1992), Dehni et al. (1995), Yao et al. (1998), Allen and Lobe (1999)) and persistent expression of Hes1 or TLE1 in the mammalian forebrain leads to similar phenotypes characterized by a loss of neurons (Ishibashi et al. (1994), Yao et al. (2000)). More precisely, TLE1 is expressed in mitotic neural precursor cells, but its expression is down-regulated with the generation of new post-mitotic neurons, which suggests that TLE1 may play a role in the negative regulation of neuronal differentiation, like *Drosophila* Gro proteins (Dehni et al. (1995); Yao et al. (1998)). These findings, together with the demonstration that mutations that disrupt the interaction between Drosophila Hes and Groucho proteins also result in an inhibition of Hes ability to repress transcription, raised the possibility that Gro/TLE may be involved in transcriptional repression mediated by Hes1 (Parouch et al. (1994), Fisher et al. (1996), Jimenez et al. (1997)). In agreement with this, the overexpression of Gro/TLE potentiates transcriptional

repression mediated by Hes1 (McLarren et al. (2001)). In addition, the binding of Hes1 to Gro/TLE promotes the hyperphophorylation of Gro/TLE. This Hes1-induced hyperphosphorylation is correlated with a tighter association of Gro/TLE with nuclei and enhanced transcriptional repression (Nuthall et al. (2002)). A transcriptional corepressor function for TLE proteins was also demonstrated by studies that showed a functional interaction between TLE and a variety of DNA-binding factors that can mediate transcriptional repression, such as Runt-homology domain proteins (Levanon et al. (1998), Javed et al. (2000), McLarren et al. (2000)), homeodomain proteins containing engrailed-homology region 1 motifs (Eberhard et al. (2000), Muhr et al. (2001)), and winged-helix transcription factors (Wang et al. (2000), Yao et al. (2001). Thus, interactions with TLE proteins positively regulate the transcriptional repressive activity of Hes1; the latter provides a DNA-binding function while Gro/TLE provides a transcriptional repression function.

2.c.1.3 Gro/TLE family of proteins has a transcriptional repressive function

Two mechanisms are though to be involved in transcriptional repression by Gro/TLE family members. Previous studies have shown that oligomeric structures of Gro/TLE can interact with both histones (Palaparti et al. (1997), Chen et al. (1998), Flores-Saaib and Courey (2000)) and histone deacetylases (Chen et al. (1999), Choi et al. (1999), Brantjes et al. (2001), Yao et al. (2001)). In particular, it was found that Gro proteins form *in vivo* complexes with Rpd3, a *Drosophila* histone deacetylase (Chen et al. (1999)). The overexpression of Rpd3 in cultured cells potentiates repression of reporter genes containing Gal4-binding sites by Gal4-Gro chimeric proteins and mutations of Rpd3 that inhibits the ability of Rpd3 to deacetylate histones also prevents this potentiation (Chen et al. (1999)). Thus, this study suggested that histones deacetylation contributes to Gro-mediated transcriptional repression. It is proposed that this repression mechanism is the result of the

recruitment of histone deacetylases to DNA, which leads to the removal of acetyl groups from lysine residues in the amino-terminal domains of core histones (see reviews: Workman and Kingston (1998), Ayer (1999)). Therefore, the local chromatin structure would be altered to presumably prevent gene transcription since hypoacetylated chromatin correlates with repressed transcriptional states (see review Struhl (1998)). In addition, the possibility has been raised that Gro/ TLE proteins may inhibit the activity of the basal transcriptional machinery through interaction with the TFIIE factor. Yu et al. (2001) presented evidence showing that the amino-terminal Enhancer of split (AES) protein, a member of the Groucho/TLE family, interacts specifically with the basal transcription factor TFIIE in nuclear extract. This possibility is consistent with the observation that the protein TUP1, a general co-repressor thought to represent the functional analog of Gro/TLE in yeast (Grbavec et al. (1999), Flores-Saaib and Courey (2000)) interacts *in vitro* and *in vivo* with Srb7 subunit, a RNA polymerase II holoenzyme component (Gromoller et al. (2000)). These observations suggest that the interaction between TUP1 and holoenzyme components interferes with the basal transcriptional machinery. Overall, these findings suggest that oligomers of Gro/TLE have a transcription repression function by recruiting histones deacetylases to DNA and/or by inhibiting the basal transcriptional machinery.

2.c.2 Passive transcriptional repression

Passive transcriptional repression by Hes factors is a negative regulatory process that involves protein sequestration. For example, this mechanism takes place when Hes1 inhibits bHLH activators such as Mash1, Math1 and MyoD through a mechanism that inhibits their binding to the E box. This is achieved through the interaction of Hes1 with the ubiquitous bHLH factors like E47/E12, which are required heterodimer partners of tissue-specific bHLH factors such as MyoD, Mash1 and Math1 (Sasai et al. (1992), Akazawa et al. (1995)).

Hes1 can form non-functional heterodimer with these bHLH factors thereby disrupting the formation of functional heterodimers such as MyoD-E47 and Mash1-E47 (Sasai et al. (1992); Hirata et al. (2000)). This is supported by studies done in PC12 cells where the expression of a DNA-binding deficient form of Hes1 was almost as effective in repressing Mash1 mediated activation of the E box containing promoter as wild-type Hes1 (Castella et al. (1999)). However, it is possible that in this experiment Hes1 was interacting with other not yet identified Mash1 cofactors in order to achieve repression. This passive repression function has also been demonstrated in vivo in *Drosophila* for members of the Hairy-related proteins (Oellers et al. (1994), Dawson et al. (1995), Nakao et al. (1996)). Recent work in *Drosophila* by Giagtzoglou (2003) has provided insights on the molecular mechanisms underlying passive transcriptional repression by Hes factors. These authors have shown that *Drosophila* Enhancer of split proteins interact simultaneously with co-repressor Groucho and proneural achaete-scute proteins (Giagtzoglou et al. (2003)). As a result, transcriptional repression of proneural bHLH target genes may involve the recruitment of Groucho to the E box promoter region at which proneural bHLH are bound. Hes proteins would not require binding directly to DNA to mediate transcriptional repression of proneural protein function, but could do so by interacting directly with proneural bHLH proteins and recruit Groucho/TLE factors. Together these findings demonstrate that Hes proteins are also able to repress transcription of proneural genes by forming non-functional heterodimers with bHLH E protein, which results in an indirect inhibition of the neurogenic activity of E-proneural protein heterodimers.

3 THE HES FAMILY MEMBER, HES6 PROMOTES NEURONAL DIFFERENTIATION

3.a Spatial and temporal expression pattern of Hes6 suggests a neurogenic role

Recently, a novel member of the Hes family of protein, Hes6, was identified (Bae et al. (2000), Koyano-Nakagawa et al. (2000), Pissara et al. (2000), Vasiliauskas and Stern (2000)). Hes6 was first isolated based on its shared structural features with Hes proteins, such as the conserved proline residue in the basic region of the bHLH domain and the carboxy-terminal WRPW motif. These structural similarities suggested that Hes6 might play a role in the differentiation process in the developing nervous system like Hes1 and Hes5. In agreement with this possibility, expression studies in mouse embryos showed that *Hes6* mRNA expression is observed in most of the developing nervous system and reaches high levels by E12.5, when it is detected in the telencephalon and diencephalon, as well as many other regions of the nervous system (Bae et al. (2000), Koyano-Nakagawa et al. (2000), Pissarra et al. (2000), Vasiliauskas and Stern (2000)). In the developing cortex, high levels of *Hes6* are detected in the ventricular zone containing dividing neural precursor cells, as well as in the cortical plate containing differentiated cells. This is in contrast to Hes1, which is preferentially expressed in precursor cells in the ventricular zone and is downregulated during the transition to neurons (Sasai et al. (1992), Bae et al. (2000), Pissarra et al. (2000)). During the development of the eye, Hes6 is expressed at high levels in two layers of the neural retina; the ventricular layer containing undifferentiated cells and the ganglion cell layer containing projection neurons (Bae et al. (2000)). In the amphibian Xenopus laevis, Hes6 expression was also detected in neurogenic regions, where low levels of expression are observed in the ventricular zone and higher levels in the intermediate zone, which contains newly differentiated neurons (Koyano-Nakagawa et al. (2000)). Thus, the expression pattern of Hes6 in both neural precursors and neurons supports a role for this factor during neurogenesis.

Expression studies in the *Xenopus* have shown further that Hes6 is temporally downstream of Xngn1, a bHLH proneural protein, and upstream of Scg10, a marker for terminally differentiated neurons of the neural tube (Koyano-Nakagawa et al. (2000)). It was also observed that Hes6 expression pattern overlaps spatially and temporally with NeuroD, a neuronal differentiation bHLH factor, suggesting a role for Hes6 during the regulation of the transition of determined progenitors into differentiated neurons (Koyano-Nakagawa et al. (2000)).

To address the involvement of Hes6 in neurogenesis, Hes6 was ectopically expressed in the developing mouse retina. This resulted in the induction of rod photoreceptor cell differentiation at the expense of other cell types, suggesting that Hes6 promotes neuronal differentiation (Bae et al. (2000)). In agreement with this, injection of *Hes6* mRNA in *Xenopus* embryos resulted in increased numbers of cells expressing the neuronal differentiation marker N-tubullin, indicating that Hes6 promotes neuronal differentiation (Koyano-Nakagawa et al. (2000)). The overexpression of a mutant Hes6 that lacks the C-terminal WRPW motif was still able to promote the formation of primary neurons, suggesting that Hes6 does not need to interact with Gro/TLE corepressors to promote neurogenesis (Cossins et al. (2002), Koyano-Nakagawa et al. (2000)). In sum, spatial and temporal expression analyses as well as ectopic expression studies suggest that Hes6 acts as a positive regulator of neuronal differentiation.

3.b Regulation of Hes6 by proneural bHLH proteins

Previous studies in *Xenopus* embryos have shown that, unlike Hes1 and Hes5, Hes6 expression is not activated by the antineurogenic Notch signalling pathway (Koyano-Nakagawa et al. (2000)). More specifically, *Hes6* RNA expression is not upregulated by an activated form of the *Xenopus* Notch receptor (ICD) nor blocked by the overexpression of a

DNA-binding mutant of *Xenopus* Su(H) (Koyano-Nakagawa et al. (2000)). In agreement with the hypothesis that Hes6 does not act as a Notch effector, Hes6 expression rather appears to be positively regulated by proneural proteins. The injection of mRNAs encoding proneural bHLH proteins Xngn1, Xash3 and Xath3 induces Hes6 expression. Moreover, in *Ngn1* knockout mice, Hes6 expression is lost in the proximal cranial ganglia whose development is dependent on *Ngn1* (Ma et al. (1998), Koyano-Nakagawa et al. (2000)). Taken together, these observations from both mouse and *Xenopus* suggest that proneural bHLH proteins activate Hes6 expression during the development of the nervous system.

3.c Mechanisms underlying Hes6 function

3.c.1 Hes6 activity is independent of DNA-binding

Insights into the mechanisms used by Hes6 to promote neurogenesis was first provided by the studies of Bae et al. (2000) showing that Hes6 inhibits the transcriptional repressor activity of Hes1 at an N box reporter (Bae et al. (2000)) and also reduces the ability of Hes1 to inhibit the transcriptional activation mediated by Mash1-E47 heterodimers (Bae et al. (2000)). These observations first suggested that Hes6 antagonizes Hes1 function. One hypothesis is that Hes6 might bind to the same DNA-binding site as Hes1, thus competiting with Hes1 for DNA binding. This hypothesis was not consistent, however, with the demonstration that Hes6 does not bind to the E or N box motifs recognised by other Hes proteins (Bae et al. (2000), Koyano-Nakagawa et al. (2000)). Moreover, the overexpression in *Xenopus* embryos of a mutated form of Hes6 that lacks the basic arm of the bHLH domain, and thus is unable to bind to DNA, resulted in a promotion of primary neurogenesis similar to that promoted by wild type Hes6 (Koyano-Nakagawa et al. (2000), Cossins et al. (2002)). Together, these results suggest that Hes6 function is independent of DNA-binding. The inability of Hes6 to bind to N- or E-boxes was proposed to derive from

the fact that the loop region of Hes6 is five amino acid shorter than that of Hes1 and Hes2 and four amino acid shorter than Hes3 and Hes5 (Bae et al. (2000), Koyano-Nakagawa et al. (2000)). Interestingly, however, Cossins et al. (2002) found that, at least *in vitro*, Hes6 binds to a different DNA sequence referred to as the Enhancer of Split E box (ESE box) (Jennings et al. (1999)). Hes6 has the ability to represse transcription at a promoter containing ESE boxes to a degree similar to Hes1 in reporter assays in transfected cells (Bae et al. (2000), Cossins et al. (2002)). However, the physiological significance of these observations remains to be determined. Together, these observations suggest that during neural development the functions of Hes6 are DNA-binding independent. However, binding to DNA (ESE boxes) may be important during other functions of Hes6.

3.c.2 Hes6 interacts with other Hes proteins.

The finding that Hes6 has a negative effect on the transcription functions of Hes1 is consistent with the observation that Hes6 binds to mouse Hes1 and *Xenopus* XHairy 2A and Xhairy 1 proteins in *in vitro* assays (Bae et al. (2000), Koyano-Nakagawa et al. (2000)). This suggests that Hes1:Hes6 dimerization may be responsible for the formation of inactive Hes1-containing complexes. However, virtually nothing is known about the molecular mechanisms underlying this possible scenario.

Taking into consideration that Hes6 1) forms heterodimers with Hes1 in transfected non-neural cells (Bae et al. (2000), Koyano-Nakagawa et al. (2000)), 2) it cannot bind canonical Hes DNA-binding sites (Bae et al. (2000), Koyano-Nakagawa et al. (2000)), and that 3) it does not interfere with the ability of Hes1 to bind to N-boxes, but reduces Hes-1 mediated repression in transfected non-neural cells (Bae et al. (2000)), we formulated the following hypothesis: **Hes6 promotes neuronal differentiation by acting as a negative regulator of Hes1.** We have therefore investigated this possibility, as well as the underlying

molecular mechanisms. Chapter 2 of this thesis will present the results of studies that have addressed this important hypothesis.

CHAPTER 2: HES6 PROMOTES CORTICAL NEUROGENESIS AND INHIBITS HES1 TRANSCRIPTION REPRESSION ACTIVITY BY MULTIPLE MECHANISMS

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ABSTRACT

Hes1 is a mammalian basic helix loop helix transcriptional repressor that inhibits neuronal differentiation together with corepressors of the Groucho (Gro)/TLE family. The interaction of Hes1 with Gro/TLE is mediated by a WRPW tetrapeptide present in all Hes family members. Contrary to Hes1, the related protein Hes6 promotes neuronal differentiation. Little is known about the molecular mechanisms that underlie the neurogenic activity of Hes6. It is shown here that Hes6 antagonizes Hes1 function by two mechanisms. Hes6 inhibits the interaction of Hes1 with its transcriptional corepressor Gro/TLE. Moreover, it promotes a proteolytic degradation of Hes1. This effect is maximal when both Hes1 and Hes6 contain the WRPW motif and is reduced when Hes6 is mutated to eliminate a conserved site (Ser183) that can be phosphorylated by protein kinase CK2. Consistent with these findings, Hes6 inhibits Hes1-mediated transcriptional repression in cortical neural progenitor cells and promotes the differentiation of cortical neurons, a process normally inhibited by Hes1. Mutation of Ser183 impairs the neurogenic ability of Hes6. Taken together, these findings clarify the molecular events underlying the neurogenic function of Hes6 and suggest that this factor can antagonize Hes1 activity by multiple mechanisms.

INTRODUCTION

In the developing mammalian central nervous system (CNS), differentiated neuronal and glial cells derive from multipotent neural progenitor cells located in the proliferative zone of the neural tube. The commitment of these progenitor cells to the neuronal lineage is regulated by the antagonistic activities of a number of positively and negatively acting transcription factors containing the basic helix-loop-helix (bHLH) DNA-binding and dimerization motif (reviewed in 2, 18). Neurogenic bHLH factors include several evolutionarily conserved molecules related to the proneural proteins Atonal and Achaete-Scute of Drosophila (8, 13, 20). They function by forming heterodimers with the ubiquitous bHLH protein E47. These dimers bind to DNA sequences commonly referred to as E boxes (CANNTG) and transactivate the expression of genes that promote the acquisition of the neuronal fate (17, 32).

Antineurogenic bHLH factors include members of the Hairy/Enhancer of split (Hes) family (1, 26, 32). Contrary to proneural proteins, Hes factors like Hes1 and Hes5 mediate transcriptional repression and bind preferentially to DNA sequences referred to as N boxes (CACNAG) (32). They are thought to inhibit neuronal differentiation by antagonizing the neurogenic activity of the proneural proteins via multiple mechanisms, including a direct involvement in the negative regulation of proneural gene expression (4, 21) and an inhibition of the activity of E47·proneural protein heterodimers (1, 3, 32). Genetic perturbations that alter the normal balance of the activities of proneural and antineurogenic bHLH proteins have dramatic effects on CNS development *in vivo*, underscoring the importance of understanding how the functions of these factors are normally regulated (8, 16, 26, 36).

The *Hes1* gene is initially expressed in proliferating neural progenitor cells and becomes down-regulated during the progenitor-to-neuron transition (32). Persistent expression of Hes1 inhibits neuronal development, whereas disruption of *Hes1* function

results in the premature differentiation of neuronal cells and the up-regulation of proneural genes (15, 16, 36). These observations indicate that Hes1 acts in neural progenitor cells to control the timing of neuronal differentiation. Molecular mechanisms that contribute to the positive or negative regulation of Hes1 activity in neural progenitor cells are beginning to be elucidated. In particular, studies in both invertebrate and vertebrate species show that antineurogenic Hes proteins are coexpressed, and directly interact, with general transcriptional corepressors of the Groucho/Transducin-like Enhancer of split (Gro/TLE) family (7, 12, 24, 25, 29, 34, 40). This interaction is mediated by a WRPW tetrapeptide motif present at the carboxy-terminus of all Hes proteins (7, 11, 24). Mutations that disrupt the Hes•Gro/TLE interactions impair the ability of Hes proteins to mediate transcriptional repression (7, 24, 29). Moreover, Hes1 activates phosphorylation mechanisms that promote the transcription repression function of Gro/TLE (25). Together, these observations identify Gro/TLE proteins as positive regulators of Hes activity and suggest that Hes1 acts by recruiting hyperphosphorylated Gro/TLE to specific DNA sites where the latter mediate transcriptional repression (25).

Another protein that has recently been implicated in the regulation of Hes1 activity is the related Hes family member, Hes6 (3, 19). The *Hes6* gene is expressed throughout the developing CNS, where it is found in both undifferentiated neural progenitors and differentiated neurons (3, 19, 30, 38). Contrary to Hes1, Hes6 acts as a positive regulator of neuronal differentiation in both murine retinal explants and *Xenopus* embryos (3, 19). Although little is known about the molecular mechanisms underlying the neurogenic ability of Hes6, a number of observations suggest that Hes6 may promote neurogenesis by antagonizing the function of Hes1. Studies in transfected non-neural cells show that Hes6 can heterodimerize with Hes1 and can inhibit the ability of Hes1 to both repress transcription from promoters containing N box sequences and suppress the activity of

E47·proneural protein heterodimers (3). In addition, Hes6 does not require an intrinsic DNA-binding ability to promote neurogenesis because mutation of the basic arm of its bHLH domain does not abolish its neurogenic ability *in vivo* (19). Together, these observations suggest that Hes6 may promote neuronal differentiation via DNA binding-independent events that involve a negative regulation of Hes1 function in the CNS. Virtually nothing is known, however, about the molecular mechanisms underlying this inhibitory effect.

Here we describe experiments showing that Hes6 negatively regulates Hes1 activity by at least two mechanisms. Hes6 inhibits the interaction of Hes1 with Gro/TLE. In addition, it promotes a proteolytic degradation of Hes1. This effect is maximal when both Hes1 and Hes6 contain the WRPW motif, and is reduced by a point mutation (S183A) that removes a consensus site for phosphorylation by protein kinase CK2. In agreement with these findings, Hes6 inhibits Hes1-mediated transcriptional repression in cortical neural progenitor cells and promotes their neuronal differentiation. Moreover, the S183A mutation attenuates Hes6 phosphorylation by protein kinase CK2 and impairs Hes6 ability to promote neuronal differentiation. Taken together, these findings identify novel mechanisms through which Hes6 may act as a negative regulator of Hes1 activity and a positive regulator of neuronal differentiation.

MATERIALS AND METHODS

Plasmids. Polymerase chain reaction (PCR) was used to amplify the sequences encoding full-length Hes6 (oligonucleotide primers *Hes6-1*, 5'-GACCATGGCTCCGTCCCA, and *Hes6-2*, 5'-TCACCAAGGCCTCCACACACTC) or Hes6□WRPW (oligonucleotide primers *Hes6-1* and *Hes6-3*, 5'-TCACACACTCTGAGCCCGGCGAGC) using the full-length *Hes6* cDNA Image clone W66929 as template (5). The sequence encoding a truncated form of Hes6 lacking the first 13 amino acids (Hes6(14-224)) was also amplified by PCR (oligonucleotide primers *Hes6-4*, 5'-TCAGGAGGATGAGGACCGCTGGGAA, and *Hes6-2*); Hes6 and Hes6(14-224) behaved equally in our studies. PCR products were subcloned into the pcDNA3-GAL4bd vector digested with BamHI (followed by filling-in with Klenow DNA polymerase) or into the pCMV2-HA plasmid digested with EcoRV or SmaI. The pCMV2-HA-Hes6(S183A) plasmid was obtained by first generating the sequence encoding the indicated point mutation using a PCR-based strategy (the mutated oligonucleotide primers were *Hes6-5F*, 5'-GACCTGTGTGCTGACCTAGAGGAGAT, and *Hes6-5R*, 5'-TCTAGGTCAGCACACA GGTCGT), followed by subcloning into pBluescript plasmid and DNA sequencing. The verified mutant sequence was then subcloned into pCMV2-HA-Hes6 digested with SmaI, replacing the wild type sequence. Constructs for the bacterial expression of fusion proteins of GST and Hes6 or Hes6(S183A) were obtained by digesting pCMV2-HA-Hes6 or pCMV2-HA-Hes6(S183A) with BglII and BamH1, followed by subcloning into pGEX1 digested with BamH1. The pGEX1-Hes1 DNA has been described (23). Constructs pEBG-Hes6 and pEBG-Hes6ΔWRPW were generated by digesting pcDNA3-GAL4bd-Hes6 or pcDNA3-GAL4bd-Hes6ΔWRPW, respectively, with EcoRI, followed by filling-in with Klenow DNA polymerase and subcloning into the filled-in ClaI site of pEBG to generate plasmids for the expression of fusion proteins of GST and Hes6 or Hes6□WRPW in mammalian cells. Plasmid pCMV2-FLAG-Hes1ΔWRPW:Gro/TLE1 was generated by first

subcloning the region encoding Hes1ΔWRPW (obtained by PCR amplification with primers *Hes1-1*, 5'-AATGCCAGCTGATATAATGGAG, and *Hes1-2*, 5'-ACATGGAGTCCGCAG TGAGCGA) into pCMV2-FLAG digested with EcoRV. This was followed by in-frame ligation of the sequence encoding Gro/TLE1 (also obtained by PCR with primers *Gro/TLE1-1*, 5'-GGATGTTCCCGCAGAGCCGG, and *Gro/TLE1-2*, 5'TCAGTAGATGA CTTCATAGAC) into an XbaI site located downstream of the last codon of *Hes1*. Ligation products were analyzed and confirmed by sequencing. Plasmids pCMV2-FLAG-Hes1, pCMV2-FLAG-Hes1ΔWRPW, pEBG-Hes1, and pEBG-Hes1ΔWRPW have been described previously (12, 23, 24). Plasmids pFOX-Luc1, pFOX-ngn3p-Luc1 (containing a portion of the *neurogenin3 (ngn3)* promoter extending ~2.6 kbp upstream of the transcription start site), and pFOX-ΔN-box-ngn3p-Luc1 (containing a mutated version of the ~2.6 kbp *ngn3* promoter lacking the Hes1 binding sites located within 200 bp proximal to the transcription start site) have been described (21).

Transient transfections, protein-protein interaction assays, and Western blotting analysis. Human 293A cells were cultured and transfected using the SuperFect reagent (Qiagen) as described previously (23-25). When appropriate, transfected cells were incubated for 6 hours in the presence of 10 μM MG132 (Calbiochem) prior to cell lysis. Treatment of cell lysates with calf intestinal phosphatase was performed as described (14). To examine the effect of Hes6 on Hes1 stability, cells were transfected for 36 hr with pCMV2-FLAG-Hes1/Hes1ΔWRPW (50 ng/transfection) in the absence or presence of Hes6, HesΔWRPW, or Hes6(S183A) expression plasmids (200-800 ng/transfection). To examine the effect of Hes6 on the Hes1•Gro/TLE interaction, cells were transfected for 24 hr with Hes1- or Hes1ΔWRPW expression plasmids (100-200 ng/transfection) in the absence or presence of Hes6- or Hes6ΔWRPW expression plasmids (100-200 ng/transfection). Cell lysates were prepared and GST coprecipitation (23, 24), immunoprecipitation (14, 40), and Western blotting (6, 25, 28) studies were performed as

described previously. The antibodies used were as follows: panTLE (6, 28, 34), anti-GST and anti-GAL4bd (Santa Cruz Biotechnology), anti-HA (Roche), or anti-FLAG (Sigma).

In vitro phosphorylation of bacterially purified Hes proteins. Fusion proteins of GST and Hes6 or Hes6(S183A) were purified from bacteria as described (12, 23). Roughly 50 ng of each fusion protein were resuspended in buffer A (50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Triton X-100, 200 μm ATP) containing 200 μCi/ml [γ-^{32}P]ATP in the presence of 0.5 U of purified protein kinase CK2 (New England Biolabs)/μl for 15 min at 30 °C. Reactions were terminated by the addition of 2X SDS-PAGE sample buffer and incubation at 65 °C for 5 min. After gel electrophoresis, proteins were transferred to nitrocellulose and exposed to film. After autoradiography, membranes were subjected to Western blotting with anti-GST antibodies.

Telencephalic neural progenitor cell cultures. Primary neural progenitor cell cultures were established from dorsal telencephalic cortices dissected from embryonic day (E) 13.5 mouse embryos as described previously (10, 33). Cells were seeded into either 4-well chamber slides (Nalge Nunc Int.) for immunocytochemical studies or 6-well dishes (BD Labware) for transcription assays. All chambers and dishes were coated with 0.1% poly-D-lysine and 0.2% laminin (BD Biosciences). Cells were cultured in Neurobasal medium supplemented with 1% N2, 2% B27, 0.5 mM Glutamine, 1% penicillin & streptomycin (Invitrogen), and 40 ng/ml FGF2 (Collaborative Res.).

Transient transfection/transcription studies in neural progenitor cells. Approximately $1.5x10^6$ cells/ml were seeded at the start of the experiments. After twenty-four hr *in vitro* ("day 1"), when ~90% of the cultured cells were mitotic (10, 25, 35), transfections were performed by mixing the appropriate combinations of plasmids (total amount of DNA was adjusted to 2.0 μg/well in each transfection) with OptiMEM medium (Invitrogen). An equal volume of OptiMEM medium was mixed separately with Lipofectamine 2000 reagent (Invitrogen; 1.25 μl/μg of DNA) and then combined with the DNA mixture and incubated for 20 min. The DNA/Lipofectamine 2000 mix was then

added dropwise to each well. In each case, a pRSV-β-galactosidase DNA was cotransfected to provide a means of normalizing the assays for transfection efficiency. Twenty-four hours after transfection, cells were harvested and luciferase and β-galactosidase activities were determined as described (23-25). Results were expressed as mean values ± S.D.

Immunocytochemical analysis of differentiating neural progenitor cells. Approximately $4x10^5$ cells/ml were seeded at the start of the experiments. After forty-eight hr *in vitro*, cells were transfected as described above using plasmids encoding either enhanced green fluorescent protein (GFP) alone (0.2 μg/well), or combinations of GFP and Hes6, Hes6ΔWRPW, or Hes6(S183A) (0.5 μg/well of either Hes6 plasmid). Total amount of DNA was adjusted to 1.0 μg. Cells were allowed to differentiate until day 4-5 *in vitro*, when they were fixed and subjected to double-label immunocytochemical analysis of the expression of GFP, nestin (a marker of undifferentiated neural progenitor cells), MAP2 or NeuN (markers of differentiated neurons) as described (33, 35). Anti-nestin (BD-Pharmingen), -MAP2 (Sigma), or -NeuN (Chemicon) antibodies were used. Digital image acquisition and analysis were performed with the Northern Eclipse software (Empix Inc.). Results were expressed as mean values ± S.D.

RESULTS

Promotion of cortical neurogenesis by Hes6.

Hes6 was shown to promote neuronal differentiation in *Xenopus* embryos and mouse retinal explants (3, 19). To determine whether Hes6 might also promote the development of cortical neurons, we transfected exogenous Hes6 in primary cultures of neural progenitor cells isolated from the dorsal telencephalon of E13.5 mouse embryos. These cortical progenitors endogenously express Hes6 (Ref. 3 and data not shown), as well as Hes1 and Gro/TLE (6, 32, 40). Enhanced GFP was coexpressed to mark the transfected cells. Exogenous Hes6 expression led to a significant increase in the number of differentiated neurons when compared to GFP alone, as revealed by immunocytochemistry with antibodies against the neuronal-specific protein MAP2 (Fig. 1A and Fig. 1B, cf. bars 1 and 2). This increase was correlated with a decrease in the number of undifferentiated neural progenitor cells expressing the protein nestin (Fig. 1C, cf. bars 1 and 2). These results thus show that Hes6 promotes cortical neuronal differentiation. Since previous studies have shown that the neurogenic ability of *Xenopus* Hes6 does not require its carboxy-terminal WRPW motif involved in Gro/TLE binding (19), we next examined if Hes6ΔWRPW, a truncated form lacking this motif, would also promote cortical neuronal differentiation. Exogenous Hes6ΔWRPW also caused an increase in the number of differentiated neurons, although less effectively than Hes6 (Fig. 1B, cf. lanes 1-3). Hes6 and Hes6ΔWRPW were expressed at equivalent levels (Fig. 3A below). Together, these findings strongly suggest that Hes6 promotes the differentiation of cortical progenitor cells into postmitotic neurons. They suggest further that its WRPW motif is not required for, but contributes to, a maximal neurogenic effect. This is consistent with the finding that although both Hes6 and

Hes6ΔWRPW can promote neurogenesis in *Xenopus* embryos, the former elicited a more robust neurogenic effect than the latter (19).

Comparison of the interaction of Hes6 or Hes1 with Gro/TLE.

To elucidate the molecular mechanisms underlying the neurogenic activity of Hes6, we tested whether this function might involve an inhibition of the anti-neurogenic activity of Hes1. Both Hes1 and Hes6 bind to Gro/TLE (9, 12, 24, 25) and are coexpressed with the latter in a number of tissues (3, 6, 9, 12, 32, 34, 39). In particular, *Hes1* and *Hes6* are coexpressed in neural progenitor cells but not in differentiated neurons where *Hes6* continues to be expressed while *Hes1* is down-regulated (3, 19, 32). This suggested that Hes6 might act as a negative regulator of Hes1 activity in neural progenitors by competing with Hes1 for binding to Gro/TLE, thus 'titrating away' the corepressor function that Gro/TLE provides to Hes1. To examine this possibility, we first tested if Hes6 had a higher affinity than Hes1 for Gro/TLE. 293A cells that express endogenous Gro/TLE (Fig. 2A, lanes 1-4) were transfected with plasmids encoding either GST-Hes6 or GST-Hes1. The precipitation of equivalent amounts of these fusion proteins (Fig. 2B, cf. lanes 5 and 7) resulted in the coprecipitation of equivalent amounts of endogenous Gro/TLE (Fig. 2A, cf. lanes 5 and 7). In contrast, expression of fusion proteins of GST and truncated forms of Hes6 or Hes1 lacking the WRPW motif (Fig. 2B, lanes 6 and 8) did not result in the coprecipitation of Gro/TLE (Fig. 2A, lanes 6 and 8), consistent with the demonstrated requirement for this motif for Gro/TLE binding (24). These findings show that Hes1 and Hes6 interact with Gro/TLE with similar affinities when they are expressed at equivalent levels.

Effect of Hes6 on the interaction of Hes1 with Gro/TLE.

To directly test if Hes6 might compete with Hes1 for Gro/TLE binding, we performed Hes1·Gro/TLE coimmunoprecipitation studies in the absence or presence of Hes6. 293A cells were transfected with FLAG epitope-tagged Hes1, followed by immunoprecipitation with anti-FLAG antibodies. In the absence of HA-Hes6 (Fig. 3A, lane 1), Gro/TLE coimmunoprecipitated efficiently with Hes1 (Fig. 3E, lane 1, see arrow). When Hes6 was coexpressed with Hes1 (Fig. 3A, lane 2), we observed a significant decrease in the amount of Gro/TLE that coimmunoprecipitated with Hes1 (Fig. 3E, cf. lanes 1 and 2, see arrow). Under these conditions (see Materials and Methods), Hes6 expression did not cause a significant decrease in the level of transfected Hes1 (Figs. 3B and D, cf. lanes 1 and 2) and had no negative effect on the expression of endogenous Gro/TLE (Fig. 3C), suggesting that the decreased Gro/TLE coimmunoprecipitation was not simply the result of decreased levels of these proteins. In this and succeeding figures, the relative intensities of the Hes1 and Hes6 immunoreactive bands do not reflect the actual relative amounts of these factors because different antibodies were used for each protein and blots were not developed for equal lengths of time. To test if the reduction in Hes1·Gro/TLE coimmunoprecipitation resulted from a titration effect mediated by Hes6 homodimers, the same assays were performed using Hes6ΔWRPW (Fig. 3A, lane 3). This protein also caused a decrease in Gro/TLE coimmunoprecipitation with Hes1, although this reduction was not as robust as with Hes6 (Fig. 3E, cf. lanes 1 and 3, see arrow). Coexpression of Hes6ΔWRPW did not affect the levels of Hes1 or Gro/TLE (Figs. 3B and C). Similar studies were performed using fusion proteins of Hes6 and the DNA-binding domain of GAL4 (GAL4bd). Expression of increasing amounts of GAL4bd-Hes6 (Fig. 4A, lanes 2 and 3) led to a significant inhibition of the coimmunoprecipitation of Gro/TLE with Hes1 (Fig. 4D, cf. lanes 1-3), without

significantly affecting the expression of either Hes1 (Fig. 4B, lanes 1-3; see arrow) or Gro/TLE (Fig. 4C, lanes 1-3). GAL4bd-Hes6ΔWRPW had a similar effect, although it was somewhat less effective than GAL4bd-Hes6 (Fig. 4D, lanes 4 and 5).

To extend these observations, cells were transfected with Hes1ΔWRPW, followed by immunoprecipitation with anti-FLAG antibodies. As expected, in the absence of cotransfected Hes6, Gro/TLE did not coimmunoprecipitate with Hes1ΔWRPW (Fig. 3E, lane 7). In contrast, Gro/TLE coimmunoprecipitated with Hes1ΔWRPW when the latter was cotransfected with Hes6 (Fig. 3E, lane 4), but not with Hes6ΔWRPW (Fig. 3E, lane 5). As previously reported (3), Hes1 and Hes6 proteins heterodimerized with each other under the experimental conditions of these assays (data not shown). Expression of Hes6 alone followed by immunoprecipitation with anti-FLAG antibodies did not result in Gro/TLE coprecipitation (Fig. 3E, lane 6). The expression of Hes1ΔWRPW was not affected by Hes6 expression (Figs. 3B and D). Taken together, these findings demonstrate that Hes6 can antagonize the interaction of Hes1 with Gro/TLE. The WRPW motif of Hes6 is not necessary for this effect, suggesting that this is not solely the result of a competition by Hes6 homodimers for Gro/TLE binding.

Effect of Hes6 on the stability of Hes1.

During the course of our transfection experiments, we noted that under appropriate conditions (see Materials and Methods) the coexpression of increasing levels of Hes6 caused a gradual decrease of FLAG-Hes1 immunoreactivity (Figs. 5A and B, cf. lanes 1-3). A similar effect was observed when Hes6ΔWRPW was expressed (Figs. 5A and B, cf. lanes 1, 4 and 5), although this truncated form appeared to cause a smaller decrease in Hes1 levels compared with Hes6. The expression of Hes1ΔWRPW was also reduced in the presence of Hes6, but

not as significantly as in the case of Hes1 (Fig. 5A, cf. lanes 1-3 and 6-8). In contrast, Hes6ΔWRPW had no significant effect on Hes1ΔWRPW levels (Fig. 5A, cf. lanes 6, 9 and 10). These findings were specific because the levels of endogenous Gro/TLE were not affected by either Hes6 or Hes6ΔWRPW (Fig. 5C). To corroborate these results and exclude any effects due to the presence of the HA epitope on Hes6, similar studies were performed using GAL4bd-Hes6. Expression of both GAL4bd-Hes6 and GAL4bd-Hes6ΔWRPW also caused a decrease in Hes1 immunoreactivity compared to the expression of GAL4bd alone (data not shown). These combined observations suggest that Hes6 promotes mechanisms that negatively regulate the stability of Hes1.

To elucidate these mechanisms further, we tested if the stability of Hes6 and/or Hes1 might be increased by inhibition of the 26S proteasome. Unexpectedly, we observed a decrease in both HA-Hes6 and GAL4bd-Hes6 immunoreactivity when cells were treated with the protease inhibitor MG132 (Figs. 6A and B, cf. lanes 2 and 3). The proteasome inhibitor lactacystin also caused a similar decrease in Hes6 immunoreactivity (data not shown). This effect was specific because it was not observed when Hes6ΔWRPW was tested (Figs. 6A and B, cf. lanes 4 and 5). Moreover, MG132 had no effects on the expression of either Hes1 (Fig. 6C, lanes 1 and 2) or Gro/TLE (Fig. 6C, lanes 3 and 4). We also observed that the decrease in full-length HA-Hes6 or GAL4bd-Hes6 was not correlated with the appearance of smaller immunoreactive species. In particular, we did not observe bands migrating near or above the position where GAL4bd (~19 kDa, see Fig. 4A above) migrates, suggesting that MG132 treatment caused extensive degradation of the Hes6 proteins. These combined findings show that Hes6 is susceptible to proteolytic mechanisms that can be mimicked or activated (rather than suppressed) by treatment with MG132. These mechanisms depend on the presence of the WRPW motif, perhaps because Hes6 is more

prone to degradation when it is competent to associate with Gro/TLE or because the WRPW motif unmasks sites that are involved in degradation pathways.

These observations raised the possibility that the susceptibility of Hes6 to proteolytic degradation might be correlated with its negative effect on the stability of Hes1. To test this, Hes1 was expressed in the absence or presence of increasingly high levels of Hes6. We found that the gradual decrease in Hes1 stability induced by transfecting increasing amounts of *Hes6* DNA (Fig. 6D) was not correlated with a gradual increase in Hes6 immunoreactivity (Fig. 6E). In contrast, when Hes6 was transfected in the absence of Hes1, we observed the expected correlation between higher amounts of DNA and increasing protein levels (Fig. 6F). Taken together, these results show that Hes6 promotes a degradation of Hes1 in a dose-dependent manner. They suggest further that Hes6 may become increasingly unstable when it is bound to Hes1. This in turn raises the possibility that Hes1 becomes targeted for degradation due to its association with Hes6. This process is maximally effective when both Hes1 and Hes6 contain the WRPW motif involved in Gro/TLE binding.

Inhibition of Hes1-mediated transcriptional repression by Hes6 in telencephalic neural progenitor cells.

The previous results show that Hes6 can negatively regulate both Hes1 stability and its interaction with Gro/TLE. Since these effects are predicted to impair Hes1-mediated transcriptional repression, we next tested the possibility that Hes6 might suppress the ability of Hes1 to act as a transcriptional repressor in a cellular context where these proteins are normally coexpressed. Primary cultures of cortical neural progenitor cells were established and transfected with a reporter plasmid containing the *luciferase* gene under the control of the *ngn3* promoter. Hes1 has been shown previously to specifically bind to this promoter and repress its activity (21). We found that the *ngn3* promoter drove strong expression of the

reporter gene in transfected neural progenitors, and that Hes1 significantly suppressed transcription from this promoter (Fig. 7A, cf. bars 1 and 2). When increasing amounts of Hes6 were cotransfected, Hes1-mediated repression was progressively reduced (Fig. 7A, cf. bars 2-4). Expression of Hes6ΔWRPW also resulted in an inhibition of Hes1-mediated repression (Fig. 7A, bars 5 and 6). Control experiments showed that neither Hes6 nor Hes6ΔWRPW had an activating effect on the *ngn3* promoter when transfected in the absence of Hes1 (Fig. 7A, bars 12 and 13). Moreover, no significant effects were observed when the *ngn3* promoter was mutated to delete the Hes1-binding sites present within its proximal region (21) (Fig. 7B). These results show that Hes6 has the ability to inhibit transcription repression mediated by Hes1 in neural progenitor cells.

We then investigated if this inhibitory effect was the result of either a promotion of Hes1 degradation or the prevention of Hes1·Gro/TLE complex formation (or a combination of both). We hypothesized that transcriptional repression mediated by a chimeric protein in which Hes1 was constitutively associated with Gro/TLE might be suppressed by Hes6 if that involved a proteolysis of Hes1, but not if it required an inhibition of Hes1·Gro/TLE interaction. A fusion protein was engineered (Hes1ΔWRPW:Gro/TLE1) in which the WRPW motif of Hes1 was removed and the entire sequence of Gro/TLE1 subcloned in its place. This chimeric protein repressed transcription driven by the *ngn3* promoter in neural progenitor cells and its repressive activity was comparable to that of Hes1 (Fig. 7A, cf. bars 2 and 7). We found that cotransfection of increasing amounts of Hes6 had a de-repression effect on Hes1ΔWRPW:Gro/TLE1, although this was somewhat weaker than its inhibitory effect on Hes1 (Fig. 7A, cf. bars 2-4 and 7-9). These findings indicate that Hes6 can antagonize Hes1 transcriptional repression activity even when the latter is constitutively bound to Gro/TLE, strongly suggesting that an inhibition of the

Hes1•Gro/TLE interaction is not the only mechanism utilized by Hes6 to suppress Hes1. In turn, this implicates mechanisms involving the promotion of Hes1 degradation in this event. Importantly, although Hes6ΔWRPW had an inhibitory effect on Hes1 (Fig. 7A, cf. bars 2, 5, and 6) it had not no significant effect on Hes1ΔWRPW:Gro/TLE1 (Fig. 7A, cf. bars 7, 10, and 11).

In agreement with these findings, examination of the expression of Hes1ΔWRPW:Gro/TLE1 using antibodies against the amino-terminal FLAG epitope showed that Hes6 caused a significant reduction in immunoreactivity compared to controls (Fig. 8A, cf. lanes 1 and 2), indicating that Hes6 promotes a degradation of Hes1ΔWRPW:Gro/TLE1. Both GAL4bd-Hes6 and HA-Hes6 had the same effect on the expression of Hes1□ WRPW:Gro/TLE1 (data not shown). In contrast, Hes6ΔWRPW did not affect the expression of this fusion protein (Figs. 8A and B, cf. lanes 1 and 3), consistent with the lack of a negative effect of Hes6ΔWRPW on the transcription repression ability of Hes1ΔWRPW:Gro/TLE1 described above. Reprobing with anti-Gro/TLE antibodies directed against the carboxy-terminal domain of this fusion protein confirmed that Hes6ΔWRPW did not decrease the expression of Hes1ΔWRPW:Gro/TLE1 like Hes6 did (Fig. 8C, cf. lanes 1-3). Moreover, using this antibodies we noticed that coexpression of Hes6 was not correlated with detectable immunoreactive species migrating between endogenous Gro/TLEs (Fig. 8C, see arrowhead) and full-length Hes1ΔWRPW:Gro/TLE1 (Fig. 8C, see arrow) or lower forms of smaller size. These observations suggest that Hes6 expression caused a general proteolysis of the Hes1ΔWRPW:Gro/TLE1 fusion protein and not solely a confined degradation of its amino-terminal portion. Taken together, these findings show that Hes6 inhibits Hes1-mediated transcriptional repression in neural progenitor cells and

strongly suggest that the promotion of Hes1 proteolysis by Hes6 is important for this inhibitory effect.

Involvement of Ser183 in the ability of Hes6 to promote Hes1 degradation and neuronal differentiation.

Previous studies (37) have shown that the *Drosophila* Hes family members, Enhancer of split m5, m7, and m8 contain an evolutionarily conserved sequence motif characterized by a carboxy-terminal consensus site for phosphorylation by protein kinase CK2, defined as (S/T)(D/E)X(D/E), preceded at a short distance by the sequence SP(A/V)SS. This sequence, hereafter referred to as the "SPXSS-SDXE motif" is located within a region with a high PEST score (37). PEST-rich sequences behave as cis-acting signals that regulate protein turnover and have been suggested to be activated via phosphorylation (27, 31*). Drosophila* m5, m7, and m8 proteins were shown to associate with, and be phosphorylated by protein kinase CK2 at their conserved SPXSS-SDXE sequences. This phosphorylation is believed to activate their PEST domains and result in decreased stability (37).

Using the program PESTfind (http://at.embnet.org/embnet/tools/bio/PESTfind), we identified a conserved potential PEST sequence at the carboxy-terminus of mouse and human Hes6 proteins (Fig. 9A; PEST score, +13.02; PEST scores greater than +5 are considered significant). This region contains a conserved sequence similar to the SPXSS-SDXE motif found in the PEST domain of *Drosophila* m5, m7, and m8 (Fig. 9A). This raised the possibility that Hes6 might be phosphorylated by protein kinase CK2 and that this event may regulate its stability through modulation of PEST sequence activity. To test this, we first determined whether Hes6 is a phosphorylated protein. Lysates from cells transfected with Hes6 were incubated in the absence or presence of calf intestinal phosphatase, followed by gel electrophoresis. After this treatment, Hes6 exhibited a faster electrophoretic mobillity,

indicating that it is a phosphorylated protein (Fig. 9B, cf. lanes 1 and 2). In addition, purified protein kinase CK2 directly phosphorylated a fusion protein of GST and Hes6 isolated from bacteria (Figs. 9C and D, lane 2). Importantly, a S183A mutation within the SPXSS-SDXE motif significantly attenuated phosphorylation of Hes6 by protein kinase CK2 even when Hes6(S183A) was present at higher levels than wildtype Hes6 (Figs. 9C and D, cf. lanes 2 and 4). Hes1, which does not contain an SPXSS-SDXE motif, was not phosphorylated by protein kinase CK2 (Fig. 9E, lane 1) even when expressed at significantly higher levels than Hes6 (Fig. 9F, cf. lanes 1 and 2). Taken together, these findings identify Hes6 as a specific target of protein kinase CK2 and strongly suggest that this kinase can phosphorylate Hes6 at Ser183.

Based on these observations, we tested if Ser183 might be important for the ability of Hes6 to cause a reduced stability of Hes1. 293A cells were transfected with Hes1 alone or in the presence of Hes6 or Hes6(S183A). Hes6 caused a dramatic decrease in Hes1 expression whereas Hes6(S183A) had a weaker, although still detectable, effect (Figs. 10A and B, cf. lanes 1-3). These findings suggest that phosphorylation of Ser183 plays a positive role in the ability of Hes6 to promote Hes1 degradation. In turn, this raised the possibility that Hes6(S183A) might have a weaker neurogenic activity than wild type Hes6 due to its reduced ability to decrease Hes1 stability. To examine this possibility, cortical progenitor cells were transfected with Hes6 or Hes6(S183A) and the transfected cells were examined for the expression of markers of either proliferating cells (the Ki67 protein) or differentiated neurons (the NeuN protein), as described (33). We found that exogenous Hes6 led to the differentiation of supernumerary neurons (Fig. 10C, cf. bars 1 and 2) and a decrease in undifferentiated progenitors (Fig. 10D, cf. bars 1 and 2). In contrast, Hes6(S183A) did not promote similar effects (Figs. 10C and D, bar 3). Taken together, these findings identify an important role for Ser183 in the neurogenic activity of Hes6 and show a correlation between

phosphorylation of this residue by protein kinase CK2 and the ability of Hes6 to negatively regulate Hes1 functions and promote neuronal development.

DISCUSSION

Involvement of Hes6 in neuronal differentiation.

Previous studies in mouse and *Xenopus* have revealed that *Hes6* expression is correlated with the transition of neural progenitor cells to the neuronal fate (3, 19, 30, 38). In *Xenopus*, *Hes6* activation follows the expression of neuronal determination genes, like *ngn* family members, and overlaps with neuronal differentiation genes like *NeuroD* (19). In mice, *Hes6* expression was detected in both the proliferative zone containing neural progenitor cells and areas containing postmitotic neurons (3). Taken together with the demonstration that *Xenopus Hes6* expression is not activated by the Notch signaling pathway, which plays an antineurogenic role, but rather appears to be driven by neurogenic bHLH proteins (19), these observations first suggested an involvement of Hes6 in mechanisms that positively regulate neurogenesis. This possibility was confirmed by ectopic expression studies in *Xenopus* embryos and murine retinal explants that revealed that Hes6 promotes neuronal differentiation (19). Importantly, those studies also suggested that Hes6 may act primarily by promoting the differentiation of progenitors that already express proneural proteins, perhaps by antagonizing the functions of inhibitors of the latter. By removing this inhibition Hes6 may allow proneural proteins to perform more effectively their neurogenic functions leading to enhanced neuronal differentiation. In an effort to clarify how Hes6 may antagonize inhibitory activities that negatively regulate proneural protein functions, we have focused on the Hes1 protein, a well characterized member of a family of bHLH proteins that act as inhibitors of proneural proteins in both invertebrates and vertebrates (18). In particular, Hes1 inhibits transcription from proneural gene promoters (4, 21) and the expression of proneural genes is prematurely activated in *Hes1* nullizygous mice (16), suggesting that Hes1 acts as a negative regulator of proneural proteins *in vivo*. *Hes1* and *Hes6* are coexpressed in differentiating neural progenitor cells (3, 19, 32), and they can heterodimerize in transfected

cells and *in vitro* (3). Moreover, Hes6 was shown to reduce the ability of Hes1 to repress transcription from an artificial promoter in NIH3T3 cells (3). These observations raised the possibility that Hes6 acts as a negative regulator of the antineurogenic activity of Hes1. However, they did not clarify the molecular mechanisms that underlie this function. To address this important question, we have performed a combination of molecular and cellular investigations that have characterized two complementary mechanisms that Hes6 may utilize to negatively regulate Hes1 activity and positively regulate neuronal differentiation.

Inhibition of Hes1·Gro/TLE interaction by Hes6.

Our studies have shown that the interaction of Hes1 with its transcriptional corepressor Gro/TLE is reduced when Hes6 is coexpressed at levels that do not have a significant effect on the stability of either Hes1 or Gro/TLE. This effect is unlikely to result solely from a competition for Gro/TLE between Hes6 and Hes1 homodimers because a truncated form of Hes6 that is unable to bind to Gro/TLE also inhibits the interaction of Hes1 with the latter. Our finding that Hes1ΔWRPW·Hes6 heterodimers, which have only one WRPW motif, appear to interact with Gro/TLE like Hes1·Hes6 heterodimers, which have both WRPW motifs, suggests instead that Hes1·Hes6 heterodimers interact more poorly with Gro/TLE than homodimers of either protein. Reasons for this reduced affinity may include the fact that the folding of these heterodimers may not allow a proper alignment of the WRPW motifs of Hes1 and Hes6. Gro/TLE proteins exist as tetramers, so the correct alignment of WRPW motifs may be critical for the establishment of a strong interaction between Hes factors and Gro/TLE. A weaker association may be caused by differential post-translational modifications, including phosphorylation of Ser183 of Hes6 (see below for further discussion). Alternatively, other cofactors that may interact selectively with either

Hes1 or Hes6 may not allow a strong interaction between Gro/TLE and Hes1·Hes6 heterodimers. In either case, the formation of Hes1·Hes6 heterodimers that interact poorly with Gro/TLE is likely to prevent/reduce the interaction of Hes1 homodimers with Gro/TLE thereby depriving Hes1 of its critical transcriptional corepressor and negatively regulating its functions. As will be discussed below, this situation may lead, under conditions of increasing Hes6 expression, to an additional mechanism of Hes1 suppression, namely the targeting of Hes1·Hes6 dimers for proteolytic degradation.

Regulation of Hes1 stability by Hes6.

Our investigations have shown for the first time that expression of increasing amounts of Hes6 causes a gradual decrease of Hes1 stability resulting in a loss of full-length protein. This finding raises the interesting possibility that Hes6 may act as a negative regulator of Hes1 activity by regulating the stability of the latter. Such a situation may occur, for instance, in determined neural progenitor cells in which increased proneural protein activity may promote an up-regulation of Hes6 expression. In turn, Hes6 may cause an inactivation of Hes1 by affecting its turnover, thereby contributing to the mechanisms that will drive those progenitors into the neuronal lineage. Such a situation might explain not only the ability of Hes6 to suppress Hes1-mediated repression but also the previous observation that Hes6 can also suppress the ability of Hes1 to inhibit the activity of E2A·proneural protein heterodimers (3). Inhibition of proneural protein activity by Hes1 is thought to involve the formation of heterodimers between Hes1 and ubiquitous bHLH proteins like E47, thus titrating away the latter from the proneural proteins. A proteolytic degradation of Hes1 would therefore be expected to prevent these interactions and inhibit this effect.

We have shown that Hes6 is intrinsically susceptible to proteolytic degradation events that can be uncovered by exposure to the protease inhibitor MG132. The mechanisms underlying the effect of MG132 on Hes6 are still unclear and likely involve indirect effects resulting from either the MG132-mediated activation of genes that encode factors that may destabilize Hes6, the inhibition of proteolytic pathways that may normally degrade factors that reduce Hes6 stability, or the inhibition of pathways leading to the expression of factors that render Hes6 more stable. Regardless of the exact nature of the events induced by MG132, the observation that Hes6 is prone to proteolytic degradation is in agreement with the presence of an evolutionarily conserved PEST domain containing an SPXSS-SDXE subdomain that includes a resident consensus protein kinase CK2 phosphorylation site at Ser183. The presence of PEST domains is characteristic of proteins that undergo increased turnover, and phosphorylation of PEST sequences by protein kinase CK2 was shown to negatively affect intrinsic protein stability (22, 27, 31). The *Drosophila* Hes family members Enhancer of split m5/7/8 share with Hes6 a similar SPXSS-SDXE motif within a carboxy-terminal region characterized by a high PEST score. They were shown to bind directly to protein kinase CK2 and be phosphorylated by this kinase at their conserved SDXE site. This phosphorylation is believed to decrease their stability (37). In agreement with those results, we have shown that Hes6, but not Hes1, is phosphorylated by protein kinase CK2 at Ser183 within the SDXE motif, suggesting a previously unrecognized relatedness of Hes6 to the m5/7/8 subgroup of *Drosophila* Enhancer of split proteins.

Our studies have also shown that maximal Hes6-mediated degradation of Hes1 is correlated with a decreased stability of Hes6 itself. This observation suggests that the formation of Hes1·Hes6 heterodimers may increase the intrinsic susceptibility of Hes6 to degradation causing the 'recruitment' of Hes1 into the same proteolytic mechanisms. Although the molecular events underlying such a process remain to be fully elucidated, our

investigations have revealed important roles for both the protein kinase CK2 phosphorylation site at Ser183 of Hes6 and the WRPW motif. We have shown that mutation of Ser183 into Ala attenuates, albeit does not abrogate, the destabilizing effect of Hes6 on Hes1. This finding suggests that the SPXSS-SDXE motif of Hes6 and its resident Ser183 may contribute to the mechanisms that activate the PEST domain of Hes6. Heterodimerization with Hes1 may render this region more accessible to such mechanisms thereby promoting the degradation of Hes6 and Hes1. Alternatively, the phosphorylation of Ser183 may cause a misalignment of the WRPW motifs of Hes1 and Hes6 when these factors heterodimerize, leading to a conformation that results in sub-optimal Gro/TLE binding compared to homodimers of either protein. This may lead to the formation of misfolded Hes1·Hes6·Gro/TLE ternary complexes that may be recognized as defective and targeted for removal via proteolytic degradation. Mutation of Ser183 may allow Hes1·Hes6 dimers to interact better with Gro/TLE resulting in the formation of properly folded complexes of increased stability.

The possibility that enhanced proteolysis of Hes1 and Hes6 may be caused by their association into incorrectly folded complexes is also suggested by our observation that the formation of Hes1·Hes6 heterodimers does not appear to be sufficient to activate proteolytic degradation of these proteins by itself, because removal of the WRPW motif from either or both Hes1 and Hes6 progressively attenuates Hes1 degradation promoted by Hes6. Moreover, Hes6ΔWRPW had no detectable effect on the stability of the chimeric protein Hes1ΔWRPW:Gro/TLE1, in contrast to the significant degradation induced by full-length Hes6. In addition, heterodimers of Hes1 and Hes6 do not efficiently coimmunoprecipitate with Gro/TLE, regardless of whether they contain one or two WRPW motif, suggesting that they may not be able to form stable complexes. Since removal of the WRPW motif does not

impair the ability of Hes1 and Hes6 to heterodimerize (data not shown), these observations suggest that heterodimers of Hes1 and Hes6 may be more susceptible to degradation if they are associated with Gro/TLE through their WRPW motifs. Heterodimers lacking this motif, and thus unable to interact with Gro/TLE, may be able to fold more properly and avoid extensive degradation. Based on these combined observations, we propose that Hes1·Hes6 heterodimers are prone to increased degradation when they form complexes with Gro/TLE. This situation may due to specific structural features of these proteins that may not allow the formation of properly folded complexes with Gro/TLE, in turn resulting in the activation of proteolytic mechanisms involving Ser183 of Hes6. Conversely, it may be phosphorylation of Ser183 that causes a misfolding of the carboxy-termini of these heterodimers and an impaired ability to bind to Gro/TLE, resulting in degradation as a secondary effect to remove the misfolded complexes. Future studies will be aimed at distinguishing between these possibilities. In either case, it appears that Ser183 plays an important role in Hes6 functions, as further indicated by the inability of Hes6(S183A) to promote neuronal differentiation (see below for further details).

We recognize that other mechanisms are also possible. For instance, the WRPW motif of Hes6 may promote the instability of Hes1·Hes6 heterodimers in a Gro/TLE-independent manner, possibly by acting as a binding site for proteins other than Gro/TLE resulting in the direct or indirect recruitment of proteolytic enzymes. However, it remains to be determined whether the WRPW motif mediates interactions with proteins other than Gro/TLE. In addition, we cannot rule out the possibility that the destabilizing effect of Hes6 on Hes1 is the result of the activity of Hes6 as a transcriptional repressor. Hes6 may directly suppress the expression of factors that promote the stability of Hes1. This seems unlikely, however, because Hes6ΔWRPW, which can not recruit the Gro/TLE corepressor and was

shown to be unable to mediate transcriptional repression when fused to GAL4bd (9), also promotes Hes1 degradation. In addition, the *in vivo* neurogenic activity of Hes6 does not appear to be DNA-binding dependent, arguing against mechanisms that are solely based on direct transcriptional functions (19). It remains possible, though, that Hes6 mediates as yet uncharacterized transcriptional mechanisms that may affect Hes1 expression in a dose-dependent manner.

Characterization of the molecular mechanisms underlying the suppression of Hes1-mediated transcriptional repression by Hes6.

To begin to elucidate if different mechanisms of Hes1 inhibition are used by Hes6 in combination (to achieve maximal effects) or separately (perhaps depending on particular cellular and/or developmental conditions), we have examined the effect of Hes6 on the ability of Hes1 to mediate transcriptional repression in cortical progenitor cells, where these proteins are coexpressed. We have found that Hes6 suppresses Hes1-mediated repression. Both Hes6 and Hes6ΔWRPW have a similar inhibitory effect. This observation does not suggest that the suppression of Hes1 activity derives from the Hes6-mediated repression of a gene(s) encoding a positive regulator(s) of Hes1, because previous studies have shown that Hes6 requires its WRPW motif to repress transcription when targeted to DNA as a fusion protein with GAL4bd (9). Moreover, this finding also argues against a mechanism involving solely a competition for Gro/TLE between Hes1 and Hes6 homodimers. To determine if Hes1 suppression was the result of the inhibition of the interaction of Hes1 with Gro/TLE or the promotion of Hes1 proteolysis (or a combination of both), we have examined the effect of Hes6 on a chimeric protein in which Hes1 is constitutively bound to Gro/TLE. This fusion protein represses transcription in cortical progenitor cells like full-length Hes1, and its repressive ability should not be affected by conditions that would otherwise inhibit

Gro/TLE recruitment. Our investigations have revealed that Hes6 still has an inhibitory effect on Hes1ΔWRPW:Gro/TLE1, although this is weaker than its effect on Hes1. These findings thus suggest that the promotion of Hes1 degradation plays an important role in the inhibitory effect of Hes6 on Hes1-mediated repression. In agreement with this possibility, we have found that Hes6ΔWRPW, which does not promote a significant proteolysis of Hes1ΔWRPW:Gro/TLE1, does not have a negative effect on repression mediated by the latter. Together, these findings clarify mechanisms that underlie the ability of Hes6 to act as a negative regulator of Hes1 in cortical neural progenitor cells.

Promotion of cortical neurogenesis by Hes6.

To determine if Hes6 is involved in the regulation of neuronal differentiation in the mammalian forebrain, we have examined the consequence of exogenous Hes6 expression in primary cultures of cortical neural progenitor cells. In our studies, Hes6 induced a decrease in the number of undifferentiated progenitor cells and an increase in the number of differentiated neurons arising from these progenitors, showing that Hes6 promotes cortical neuronal differentiation. This effect is likely the result of the recruitment of supernumerary progenitors into the neuronal lineage. Because neural progenitor cells of the dorsal telencephalon express proneural proteins like Ngn1 and -2, our results are consistent with previous studies in *Xenopus* suggesting that Hes6 promotes the neuronal differentiation of Ngn-expressing neural progenitor cells (19). Based on these results and our demonstration that Hes6 efficiently suppresses Hes1-mediated transcriptional repression in cortical progenitors, we propose that the inhibition of Hes1 activity is at least one of the mechanisms utilized by Hes6 to promote neuronal differentiation. In possible agreement with this, we have found that the mutated protein Hes6(S183A) had an attenuated negative effect on the

stability of Hes1 when compared to wildtype Hes6 and did not promote neuronal differentiation. These observations suggest a correlation between reduced ability to promote Hes1 degradation and reduced Hes6 neurogenic activity. We found that Hes6(S183A) was able to cause a detectable decrease of Hes6 stability in 293A cells but failed to promote the neuronal differentiation of cortical progenitors. This situation may reflect the fact that the observed residual levels of Hes1 may be sufficient to inhibit neuronal differentiation or that Hes6(S183A) may have a weaker effect on Hes1 in neural progenitors compared to 293A cells. It is entirely possible, however, that additional mechanisms involving Ser183 may be important for the neurogenic activity of Hes6. Further elucidation of the mechanisms underlying Hes6 activity will clarify important events regulating vertebrate neurogenesis.

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REFERENCE LIST

1. Akazawa, C., Y. Sasai, S. Nakanishi, and R. Kageyama. (1992) Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system. *J. Biol. Chem.* 267:21879-21885.

2. Anderson, D.J. (2001) Stem cells and pattern formation in the nervous system: the possible versus the actual. *Neuron* 30:19-35.

3. Bae, S.K., Y. Bessho, M. Hojo, and R. Kageyama. (2000) The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation. *Development* 127:2933-2943.

4. Chen, H.A, A. Thiagalingam, H. Chopra, M.W. Borges, J.N. Feder, B.D. Nelkin, S.B. Baylin, and D.W. Ball. (1997) Conservation of the Drosophila lateral inhibition pathway in human lung cancer: a hairy-related protein (HES-1) directly represses achaete-scute homolog-1 expression. *Proc. Natl. Acad. Sci.* USA 94:5355-5360.

5. Cossins, J., A.E. Vernon, Y. Zhang, A. Philpott, and P.H. Jones. (2002) Hes6 regulates myogenic differentiation. *Development* 129:2195-2207.

6. Dehni, G., Y. Liu, J. Husain, and S. Stifani. (1995) *TLE* expression correlates with mouse embryonic segmentation, neurogenesis, and epithelial determination. *Mech. Dev.* 53:369-381.

7. Fisher, A. L., S. Ohsako, and M. Caudy. (1996) The WRPW motif of the Hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. *Mol. Cell. Biol.* 16:2670-2677.

8. Fode, C., Gradwohl, X. Morin, A. Dierich, M. LeMeur, C. Goridis, and F. Guillemot. (1998) The bHLH protein NEUROGENIN2 is a determination factor for epibranchial placode-derived sensory neurons. *Neuron* 20:483-494.

9. Gao, X., T. Chandra, M.O. Gratton, I. Quelo, J. Prud'homme, S. Stifani, and R. St-Arnaud. (2001) HES6 acts as a transcriptional repressor in myoblasts and can induce the myogenic differentiation program. *J. Cell Biol.* 154:1161-1171.

10. Ghosh, A, and M.E. Greenberg. (1995) Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. *Neuron* 15:89-103.

11. Grbavec, D., and S. Stifani. (1996) Molecular interaction between TLE1 and the carboxyl-terminal domain of HES-1 containing the WRPW motif. *Biochem. Biophys. Res. Commun.* 223:701-705.

12. Grbavec, D., R. Lo, Y. Liu, and S. Stifani. (1998) Transducin-like Enhancer of split 2, a mammalian homologue of Drosophila Groucho, acts as a transcriptional repressor, interacts with Hairy/Enhancer of split proteins, and is expressed during neuronal development. *Eur. J. Biochem.* 258:339-349.

13. Guillemot, F., L.C. Lo, J.E. Johnson, A. Auerbach, D.J. Anderson, and A.L. Joyner. (1993) Mammalian *achaete-scute* homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell* 75:463-476.

14. Husain, J., R. Lo, D. Grbavec, and S. Stifani. (1996) Affinity for the nuclear compartment and expression during cell differentiation implicate phosphorylated Groucho/TLE1 forms of higher molecular mass in nuclear functions. *Biochem. J.* 317:523-531.

15. Ishibashi, M., K. Moriyoshi, Y. Sasai, K. Shiota, S. Nakanishi, and R. Kageyama. (1994) Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. *EMBO J.* 13:1799-1805.

16. Ishibashi, M., S.L. Ang, K. Shiota, S. Nakanishi, R. Kageyama, and F. Guillemot. (1995) 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. *Genes Dev.* 9:3136-3148.

17. Johnson, J.E., S.J. Birren, T. Saito, and D.J. Anderson. (1992) DNA binding and transcriptional regulatory activity of mammalian achaete-scute homologous (MASH) proteins revealed by interaction with a muscle specific enhancer. *Proc. Natl. Acad. Sci.* USA 89:3596-3600.

18. Kageyama, R., and S. Nakanishi. (1997) Helix-loop-helix factors in growth and differentiation of the vertebrate nervous system. Curr. Opin. Genet. Dev. 7:659-665.

19. Koyano-Nakagawa, N., J. Kim, D. Anderson, and C. Kintner. (2000) Hes6 acts in a positive feedback loop with the neurogenins to promote neuronal differentiation. *Development* 127:4203-4216.

20. Lee, J.E., S.M. Hollenberg, L. Snider, D.L. Turner, N. Lipnick, and H. Weintraub. (1995) Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science* 268:836-844.

21. Lee, J.C., S.B. Smith, H. Watada, J. Lin, D. Scheel, J. Wang, R.G. Mirmira, and M.S. German. (2001) Regulation of the pancreatic pro-endocrine gene Neurogenin3. *Diabetes* 50:928-936.

22. Liu, Z.P., R.L. Galindo, and S.A. Wasserman. (1997) A role for CKII phosphorylation of the cactus PEST domain in dorsoventral patterning of the Drosophila embryo. *Genes Dev.* 11:3413-3422.

23. McLarren, K.W., R. Lo, D. Grbavec, K. Thirunavukkarasu, G. Karsenty, and S. Stifani. (2000) The mammalian basic helix loop helix protein HES-1 binds to and modulates the transactivating function of the Runt-related factor Cbfa1. *J. Biol. Chem.* 275:530-538.

24. McLarren, K.W., F. Theriault, and S. Stifani. (2001) Association with the nuclear matrix and interaction with Groucho and RUNX proteins regulate the transcription repression ability of the basic helix loop helix factor Hes1. *J. Biol. Chem.* 276:1578-1584.

25. Nuthall, H., J. Husain, K.W. McLarren, and S. Stifani. (2002) Role for Hes1-induced phosphorylation in Groucho-mediated transcriptional repression. *Mol. Cell. Biol.* 22:389-399.

26. Ohtsuka, T., M. Ishibashi, G. Gradwhohl, S. Nakanishi, F. Guillemot., and R. Kageyama. (1999) *Hes1* and *Hes5* as Notch effectors in mammalian neuronal differentiation. *EMBO J.* 18:2196-2207.

27. Packman, L.C., K. Kubota, J. Parker, and N.J. Gay. (1997) Casein kinase II phosphorylates Ser468 in the PEST domain of the Drosophila IkappaB homologue cactus. *FEBS Lett.* 400:45-50.

28. Palaparti, A., A. Baratz, and S. Stifani. (1997) The Groucho/Transducin-like Enhancer of split transcriptional repressors interact with the genetically defined amino-terminal silencing domain of histone H3. *J. Biol. Chem.* 272: 26604-26610.

29. Paroush, Z., R.L. Finley, T. Kidd, S.M. Wainwright, P.W. Ingham, R. Brent, and D. Ish-Horowicz. (1994) Groucho is required for Drosophila neurogeneis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. *Cell* 79:805-815.

30. Pissarra, L., D. Henrique, and A. Duarte. (2000) Expression of hes6, a new member of the Hairy/Enhancer of split family, in mouse development. *Mech. Dev.* 95:275-278.

31. Rechsteiner, M., and S.W. Rogers. (1996) PEST sequences and regulation by proteolysis. *Trends Biochem. Sci.* 21:267-271.

32. Sasai, Y., R. Kageyama, Y. Tagawa, R. Shigemoto, and S. Nakanishi. (1992) Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and *Enhancer of split. Genes Dev.* 5:2620-2634.

33. Seidah, N., S. Benjannet, L. Wickham, J. Marcinkiewicz, S. Belanger Jasmin, S. Stifani, A. Basak, A. Prat, and M. Chretien. (2003) The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. *Proc. Natl. Acad. Sci.* USA 100:928-933.

34. Stifani, S., C.M. Blaumueller, N.J. Redhead, R.E. Hill, and S. Artavanis-Tsakonas. (1992) Human homologs of a Drosophila *Enhancer of split* gene product define a novel family of nuclear proteins. *Nature Genet.* 2:119-127.

35. Toma, J.G., H. El-Bizri, F. Barnabe-Heider, R. Aloyz, and F.D. Miller. (2000) Evidence that helix-loop-helix proteins collaborate with retinobloastoma tumor suppressor protein to regulate cortical neurogenesis. *J. Neurosci.* 20:7648-7656.

36. Tomita, K., M. Ishibashi, K. Nakahara, S.L. Ang, S. Nakanishi, M. Asashima, and R. Kageyama. (1996) Mammalian *hairy* and *Enhancer of split* homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. *Neuron* 16:723-734.

37. Trott, R.L., K. Madhavi, Z. Paroush, and A.P. Bidwai. (2001) *Drosophila melanogaster* casein kinase II interacts with and phosphorylates the basic helix-loop-helix proteins m5, m7, and m8 derived from the *Enhancer of split* complex. *J. Biol. Chem.* 276:2159-2167.

38. Vasiliauskas D., and C. Stern. (2000) Expression of mouse HES-6, a new member of the Hairy/Enhancer of split family of bHLH transcription factors. *Mech. Dev.* 98:133-137.

39. Yao, J., Y. Liu, R. Lo, I. Tretjakoff, A. Peterson, and S. Stifani. (2000) Disrupted development of the cerebral hemispheres in transgenic mice expressing the mammalian Groucho homologue Transducin-like Enhancer of split 1 in postmitotic neurons. *Mech. Dev.* 93:105-115.

40. Yao, J., E. Lai, and S. Stifani. (2001) The winged-helix protein brain factor 1 interacts with Groucho and Hes proteins to repress transcription. *Mol. Cell. Biol.* 21:1962-1972.

FIGURES AND LEGENDS

Figure 1. Promotion of cortical neurogenesis by Hes6.

(A) Primary cultures of E13.5 mouse embryonic cortical neural progenitor cells were transfected with plasmids encoding either GFP alone (GFP) or a combination of GFP and Hes6 (Hes6). Forty-eight hours later, cells were fixed and subjected to double-labelling analysis of the expression of GFP (left panels) or MAP2 (middle panels). The combined GFP and MAP2 staining is shown in the right panels. (B and C) Quantitation of the percentage of GFP-MAP2-double-positive cells (B) or of cells in similar double-labelling experiments conducted in parallel with antibodies against nestin (C). Results are shown as the mean ± S.D (n=5). *, P<0.01; **, P<0.001.

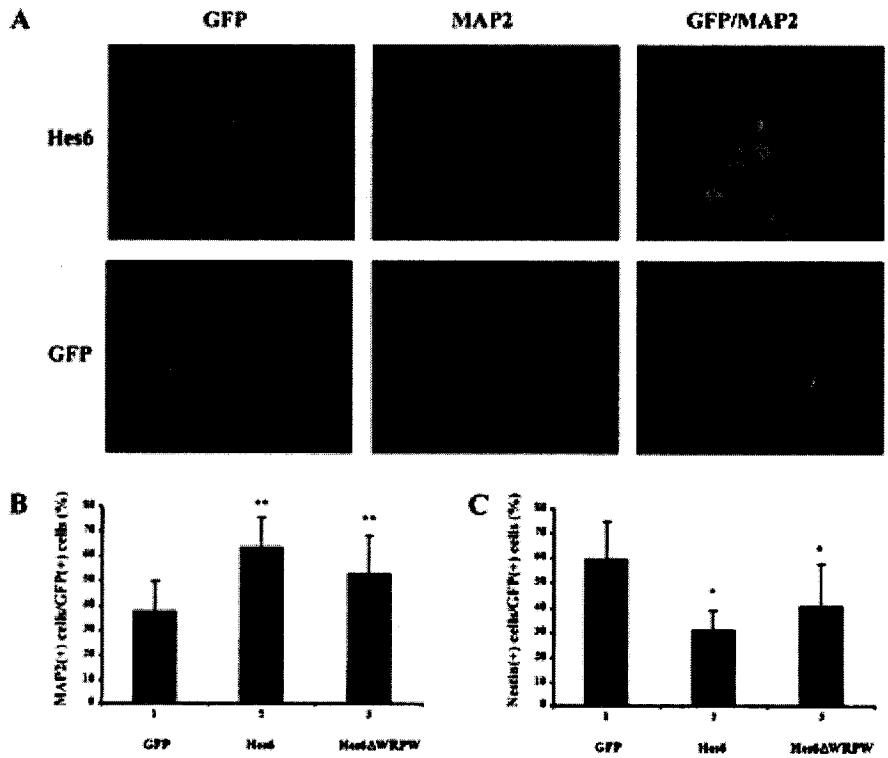

Figure 1: Promotion of cortical neurogenesis by Hes6

Figure 2. Interaction of Hes6 and Hes1 with Gro/TLE.

293A cells were transfected with plasmids encoding the indicated GST fusion proteins. Cell lysates were collected and the fusion proteins isolated on glutathione-Sepharose beads. The precipitated material (Pull-Down) was subjected to SDS-PAGE (lanes 5-8) on a 10% gel, together with one-tenth of each input lysate collected prior to incubation with glutathione-Sepharose beads (lanes 1-4). This was followed by Western blotting (WB) with either antibodies (Ab.) that recognize all Gro/TLE proteins (panTLE) (A) or anti-GST antibodies (B). Positions of size standards are indicated in kilodaltons.

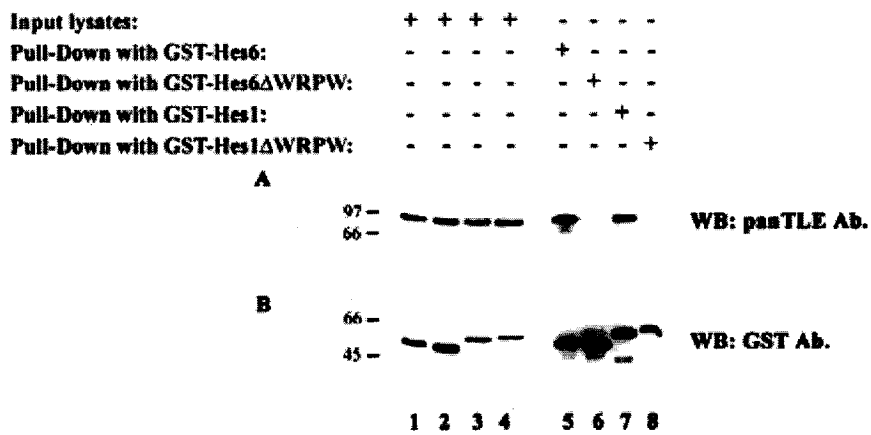

Figure 2: Interaction of Hes6 and Hes1 with Gro/TLE

Figure 3. Inhibition of the coimmunoprecipitation of Gro/TLE with Hes1 by Hes6 and Hes6ΔWRPW.

293A cells were transfected with plasmids encoding Hes1 or Hes1ΔWRPW in the absence or presence of HA-Hes6 or HA-Hes6ΔWRPW, as described in Materials and Methods. One-tenth of each cell lysate was subjected to SDS-PAGE (A-C) and the remaining lysates were subjected to immunoprecipitation (IP) with anti-FLAG antibodies (D and E). Samples were analyzed by Western blotting with anti-HA (A), anti-FLAG (B and D), or anti-Gro/TLE (C and E) antibodies. (D) The arrow points to the position of migration of Hes1. (E) The arrow points to the position of migration of Gro/TLE. The arrowhead indicates a non-specific band. IgG H., immunoglobulin G heavy chains. Positions of size standards are indicated in kilodaltons.

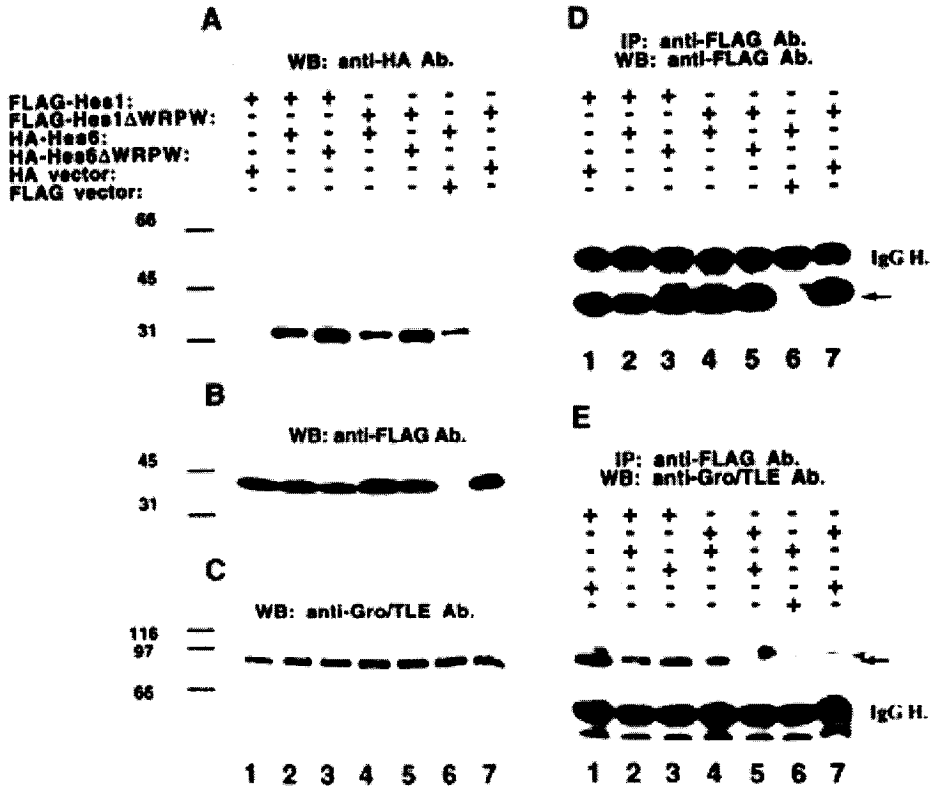

Figure 3: Inhibition of the coimmunoprecipitation of Gro/TLE with Hes1 by Hes6 and Hes6ΔWRPW

Figure 4. Inhibition of the coimmunoprecipitation of Gro/TLE with Hes1 by Hes6 and Hes6ΔWRPW.

293A cells were transfected with plasmids encoding the indicated combinations of proteins, as described in Materials and Methods. Cell lysates were collected and subjected to Western blotting (WB) with anti-GAL4bd (A) or anti-Gro/TLE (C) antibodies (Ab.), or immunoprecipitation (IP) with anti-FLAG antibodies followed by Western blotting with anti-FLAG (B) or anti-Gro/TLE (D) antibodies. The arrow in panel B points to the position of migration of Hes1. IgG H., immunoglobulin G heavy chains. Positions of size standards are indicated in kilodaltons.

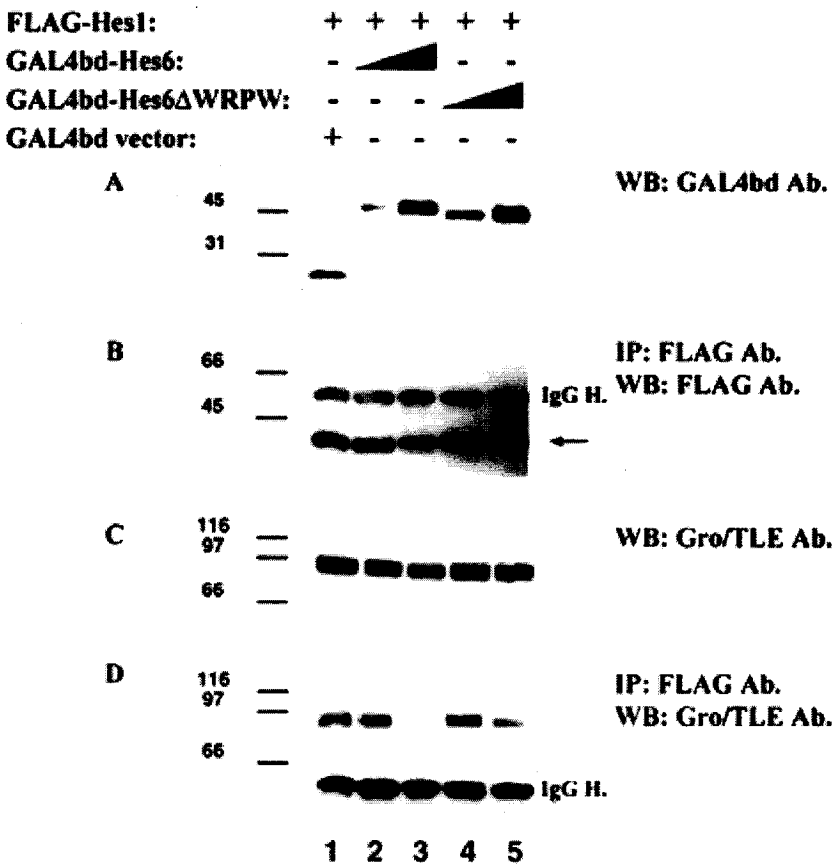

Figure 4: Inhibition of the coimmunoprecipitation of Gro/TLE with Hes1 by Hes6 and Hes6ΔWRPW

Figure 5. Effect of Hes6 expression on Hes1 stability.

293A cells were transfected with either FLAG-Hes1 or FLAG-Hes1ΔWRPW (50 ng/transfection), as indicated, in the absence (lanes 1 and 6) (HA vector) or presence of increasing amounts of HA-Hes6 or HA-Hes6ΔWRPW (200 ng/transfection in lanes 2, 4, 7, and 9, or 600 ng/transfection in lanes 3, 5, 8, and 10). Cell lysates were subjected to SDS-PAGE on an 11% gel, followed by sequential Western blotting (WB) with either anti-FLAG (A), anti-HA (B), or anti-Gro/TLE (C) antibodies (Ab.). Positions of size standards are indicated in kilodaltons.

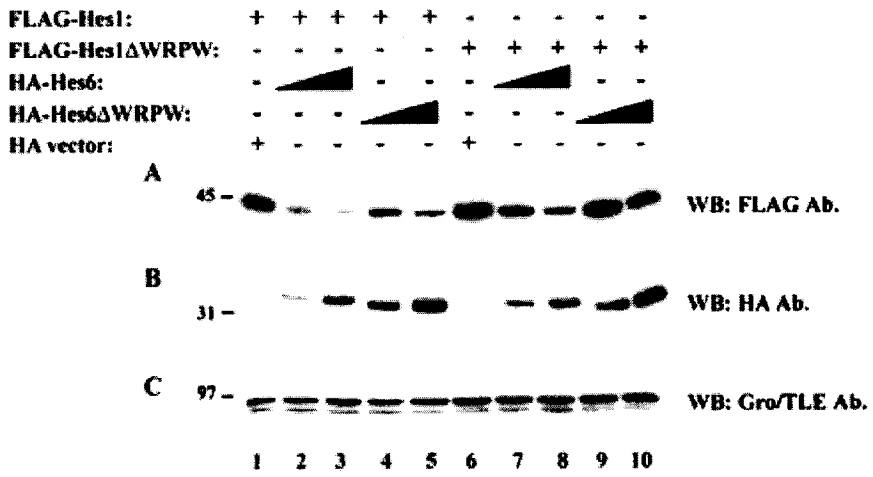

Figure 5: Effect of Hes6 expression on Hes1 stability

Figure 6. Analysis of Hes6 stability.

(A-C) 293A cells were transfected with the indicated combinations of proteins and then incubated in the absence or presence of MG132 as indicated, followed by cell lysis and Western blotting (WB) analysis. The levels of both HA-Hes6 (panel A, lanes 2 and 3) and GAL4bd-Hes6 (panel B, lanes 2 and 3) were reduced in the presence of MG132. In contrast, the levels of Hes6ΔWRPW (panel A, lanes 4 and 5), GAL4bd-Hes6ΔWRPW (panel B, lanes 4 and 5), Hes1 (panel C, lanes 1 and 2), and Gro/TLE (panel C, lanes 3 and 4) were not affected. Ab., antibodies. (D and E) 293A cells were transfected with increasing amounts of HA-Hes6 expression plasmid (400 ng/transfection in lane 2 or 800 ng/transfection in lane 3) in the presence of a constant amount of Hes1 (50 ng/transfection), followed by Western blotting with either anti-FLAG (D) or anti-HA (E) antibodies. (F) Cells were transfected with HA-Hes6 expression plasmid at 200 (lane 1), 400 (lane 2), or 800 (lane 3) ng/transfection in the absence of Hes1, followed by Western blotting with anti-HA antibodies. Positions of size standards are indicated in kilodaltons.

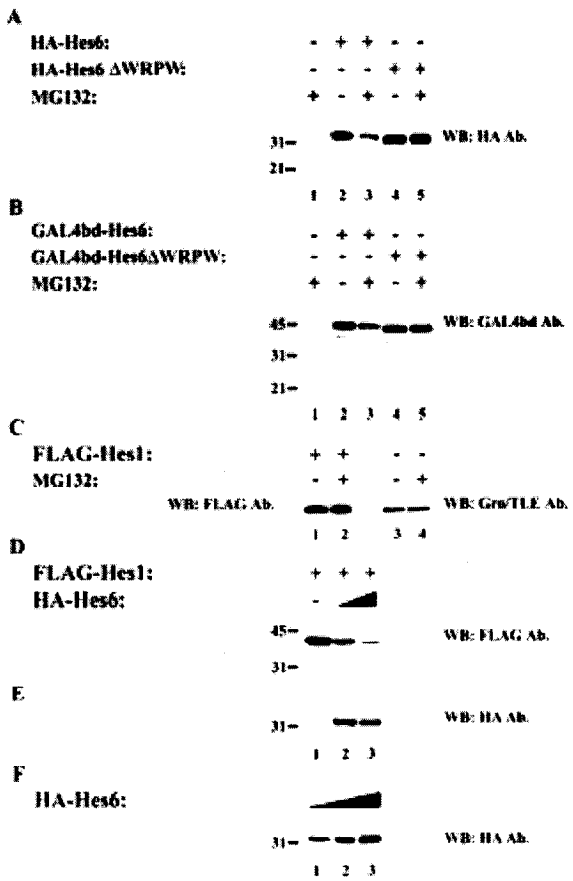

Figure 6: Analysis of Hes6 stability

Figure 7. Inhibition of Hes1-mediated transcriptional repression by Hes6 and Hes6ΔWRPW.

Primary cultures of neural progenitor cells isolated from the dorsal telencephalon of E13.5 mouse embryos were transfected with either the pFOX-ngn3p-Luc1 (A) or the pFOX-ΔN-box-ngn3p-Luc1 (B) reporter construct, as indicated, in the absence or presence of Hes1 or Hes1ΔWRPW:Gro/TLE1 and the indicated amounts (per transfection) of either HA-Hes6 or HA-Hes6ΔWRPW. The activity of the reporter gene in the absence of any expression plasmid was considered to be 100%. Luciferase activities were expressed as the mean ± S.D. of at least five independent experiments performed in duplicates. *, P<0.001; **, P<0.0001.

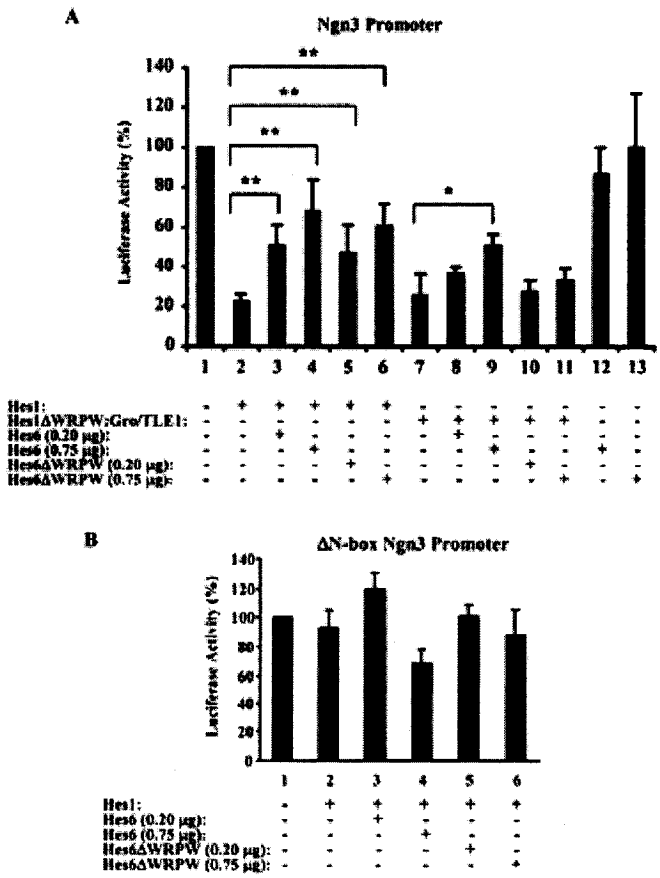

Figure 7: Inhibition of Hes1-mediated transcriptional repression by Hes6 and Hes6ΔWRPW

Figure 8. Effect of Hes6 on the expression of Hes1ΔWRPW:Gro/TLE1.

293A cells were transfected with plasmids encoding the indicated combinations of proteins, followed by preparation of cell lysates and Western blotting with either anti-FLAG (A), anti-GAL4bd (B), or anti-Gro/TLE (C) antibodies. Position of size standards are indicated in kilodaltons.

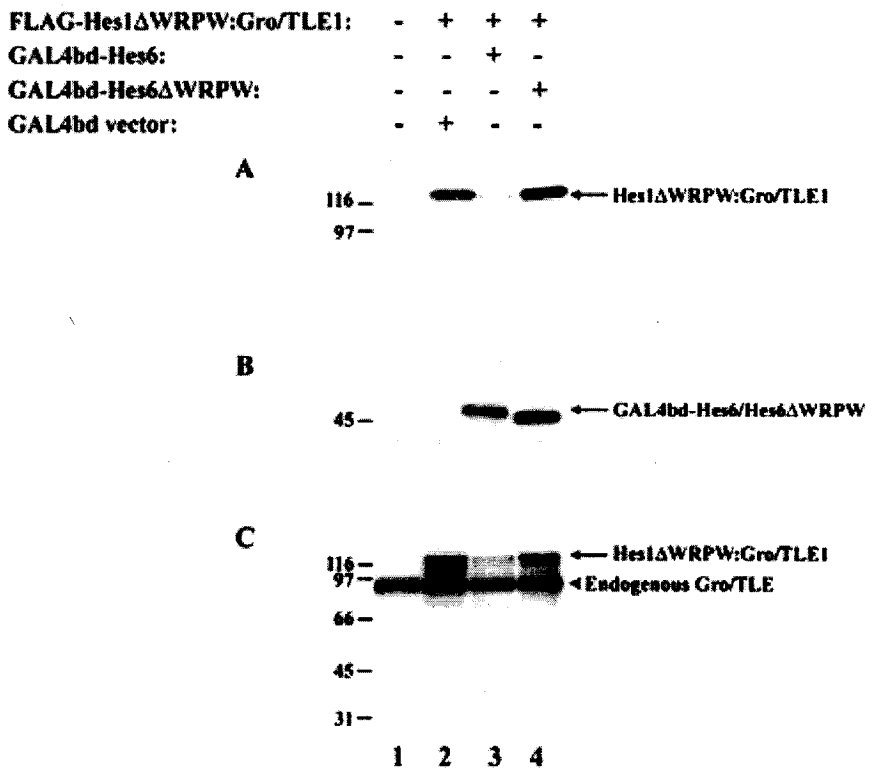

Figure 8: Effect of Hes6 on the expression of Hes1ΔWRPW:Gro/TLE1

Figure 9. Analysis of Hes6 phosphorylation.

(A) Schematic representation of the domain structure of Hes6. Indicated are the bHLH domain, the Orange domain predicted to form helices 3 and 4, the PEST region containing the SPXSS-SDXE motif and its resident Ser183, and the WRPW tetrapeptide. Shown in detail are the sequences of the SPXSS-SDXE elements from mouse and human Hes6 (3) and Drosophila Enhancer of split m5, m7, and m8 (37). Invariant residues are indicate in boldface. (B) 293A cells were transfected with HA-Hes6 and cell lysates were incubated in the absence or presence of calf intestinal phosphatase (CIP), followed by Western blotting with anti-HA antibodies. (C and D) The indicated GST fusion proteins were purified and subjected to *in vitro* phosphorylation in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of purified protein kinase CK2 (CK2), followed by autoradiography (D) and Western blotting (WB) with anti-GST antibodies (Ab.) (C). (E and F) The indicated GST fusion proteins were purified and subjected to *in vitro* phosphorylation in the presence of purified protein kinase CK2, followed by autoradiography (E) and Western blotting with anti-GST antibodies (F). Positions of size standards are indicated in kilodaltons in panel B and F.

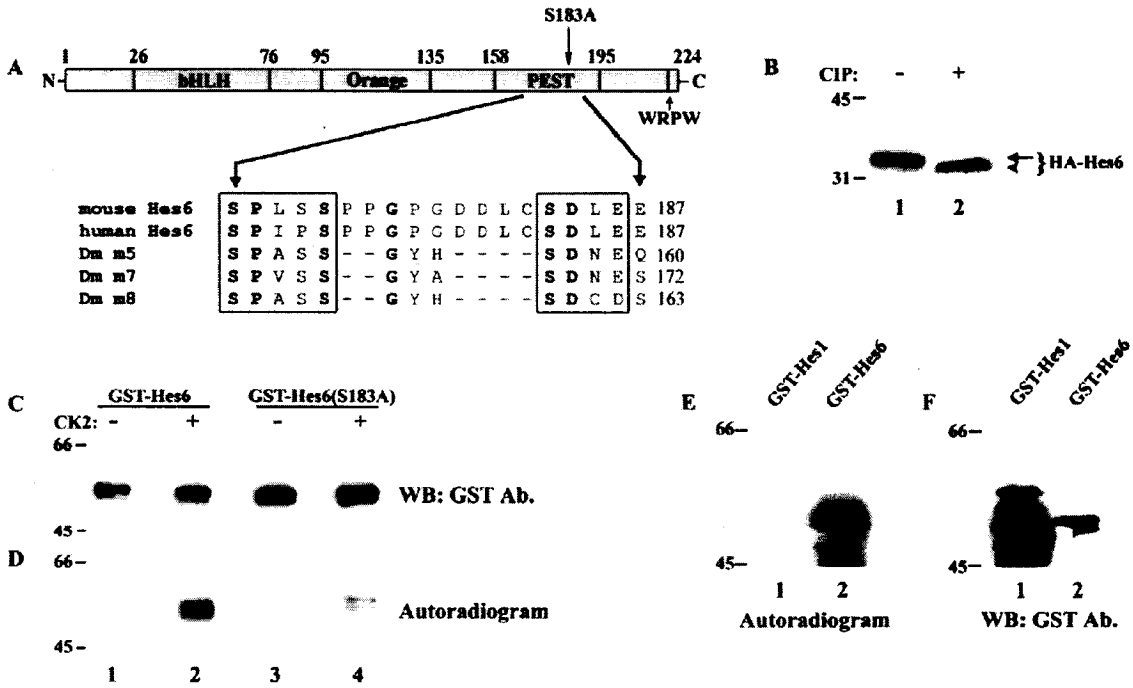

Figure 9: Analysis of Hes6 phosphorylation

Figure 10. Effects of S183A mutation on Hes6 functions.

(A and B) 293A cells were transfected with FLAG-Hes1 (50 ng/transfection) in the absence (lane 1) or presence of either HA-Hes6 (lane 2) or HA-Hes6(S183A) (lane 3) (600 ng/transfection). Cell lysates were subjected to SDS-PAGE, followed by Western blotting (WB) with either anti-FLAG (A) or anti-HA (B) antibodies (Ab.). Shown is a representative example of four separate experiments that gave the same results. Positions of size standards are indicated in kilodaltons. (C and D) E13.5 mouse embryonic cortical progenitor cells were transfected with plasmids encoding either GFP alone or a combination of GFP and Hes6 or GFP and Hes6(S183A). Forty-eight hr later, cells were fixed and subjected to double-labeling analysis of the expression of GFP, NeuN, or Ki67. Shown is the quantitation of the percentage of GFP-NeuN (C)- or GFP-Ki67 (D)-double-positive cells. Results are shown as the mean ± S.D (n=4) *, P<0.01.

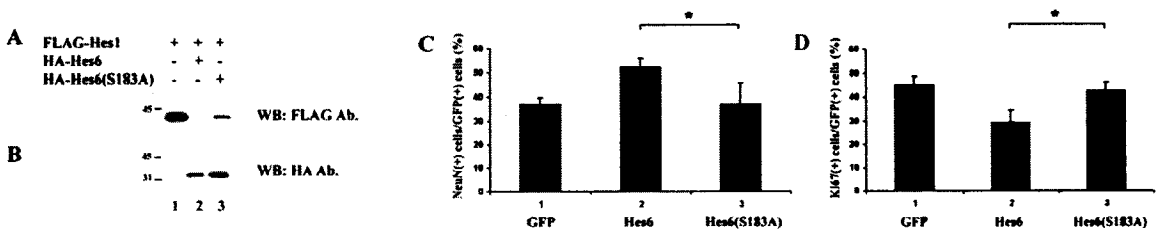

Figure 10: Effects of S183A mutation on Hes6 functions

SUMMARY AND CONCLUSIONS

Summary and future directions

The work presented in this thesis describes novel mechanisms that may underlie Hes6 function during the development of the mammalian nervous system. Our findings have shown that Hes6 is involved in the regulation of neuronal differentiation in the mammalian forebrain. Molecular and cellular investigations have identified two possible novel mechanisms by which Hes6 can antagonize Hes1 function. First, Hes6 induces a proteolysis of Hes1 that may inhibit the ability of Hes1 to bind DNA and repress transcription. This promotion of Hes1 degradation appears to play an important part in the inhibitory effect of Hes6 on Hes1-mediated transcriptional repression in cortical progenitor cells. The effect of Hes6 on Hes1 stability is maximal when both Hes1 and Hes6 contain the WRPW motif, suggesting that it is regulated by their ability to associate with Gro/TLE. In the future, it will be important to elucidate the molecular mechanisms that underlie these events. In particular, it will be interesting to perform structure/function studies to clarify the reasons why Hes6 in an unstable protein and what proteolytic enzymes are involved in Hes6 turn over. In addition, it will be important to understand why the ability to interact with Gro/TLE is correlated with a decreased stability of Hes1:Hes6 heterodimers.

Our investigations have also revealed that a point mutation (S183A) in a consensus site for phosphorylation by protein kinase CK2 reduces the ability of Hes6 to promote degradation of Hes1. Although it remains to be determined if Hes6 is phosphorylated in vivo by CK2, the in vitro phosphorylation of Hes6 by purified CK2 was reduced by the S183A mutation, showing that this residue is targeted by CK2 at least in vitro. In agreement with the possibility that S183 may play roles in Hes6 function, we have found that the ability of Hes6 to promote cortical neuronal differentiation is inhibited by mutation of S183 to a non-phosphorylatable residue. In the future, it will be important to determine whether or not

S183 is phosphorylated by CK2 in vivo and, if so, whether phosphorylation of this residue is important for the activity of Hes6 during in vivo cortical neurogenesis.

Our studies have also proposed a second mechanism by which Hes6 may inhibit Hes1 activity. This is the ability of Hes6 to interfere with the formation of complexes of Hes1 and Gro/TLE. Because the interaction with Gro/TLE is important for the transcription repression functions of Hes1, this effect of Hes6 may antagonize the transcriptional repression of Hes1 target genes. This effect is not simply the result of a competition for Gro/TLE interaction between Hes1 and Hes6 because the presence of the WRPW motif of Hes6 is not necessary. A possible hypothesis is that Hes1-Hes6 heterodimers have reduced affinity for Gro/TLE caused by an improper folding of these proteins, thus not allowing proper alignment of WRPW motifs. This may be caused by specific post-translational modifications such as the phosphorylation of Ser183 of Hes6. To test this hypothesis directly, it would be interesting to determine if the decreased stability of Hes1-Hes6 heterodimers is less dependent on the WRPW motif following mutation of Ser183 of Hes6. Also, it may be interesting to observe the effect of swapping experiments in which the SPXSS-SDXE motif of Hes6 is introduced into the C-terminus of Hes1 to determine if this affects the stability of Hes1 dimers.

Our studies have suggested that the ability of Hes6 to antagonize Hes1 function depends on the formation of Hes1-Hes6 heterodimers. In possible agreement with this, *in vitro* and *in vivo* protein-protein interaction studies performed by Koyano-Nakagawa et al. (2000) suggested that Hes6 binds more robustly to *Xenopus* hairy proteins than to itself. Thus, it is possible that Hes6 might preferentially heterodimerize rather than homodimerize. A similar situation was observed between E12 and MyoD proteins. E12 contains a so-called inhibitory domain N-terminal to the bHLH domain, which is characterized by an acid patch, DEDEDD (Sun and Baltimore, 1991). Using a mechanism not-yet identified, this sequence

prevents the formation of E12 homodimers and does not affect the ability of E12 to heterodimerize or to bind DNA (Shirakata and Paterson, 1995). Additional residues in E12 helix 1 are also involved in this inhibitory effect: two acidic residues (Glu580 and Glu 584) and a basic residue (Arg587). Conversely, MyoD contains three corresponding residues in helix 2, two basic (Arg142 and Arg146) and one acidic (Glu149), that have a role in opposing the E12 inhibitory domain thus allowing specificity in the formation of MyoD-E12 heterodimers (Shirakata and Paterson, 1995). Thus, MyoD can overcome the E12 dimerization inhibitory domain and form heterodimers with E12 through a mechanism involving, in part, charged amino acid residues in helix 2 (Shirakata and Paterson, 1995). Interestingly, Hes6 also contains an acidic patch (EDED) N-terminal to the basic arm of the bHLH domain, resembling the E12 inhibitory domain. It is therefore reasonable to hypothesize that this acidic patch could play a similar role to the E12 inhibitory domain and prevent the formation of Hes6 homodimers. Unlike MyoD, Hes1 does not contain 2 acidic residues in the helix 2 that would overcome the inhibitory domain. Other mechanisms would have to be invoked to explain Hes1-Hes6 dimerization. In the future, it would be important to examine these possibilities by characterizing the functional significance of the acidic sequence of Hes6.

Consistent with the possibility that Hes6 may preferentially heterodimerize, our results suggest that Hes6 antagonizes the ability of Hes1 to mediate transcriptional repression in cortical neural progenitor cells. Thus, by inhibiting Hes1, Hes6 may allow proneural proteins to perform their neurogenic function. In particular, Hes6 may also be able to suppress the passive transcriptional repression mediated by Hes1. This is because a proteolytic degradation of Hes1 would not only block its DNA-binding dependent functions but also functions that depend on protein-protein interactions.

Hes6 acts as a transcriptional repressor when targeted to DNA.

Although our investigations and the studies of Koyano-Nakagawa et al. (2000) have suggested that Hes6 opposes Hes1 activity and promotes neuronal differentiation through protein-protein interaction mechanisms, we cannot exclude the possibility that Hes6 may also utilize additional mechanisms that depend on DNA binding. As described previously in the literature review, Hes6 does not bind to the E or N box motifs recognized by other Hes proteins (Bae et al., 2000), but has the ability to bind to the ESE box *in vitro* (Cossins et al., 2002). This raises the question of whether, if able to bind to DNA at least under certain conditions, Hes6 might be able to regulate gene expression. By doing so, Hes6 could be able to repress the expression of factors that promote the stability of Hes1. In particular, it is possible that Hes6 might act as a transcriptional repressor due to its ability to interact with the corepressor Gro/TLE. To examine this possibility, I performed studies that demonstrated that a) Hes6 can mediate transcriptional repression when targeted to DNA as a fusion protein with the DNA-binding protein GAL4, b) the ability of GAL4-Hes6 to repress transcription depends on the WRPW motif of Hes6, and c) Hes6 interacts with Gro/TLE in a WRPW motif-dependent manner (Fig.11) (Gao et al., 2001). In spite of this, the ability of Hes6 to act as a transcriptional repressor does not seem to be relevant to neuronal development since the ectopic expression of a mutant Hes6 that lacks the C-terminal WRPW motifs is able to promote the formation of primary neurons in *Xenopus* embryos ((Koyano-Nakagawa et al., 2000; Cossins et al., 2002), and to promote cortical neuronal differentiation (Gratton et al., 2003). These results suggest that Hes6 does not need to interact with Gro/TLE corepressors to promote neurogenesis. In addition, Cossins et al. (2002) reported that ectopic expression of a Hes6DBM in which the basic arm of the bHLH domain is mutated to prevent DNA binding, resulted in a promotion of primary neurogenesis (Cossins et al., 2002). However, it is not clear whether this Hes6DBM form entered the nucleus since

a mutation in the basic arm may also have reduced its nuclear translocation. To elucidate the possibility that Hes6 acts as a transcriptional repressor in neuronal differentiation, a mutated form of Hes6 that does not bind to the ESE box but has the ability to translocate to the nucleus should be expressed in cortical progenitor cell cultures. In addition, it would be interesting to transfect into cortical progenitor cell cultures a chimeric protein of Hes6 and the transcriptional activator VP16. If this Hes6-VP16 chimeric protein still has the ability to promote neuronal differentiation this would suggest that transcriptional repression does not underlie Hes6 function in neuronal development. However, this would not discriminate between mechanisms in which Hes6 may act as a transcriptional activator or through protein-protein interactions.

In conclusion, our results have provided new insight into Hes6 functions and have suggested future investigations that will help to further elucidate the involvement of Hes6 in the mechanisms regulating mammalian neurogenesis.

Figure 11. HES6 binds TLE1 in mammalian cells.

(a) C2C12 myoblasts were cotransfected with expression vectors for GST epitope–tagged TLE1 and 6xHis epitope–tagged HES6. Cell extracts were precipitated with glutathione-Sepharose beads and analyzed by Western blotting using anti-GST (lanes 1–4) or anti-6xHis (lanes 5–8) antibodies. HES6, but not HES6-Δ, was coprecipitated with GST–TLE1. The empty GST expression vector (lanes 1 and 5) and empty HES6 expression vector, pEBVHis (lanes 2 and 6), served as negative controls for the specificity of the interaction. (b) Mammalian two-hybrid assay. 293 cells were cotransfected with the p5xGAL4UAS-SV40-luc reporter and pcDNA3-GAL4bd, pcDNA 3-GAL4bd–HES6, pcDNA3-GAL4bd–HES6-Δ, or pTLE1–VP16, alone or in combination, as indicated below the graph. Cells were collected 24 h after transfection and luciferase activity was assayed. Results are expressed as a percentage of expression relative to cells transfected with the reporter and empty vector alone. Means ± SD of three experiments are shown. (Figure reproduced from The Journal of Cell Biology, 2001, 154(6); 1161-1171. by copyright permission of The Rockefeller University Press.).

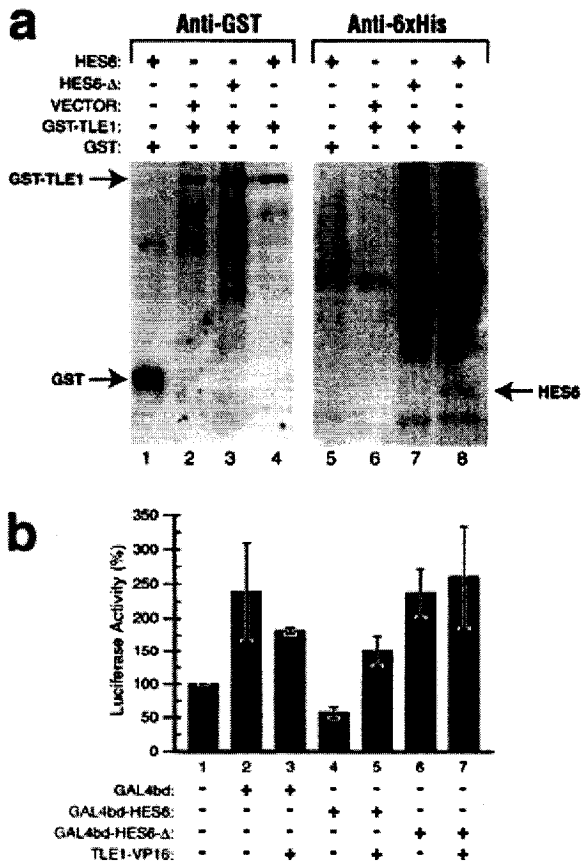

Figure 11: HES6 binds TLE1 in mammalian cells

REFERENCE LIST

Akazawa, C., Sasai, Y., Nakanishi, S., Kageyama, R. (1992) Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system. *J.Biol.Chem.* 267 (30): 21879-21885.

Akazawa, C., Ishibashi, M., Shimiza, C., Nakanishi, S., Kageyama, R. (1995) A mammalian helix-loop-helix factor structurally related to the product of *Drosophila* proneural gene *atonal* is a positive transcriptional regulator expressed in the developing nervous system. *J. Biol. Chem.* 270, 8730-8738.

Alifragis, P., Poortinga, G., Parkhurst, S.M., Delidakis, C. (1997) A network of interacting transcriptional regulators involved in *Drosophila* neural fate specification revealed by the yeast two-hybrid systme. *PNAS* 94, 13099-13104.

Allen, T., Lobe, C.G. (1999) A comparison of Notch, Hes and Grg expression during murine embryonic and post-natal development. *Cell Mol Biol* 45, 687-708.

Anderson, D.J. (2001) Stem cells and pattern formation in the nervous system: the possible versus the actual. *Neuron* 30: 19-35.

Artavanis-Tsakonas, S., Rand, M.D., Lake, R.J. (1999) Notch signaling: Cell fate control and signal integration in development. *Science* 284, 770-776.

Ayer, D.E (1999) Histone deacetylases: transcriptional repression with SINers and NuRDs. *Trends Cell Biol.* 9(5):193-8.

Bae, S., Bessho, Y., Hojo, M., Kageyama, R. (2000) The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation. *Development* 127 (13): 2933-2943.

Barrantes, I.B, Elia, A.J., Wunsch, K., Hrabe de Angelis, M.H., Mak, T.W., Rossant, J., Conlon, R.A., Gossler, A., de la Pompa, J.L. (1999) Interaction between Notch signalling and Lunatic fringe during somite boundary formation in the mouse. *Curr. Biol.* 9, 470-480

Ben-Arie, N., Bellen, H.J., Armstrong, D.L., McCall, A.E., Gorgdadze, P.R., Guo, Q., Matzuk, M.M., Zoghbi, H.Y. (1997) Math1 is essential for genesis of cerebellar granule neurons. *Nature* 390, 169-172

Bertrand, N., Castro, D.S., Guillemot, F. (2002) Proneural genes and the specification of neural cell types. *Nature Reviews* 3: 517-530.

Brantjes, H., Roose, J., van de Wetering, M., and Clevers, H. (2001) All Tcf HMG box transcription factors interact with Groucho-related co-repressors. *Nucleic Acids Res.* **29,** 1410–1419

Cabrera, C.V., Alonso, M.C. (1991) Transcriptional activation by heterodimers of the *achaete-scute* and *daughterless* gene products of *Drosophila*. *EMBO J.* 10, 2965-2973.

Campos-Ortega, J.A. (1993) Mechanisms of early neurogenesis in Drosophila melagonaster. *J. Neurobiol.* 24, 1305-1327.

Campuzano, S. (2001) Emc, a negative HLH regulator with multiple functions in *Drosophila* development. *Oncogene* 20, 8299-8307.

Casarosa, S., Fode, C., Guillemot, F. (1999) Mash1 regulates neurogenesis in the ventral telencephalon. *Development* 126, 525-534.

Castella, P., Wagner, J.A., Caudy, M. (1999) Regulation of hippocampal neuronal differentiation by the basic helix-loop-helix transcription factors HES1 and MASH-1. *J. Neurosci.Res.* 56, 229-240.

Cau, E., Casarosa, S., Guillemot, F. (2002) Mash1 and Ngn1 control distinct steps of detemrination and differentiation in the olfactory sensory neuron lineage. *Development* 129 1871-1880.

Chen, G., Nguyen, P.H., Courey, A.J. (1998) A role for Groucho tetramerization in transcriptional repression. *MCB* 18(12), 7259-7268.

Chen, G., Fernandez, J., Mische, S., and Courey, A. J. (1999) A functional interaction between the histone deacetylase Rpd3 and the corepressor Groucho in *Drosophila* development. *Genes Dev.* 13, 2218–2230.

Chen H., Thiagalingam, A., Chopra, H., Borges, M.W., Feder, J.N., Nelkin, B.D., Baylin, S.B., Ball, D.W. (1997) Conservation of the *Drosophila* lateral inhibition pathway in human lung cancer: a hairy-related protein (HES-1) directly represses acahete-scute homolog-1 expression. *PNAS* 94, 5355-5360

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., Kintner, C. (1995) Primary neurogenesis in *Xenopus* embryos regulated by a homolog of the *Drosophila* neurogenic gene *Delta. Nature* 375, 761-766.

Choi, C. Y., Kim, Y. H., Kwon, H. J., and Kim, Y. (1999) The homeodomain protein NK-3 recruits Groucho and a histone deacetylase complex to repress transcription. *J. Biol. Chem.* **274,** 33194–33197.

Cossins, J., Vernon, A.E., Zhang, Y., Philpott, A., Jones, P.H. (2002) Hes6 regulates myogenic differentiation. *Development* 129, 2195-2207.

Davis, R.L., Cheng, P.F., Lassar, A.B., Weintraub, H. (1990) The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. *Cell* 60 (5), 733-746.

Dawson, S.R., Turner, D.L., Weintraub, H., Parkhurst, S.M. (1995) Specificity for the hairy/enhancer of split basic helix-loop-helix (bHLH) proteins maps outside of the bHLH domain and suggests two separable modes of transcriptional repression. *Mol. Cell. Biol.* 15: 6923-6931.

de la Pompa, J.L., Wakeham, A., Correia, K.M., Samper, E., Brown, S., Aguilera, R.J., Nakanao, T., Honjo, T., Mak, T.W., Rossant, J., Conlon, R.A. (1997) Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* 124, 1139-1148.

Dehni, G., Liu, Y., Husain, J., Stifani, S. (1995). *TLE* expression correlates with mouse embryonic segmentation, neurogenesis, and epithelial determination. Mech. Dev. 53: 369-381.

Delidakis, C., Preiss, A., Hartley, D.A., Artavanis-Tsakonas, S. (1991) Two genetically and molecularly distinct functions involved in early neurogenesis reside within the Enhancer of split locus of Drosophila melanogaster. Genetics 129(3): 803-823.

Eberhard, D., Jiménez, G., Heavey, B., Busslinger, M. (2000) Transcriptional repression by Pax5 (BSAP) through interaction with corepressors of the Groucho family. *EMBO J.* 19, 2292-2303.

Ellenberger, T., Fass, D., Arnaud, M., Harrison, S.C. (1994) Crystal structure of transcription factor E47: E-box recognition by a basic region helix-loop-helix dimer. *Genes Dev.* 8, 970-980.

Farah, M.H., Olson, J.M., Sucic, H.B., Hume, R.I., Tapscott, S.J., Turner, D.L. (2000) Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. *Development* 127, 693-702.

Ferre-D'Amare, A.R., Prendergast, G.C., Ziff, E.B., Burley, S.K. (1993) Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain. *Nature* 363, 38-45.

Fisher, L., Ohsako, S., Caudy, M. (1996) The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. *Mol.Cell Biol.* 16 (6): 2670-2677.

Flores-Saaib, R. D., Courey, A. J. (2000) Analysis of Groucho-histone interactions suggests mechanistic similarities between Groucho- and Tup1-mediated repression. *Nucleic Acids Res.* **28,** 4189–4196.

Fode, G. Gradwohl, X. Morin, A. Dierich, M. LeMeur, C. Goridis, and F. Guillemot. (1998) The bHLH protein NEUROGENIN 2 is a determination factor for epibranchial placode-derived sensory neurons. *Neuron* 20 (3): 483-494.

Furukawa, T., Maruyama, S., Kawaichi, M., Honjo, T. (1992) The Drosophila homolog of the immunoglobulin recombination signal-binding protein regulates peripheral nervous system development *Cell* 69, 1191-1197.

Gao, X., Chandra, T., Gratton, M.-O., Quelo, I., Prud'homme, J., Stifani, S., St Arnaud, R. (2001) HES6 acts as a transcriptional repressor in myoblasts and can induce the myogenic differentiation program. *J.Cell Biol.* 154 (6): 1161-1171.

Giagtzoglou, N., Alifragis, P., Koumbanakis, K.A., Delidakis, C. (2003) Two modes of recruitment of E(spl) repressors onto target genes. *Development* 130(2): 259-270.

Gradwohl, G., Dierich, A., LeMeur, M., Guillemot, F. (2000) neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *PNAS* 97, 1607-1611.

Gratton, M.-O., Torban, E., Bélanger-Jasmin, S., Thériault, F.M., German, M.S., Stifani, S. (2003) Hes6 promotes cortical neurogenesis and inhibits Hes1 transcription repression activity by multiple mechanisms. *Mol. Cell. Biol.* 23 (19): 6922-6935.

Grbavec, D., Lo, R., Liu, Y., Greenfield, A., and Stifani, S. (1999) Groucho/ transducin-like enhancer of split (TLE) family members interact with the yeast transcriptional co-repressor SSN6 and mammalian SSN6-related proteins: implications for evolutionary conservation of transcription repression mechanisms. *Biochem. J.* **337,** 13–17.

Grbavec, D., Lo, R., Liu, Y., Stifani, S. (1998) Transducin-like Enhancer of split 2, a mammalian homologue of Drosophila Groucho, acts as a transcriptional repressor, interacts with Hairy/Enhancer of split proteins, and is expressed during neuronal development. *Eur. J. Biochem.* 258: 339-349.

Grbavec, D., Stifani, S. (1996) Molecular interaction between TLE1 and the carboxyl-terminal domain of HES-1 containing the WRPW motif. *Biochem.Biophys.Res.Commun.* 223 (3): 701-705.

Gromoller, A., Lehming, N. (2000) Srb7p is a physical and physiological target of Tup1p. *EMBO J.* **19,** 6845–6852.

Guillemot, F. (1995) Analysis of the role of basic-helix-loop-helix transcription factors in the development of neural lineages in the mouse. *Biol.Cell* 84 (1-2): 3-6.

Guillemot, F., Lo, L.C., Johnson, J.E., Auerbach, A., Anderson, D.J., Joyner, A.L. (1993) Mammalian Achaete-scute homolog is required for the early development of olfactory and autonomic neurons. *Cell* 75 (3): 463-476.

Heitzler, P., Haenlin M, Ramain P, Calleja M, Simpson P. (1996) Genes of the Enhancer of split and achaete-scute complexes are required for the regulatory loop between Notch and Delta during lateral signalling in *Drosophila. Development* 122: 161-171.

Hirata, H., Ohtsuka, T., Bessho, Y., Kageyama, R. (2000) Generation of structurally and functionally distinct factors from the basic helix-loop-helix gene *Hes3* by alternative first exons. *JBC* 275, 19083-19089.

Horton, S., Meredith, A., Richardson, J.A., Johnson, J.E. (1999) Correct coordination of neuronal differentiation events in ventral forebrain requires the bHLH factor MASH1. *Mol. Cell. Neurosci.* 14, 355-369.

Hsieh, J.J., Nofziger, D.E., Weinmaster, G., Hayward, S.D. (1997) Epstein-Barr virus immortalization: Notch2 interacts with CBF1 and blocks differentiation. *J. Virology*, 71, 1938-1945

Ishibashi, M., Ang, S.L., Shiota, K., Nakanishi, S., Kageyama, R., Guillemot, F. (1995) Targeted disruption of mammalian hairy and Enhancer of split homologs-1 (HES-1) leads to

up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. *Genes Dev.* 9, 3136-3148.

Ishibashi, M., Moriyoshi, K., Sasai, Y., Shiota, K., Nakanishi, S., Kageyama, R. (1994) Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. *EMBO J.* 13 (8): 1799-1805.

Jan, Y.N., Jan, L.Y. (1994) Neural cell fate specifications in Drosophila. *Curr. Opin. Neurobiol.* 4, 8-13.

Jarriault, S. , Brou, C. , Logeat, F. , Schroeter, E.H. , Kopan, R. and Israel, A. (1995) Signalling downstream of activated mammalian Notch. Nature, 377, 355-358

Jarriault, S. , Le Bail, O. , Hirsinger, E. , Pourquié, O. , Logeat, F. , Strong, C.F. , Brou, C. , Seidah, N.G. and Israël, A. (1998) Delta-1 activation of Notch-1 signaling results in HES-1 transactivation. Mol. Cell. Biol., 18, 7423-7431

Javed, A., Guo, B., Hiebert, S., Choi, J.Y., Green, J., Zhao, S.C., Osborne, M.A., Stifani, S., Stein, J.L., Lian, J.B., van Wijnen, A.J., Stein, G.S. (2000) Groucho/TLE/R-esp proteins associate with the nuclear matrix and repress RUNX (CBF(alpha)/AML/PEBP2(alpha)) dependent activation of tissue-specific gene transcription. *J. Cell Sci.* 113, 221-2231.

Jennings, B.H., Tyler, D.M., Bray, S.J. (1999) Target specificities of Drosophila enhancer of split basic helix-loop-helix proteins. *MCB* 19, 4600-4610.

Jimenez, G., Paroush, Z., Ish-Horowics, D. (1997) Groucho acts as a corepressor for a subset of negative regulators, including Hairy and Engrailed. *Genes Dev.* 11: 3072-3082.

Johnson, J.E., Birren, S.J., Saito, T., Anderson, D.J. (1992) DNA binding and transcriptional regulatory activity of the mammalian achaete-scute homologous (MASH) proteins revealed by interaction with the muscle-specific enhancer. *PNAS* 89, 3596-3600.

Kageyama, R., Nakanishi, S. (1997) Helix-loop-helix factors in growth and differentiation of the vertebrate nervous system. *Curr.Opin.Genet.Dev.* 7 (5): 659-665.

Koyano-Nakagawa, N., Kim, J., Anderson, D., Kintner, C. (2000) Hes6 acts in a positive feedback loop with the neurogenins to promote neuronal differentiation. *Development* 127 (19): 4203-4216.

Lee, J.C., Smith, S.B., Watada, H., Lin, J., Scheel, D., Wang, J., Mirmira, R.G., German, M.S. (2001) Regulation of the pancreatic pro-endocrine gene Neurogenin3. *Diabetes* 50: 928-936.

Lee, J.E. (1997) Basic helix-loop-helix genes in neural development. *Curr. Opin. Neurobiol.* 7, 13-20.

Levanon, D., Goldstein, R.E., Bernstein, Y., Tang, H., Goldenberg, D., Stifani, S., Paroush, Z., Groner, Y. (1998) Transcriptional repression by AML1 and LEF-1 is mediated by the TLE/Groucho corepressors. *PNAS* 95, 11590-11595

Lo, L., Dormand, E., Greenwood, A., Anderson, D.J. (2002) Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian achaete-scute and atonal homologs in cultured neural progenitor cells. *Development* 129 (7): 1553-1567.

Ma, P.C., Rould, M.A., Weintraub, H., Pabo, C.O. (1994) Crystal structure of MyoD bHLH domain-DNA complexes: perspectives on DNA recognition and implications for transcriptional activation. Cell 77, 451-459

Ma, Q., Chen, Z., del Barco Barrantes, I., de la Pompa, J.L., Anderson, D.J. (1998) neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. *Neuron* 20 (3): 469-482.

Ma, Q., Kintner, C., Anderson, D.J. (1996) Identification of neurogenin, a vertebrate neuronal determination gene. *Cell* 87, 43-52.

Massari, M.E., Murre, C. (2000) Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. Mol. Cell. Biol. 20, 429-440.

McLarren, K.W., Lo, R., Grbavec, D., Thirunavukkarasu, K., Karsenty, G., Stifani, S. (2000). The mammalian basic helix loop helix protein HES-1 binds to and modulates the transactivating function of the Runt-related factor Cbfa1. *J. Biol. Chem.* 275: 530-538.

McLarren, K.W., Theriault, F. M., Stifani, S. (2001) Association with the nuclear matrix and interaction with Groucho and RUNX proteins regulate the transcription repression activity of the basic helix loop helix factor Hes1. *J.Biol.Chem.* 276 (2): 1578-1584.

Miyata, T., Maeda. T., Lee, J.E. (1999) NeuroD is required for differentiation of the granule cells in the cerebellum and hippocampus. Genes Dev. 13, 1647-1652.

Muhr, J.E., Andersson, E., Persson, M., Jessell, T.M., Ericson, J. (2001) Groucho-mediated transcriptional repression establishes progenitor cell pattern and neuronal fate in the ventral neural tube. *Cell* 104, 861-873.

Nakamura, Y., Sakakibara, S., Miyata, T., Ogawa, M., Shimazaki, T., Weiss, S., Kageyama, R., Okano, H. (2000) The bHLH gene hes1 as a repressor of the neuronal commitment of CNS stem cells. *J. Neuroscience.* 20 (1): 283-293.

Nakao, K., Campos-Ortega, J.A. (1996) Persistent expression of genes of enhancer of split complex suppresses neural development in Drosophila. *Neuron* 16: 275-286.

Nishimura, M, Isaka, F., Ishibashi, M., Tomita, K., Tsuda, H., Nakanishi, S., Kageyama, R. (1998) Structure, chromosomal locus, and promoter of mouse *Hes2* gene, a homologue of *Drosophila* hairy and Enhancer of split. *Genomics* 49, 69-75.

Nuthall, H.N., Hussain, J., McLarren, K.W., Stifani, S. (2002) Role for Hes1-induced phosphorylation in Groucho-mediated transcriptional repression. *MCB*, 22 (2): 389-399.

Oellers, N., Dehio, M., Knust, E. (1994) bHLH protein encoded by the Enhancer of split complex of *Drosophila* negatively interferes with transcriptional activation mediated by proneural genes. *Mol. Gen. Genet.* 244: 465-473.

Ohsako, S., Hyer, J., Panganiban, G., Oliver, I, Caudy, M. (1994) Hairy function as a DNA-binding helix-loop-helix repressor of *Drosophila* sensory organ formation. Genes Dev. 8: 2743-2755.

Ohtsuka, J.M., Asakura, A., Snider, L., Hawkes, R., Strand, A., Stoeck, J., Hallahan, A., Pritchard, J., Tapscott, S.J. (2001) NeuroD2 is necessary for development and survival of central nervous system neurons. *Dev. Biol.* 234; 174-187.

Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F., Kageyama, R. (1999) Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. *EMBO J.* 18 (8): 2196-2207.

Palaparti, A., Baratz, A., and Stifani, S. (1997) Cell Cycle-dependent Phosphorylation of Groucho. *J. Biol. Chem.* **272,** 51056, 26604–26610

Paroush, Z., Finley, R.L., Kidd, T., Wainwright, S.M., Ingham, P.W., Brent, R., Ish-Horowicz, D. (1994) Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. *Cell* 79 (5): 805-815.

Perez, S.E., Rebelo, S., Anderson, D.J. (1999) Early specification of sensory neuron fate revealed by expression and function of neurogenin in the chick embryo. *Development* 126, 1715-1728

Pickles, L.M., Roe, S.M., Hemingway, E.J., Stifani, S., Pearl, L.H. (2002) Crystal Structure of the C-Terminal WD40 Repeat Domain of the Human Groucho/TLE1 Transcriptional Corepressor. *Structure.(Camb.)* 10 (6):751-761.

Pissarra, L., Henrique, D., Duarte, A. (2000) Expression of hes6, a new member of the Hairy/Enhancer-of-split family, in mouse development. *Mech.Dev.* 95 (1-2): 275-278.

Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R., Nakanishi, S. (1992) Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. *Genes Dev.* 6 (12B): 2620-2634.

Schrons, H., Knust, E., Campos-Ortega, J.A. (1992) The Enhancer of split complex and adjacent genes in the 96F region of Drosophila melanogaster are required for segregation of neural and epidermal progenitor cells. Genetics 132(2): 481-503.

Shirakata, M., Paterson, B.M. (1995) The E12 inhibitory domain prevents homodimer formation and facilitates selective heterodimerization with MyoD family of gene regulatory factors. *EMBO J.* 14(8); 1766-1772.

Sommer, L., Ma, Q., Anderson, D.J. (1996) Neurogenins, a novel family of atonal-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. *Mol. Cell. Neurosci.* 8(4), 221-241.

Struhl, K. (1998) Histone acetylation and transcriptional regulatory mechanisms. Genes Dev. 12(5): 599-606.

Sun, X.H., Baltimore, D. (1991) An inhibitory domain of E12 transcription factor prevents DNA binding in E12 homodimers but not in E12 heterodimers. *Cell* 64(2);459-470.

Takebayashi, K., Sasai, Y., Sakai, Y., Watanabe, T., Nakanishi, S., Kageyama, R. (1994) Structure, chromosomal locus, and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-1. Negative autoregulation through the multiple N box elements. *J. Biol. Chem.* 269: 5150-5156.

Takebayashi, K., Takahashi, S., Yokota, C., Tsuda, H., Nakaniski, S., Kageyama, R. (1997) Conversion of ectoderm into a neural fate by ATH-3, a vertebrate basic helix-loop-helix gene homologous to *Drosophila* proneural gene *atonal. EMBO J.* 16, 384-395.

Tietze, K., Oellers, N., Knust, E. (1992) Enhancer of splitD, a dominant mutation of *Drosophila*, and its use in the study of functional domains of a helix-loop-helix protein. *PNAS* 89, 6152-6156.

Tomita, K., Ishibashi, M., Nakahara, K., Ang, S.L., Nakanishi, S., Guillemot, F., Kageyama, R. (1997) Mammalian hairy and Enhancer of split homolog 1 regulates differentiation of retinal neuronsand its essential for eye morphogenesis. (1996) *Neuron* 16, 723-734.

Vasiliauskas, D., Stern, C.D. (2000) Expression of mouse HES-6, a new member of the Hairy/Enhancer of split family of bHLH transcription factors. *Mech.Dev.* 98 (1-2): 133-137.

Van Doren, M., Bailey, A.M., Esnayra, J., Ede, K., Posakony, J.W. (1994) Negative regulation of proneural gene activity: hairy is a direct transcriptional represor of achaete. *Genes Dev.* 8, 2729-2742.

Wainwright, S.M., Ish-Horowicz, D. (1992) Point mutations in the Drosophila hairy gene demonstrated in vivo requirements for basic, helix-loop-helix, and WRPW domains. *Mol. Cell. Biol.* 12: 2475-2483.

Wang, J.C., Waltner-Law, M., Yamada, K., Osawa, H., Stifani, S., Granner, D.K.. (2000) Transducin-like enhancer of split proteins, the human homologs of Drosophila groucho, interact with hepatic nuclear factor 3beta. *J. Biol Chem* 275, 18418-18423.

Wang, S., Sdrulla, A.D., diSibio, G., Bush, G., Nofziger, D., Hicks, C., Weinmaster, G., Barres, B.A. (1998) Notch Receptor Activation Inhibits Oligodendrocyte Differentiation. *Neuron* 21, 63-75.

Winston, R.L., Gottesfeld, J.M. (2000) Rapid identification of key amino-acid DNA contacts through combinatorial peptide synthesis. *Chem. Biol.* 7; 245-251.

Workman JL, Kingston RE. (1998) Alteration of nucleosome structure as a mechanism of transcriptional regulation. Annu Rev Biochem. 67:545-79.

Yao, J., Lai, E., and Stifani, S. (2001) The winged-helix protein brain factor 1 interacts with Groucho and Hes proteins to repress transcription. *Mol. Cell. Biol.* 21:1962-1972.

Yao, J., Liu, Y., Husain, J., Lo, R., Palaparti, A., Henderson, J., Stifani, S. (1998) Combinatorial expression patterns of individual TLE proteins during cell determination and differentiation suggest non-redundant functions for mammalian homologs of Drosophila Groucho. *Dev. Growth Differ.* 40(2): 133-146.

Yao, J., Liu, Y., Lo, R., Tretjakoff, I., Peterson, A., Stifani, S. (2000) Disrupted development of the cerebral hemispheres in transgenic mice expressing the mammalian Groucho homologue Transducin-like Enhancer of split 1 in postmitotic neurons. *Mech. Dev.* 93:105-115.

Yokota, Y. (2001) Id and development. *Oncogene* 20, 8290-8298.

Yu, X., Li, P., Roeder, R.G., Wang, Z. (2001) Inhibition of androgen receptor-mediated transcription by amino-terminal enhancer of split. *Mol. Cell. Biol.* **21,** 4614–4625.

APPENDICES

HES6 acts as a transcriptional repressor in myoblasts and can induce the myogenic differentiation program

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HES6 is a novel member of the family of basic helix–loop–helix mammalian homologues of *Drosophila* Hairy and Enhancer of split. We have analyzed the biochemical and functional roles of HES6 in myoblasts. HES6 interacted with the corepressor transducin-like Enhancer of split 1 in yeast and mammalian cells through its WRPW COOH-terminal motif. HES6 repressed transcription from an N box–containing template and also when tethered to DNA through the GAL4 DNA binding domain. On N box–containing promoters, HES6 cooperated with HES1 to achieve maximal repression. An HES6–VP16 activation domain fusion protein activated the N box–containing reporter, confirming that HES6 bound the N box in muscle cells. The expression of HES6 was induced when myoblasts fused to become differentiated myotubes. Constitutive expression of HES6 in myoblasts inhibited expression of MyoR, a repressor of myogenesis, and induced differentiation, as evidenced by fusion into myotubes and expression of the muscle marker myosin heavy chain. Reciprocally, blocking endogenous HES6 function by using a WRPW-deleted dominant negative HES6 mutant led to increased expression of MyoR and completely blocked the muscle development program. Our results show that HES6 is an important regulator of myogenesis and suggest that MyoR is a target for HES6-dependent transcriptional repression.

Introduction

Cellular differentiation is controlled by the activation or repression of specific target genes. The basic domain helix–loop–helix (bHLH)* family of transcription factors has been shown to regulate several key developmental pathways, including neurogenesis (Kageyama and Nakanishi, 1997) and myogenesis (Molkentin and Olson, 1996; Yun and Wold, 1996). The myogenic bHLH factors include MyoD, myogenin, Myf-5, and MRF-4, and elegant genetic analyses have confirmed the essential role of these factors during muscle development (Hasty et al., 1993; Nabeshima et al., 1993; Rudnicki et al., 1993; Rawls et al., 1998). To activate transcription, the myogenic bHLH factors must heterodimerize with the ubiquitous E proteins E12, E47, or HEB (for review see Massari and Murre, 2000). The myogenic bHLH/E protein heterodimers then bind their cognate DNA recognition site, the E box, defined by the consensus CANNTG (Massari and Murre, 2000). Several regulatory and structural muscle genes have been shown to contain functional E boxes within their promoter control regions (Weintraub et al., 1991; Schwarz et al., 1992; Quong et al., 1993).

Myogenic bHLH factors are expressed in proliferating, undifferentiated myoblasts, but they do not activate muscle differentiation until myoblasts exit the cell cycle. This suggests that inhibitors of the function of myogenic bHLH proteins are expressed in cycling myoblasts. Several such inhibitors have been described. The proto-oncogene c-Jun inhibits myogenesis through direct protein–protein interactions between the proto-oncogene and MyoD (Bengal et al., 1992). The HLH protein Id is devoid of a basic DNA binding domain (Benezra et al., 1990), such that it forms inactive dimers with the ubiquitous E proteins, and prevents their interaction with the myogenic bHLH factors. Id expression is very rapidly downregulated within the first 24 h upon induction of myogenic differentiation, and forced expression of Id inhibits myogenesis (Jen et al., 1992).

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*Abbreviations used in this paper: bHLH, basic domain helix–loop–helix; DM, differentiation medium; GM, growth medium; GST, glutathione *S*-transferase; MHC, myosin heavy chain; MyoR, myogenic repressor; RT, reverse transcriptase; TLE, transducin-like Enhancer of split; TPR, tetratricopeptide repeat.

Key words: bHLH; HES6; HES1; MyoR; differentiation

The activity of the myogenic bHLH factors is also directly regulated through multiple mechanisms by other bHLH transcription factors. Twist inhibits myogenesis by blocking DNA binding by MyoD, by titrating E proteins, and by inhibiting transactivation by MEF2 (Spicer et al., 1996). Mist1 is another bHLH factor affecting the myogenic differentiation program by a combination of mechanisms. Mist1 homodimers can bind E box target sites and actively repress transcription (Lemercier et al., 1998). In addition, Mist1 can also interact with MyoD to form inactive MyoD–Mist1 heterodimers (Lemercier et al., 1998). Although the Mist1 protein accumulates in myoblasts, its expression becomes undetectable 24 h after induction of myogenesis (Lemercier et al., 1997) in a manner analogous to the Id expression pattern (Jen et al., 1992).

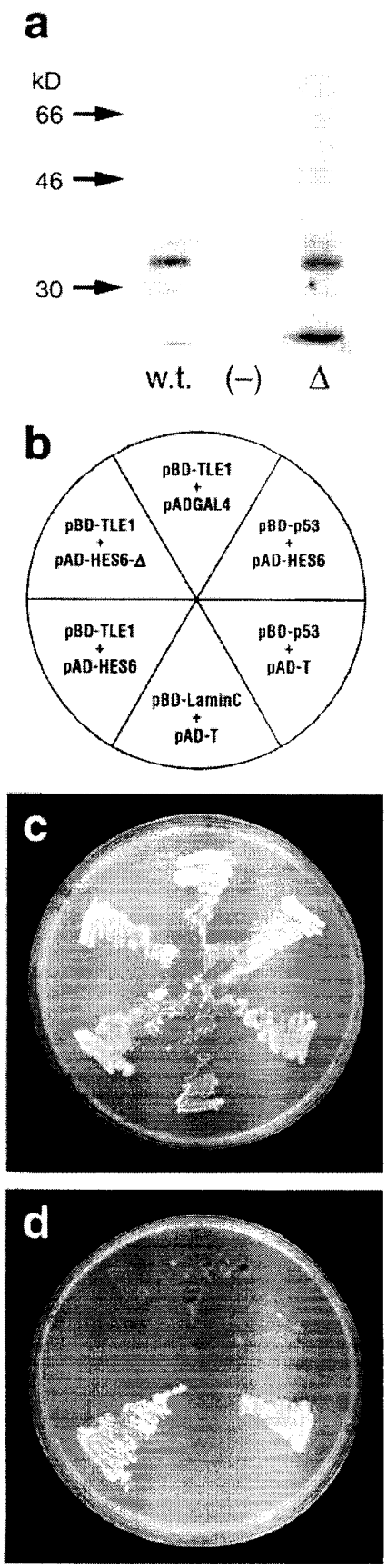
Recently, a muscle-specific bHLH protein that antagonizes the actions of MyoD has been cloned (Lu et al., 1999). This protein, myogenic repressor (MyoR), is abundantly expressed in undifferentiated myoblasts in culture, but is downregulated during differentiation. Low levels of transcript are detected after 3 d of differentiation regimen, but MyoR mRNA is undetectable by 5 d after induction of the myogenic program (Lu et al., 1999). MyoR is also specifically expressed in the developing embryo in the skeletal muscle lineage between embryonic day 10.5 (E10.5) and E16.5, and its expression is inhibited thereafter during the period of secondary myogenesis (Lu et al., 1999). MyoR forms heterodimers with E proteins, but acts as a transcriptional repressor after binding to regulatory E boxes (Lu et al., 1999). Thus, MyoR appears as a lineage-restricted transcriptional repressor of myogenesis.

Signaling through the transmembrane receptor Notch has also been shown to prevent myogenesis in tissue culture (Shawber et al., 1996; Nofziger et al., 1999; Wilson-Rawls et al., 1999), as well as in *Drosophila* embryos (Anant et al., 1998). Upon ligand binding, the intracellular domain of the Notch receptor is cleaved and freed to interact with the CBF1/KBF2/RBP-Jk transcription factors (homologues of the *Drosophila* Suppressor of Hairless proteins) (Tamura et al., 1995; Lu and Lux, 1996). The resulting complex activates the expression of candidate target genes of the HES family (mammalian *hairy* and *Enhancer of split* homologues). Indeed, constitutively active mutant Notch can activate the HES1 or HES5 promoters through CBF1 binding sites (Jarriault et al., 1998). The HES factors form homodimers that preferentially bind the sequence CACNAG, called an N box (Sasai et al., 1992; Tietze et al., 1992), but show greatly reduced affinity towards E boxes (Sasai et al., 1992). Some HES proteins can heterodimerize with the ubiquitous E proteins, but this interaction appears to titrate the E proteins and produce inactive dimers (Sasai et al., 1992). The DNA-bound HES protein dimers repress transcription by recruiting transducin-like Enhancer of split (TLE) proteins, the mammalian homologues of the *Drosophila* Groucho gene product, to specific DNA sites (Chen and Courey, 2000). The interaction between HES proteins and TLE repressors is mediated by the WRPW motif at the extreme COOH-terminal of the HES family member (Chen and Courey, 2000).

Since members of the HES family are transcriptional repressors, the net effect of activating Notch is to repress gene transcription (Artavanis-Tsakonas et al., 1995). Although the functional linkage between Notch and HES family members has been demonstrated in the regulation of neuronal differentiation using genetic manipulations (Ohtsuka et al., 1999), this link remains hypothetical in the case of muscle differentiation. HES1 has been reported to inhibit the activity of MyoD (Sasai et al., 1992), and this has been proposed as a mechanism whereby Notch inhibits myogenesis. However, more recent data have shown that Notch signaling inhibits myogenesis through an HES1-independent pathway (Shawber et al., 1996; Rusconi and Corbin, 1998; Nofziger et al., 1999; Wilson-Rawls et al., 1999). Moreover, activated forms of Notch do not upregulate the expression of HES1 in muscle cells (Shawber et al., 1996). Thus, the role of HES proteins in myoblasts remains to be determined.

Recently, a novel HES family member, HES6, was identified (Bae et al., 2000; Koyano-Nakagawa et al., 2000; Pissara et al., 2000; Vasiliauskas and Stern, 2000). HES6 is characterized by a shorter loop region within its helix–loop–helix domain, which prevents it from binding the N box (Bae et al., 2000; Koyano-Nakagawa et al., 2000). HES6 was shown to antagonize HES1 and prevent it from repressing transcription (Bae et al., 2000). By doing so, HES6 acted to promote retinal cell differentiation in explant cultures and *Xenopus* embryos (Bae et al., 2000; Koyano-Nakagawa et al., 2000). In contrast to HES1, HES6 is expressed by both undifferentiated and differentiated cells. During mouse embryogenesis, HES6 expression can be measured from E8.5 onwards, and high levels of HES6 transcripts were detected in tissues where Notch affects cell fate decisions, such as the nervous system, muscle, and thymus (Bae et al., 2000; Koyano-Nakagawa et al., 2000; Pissara et al., 2000; Vasiliauskas and Stern, 2000). During the muscle development program, HES6 was shown to be expressed during both myoblast commitment and differentiation, and is thus the only HES gene expressed throughout myogenesis in the embryo (Pissara et al., 2000; Vasiliauskas and Stern, 2000).

We examined the role of HES6 in myoblastic gene transcription and in the regulation of myoblast differentiation in culture. We report that HES6 binds TLE1 through its COOH-terminal WRPW tetrapeptide to repress transcription from N box–containing promoters in undifferentiated muscle cells. Interestingly, HES6 did not antagonize HES1 in myoblasts but cooperated in an additive manner to further repress transcription. Endogenous HES6 expression is induced during myogenesis in culture, and perturbing this pattern of expression affected the differentiation of the cells. When HES6 expression was enforced in myoblasts, MyoR expression was downregulated, and differentiation occurred even in the presence of high serum concentration. On the other hand, interfering with endogenous HES6 function by expressing a WRPW-deleted dominant negative HES6 mutant induced MyoR and inhibited myogenesis. Thus, although HES6 was seen to promote myoblast differentiation as it was reported to promote neurogenesis (Bae et al., 2000; Koyano-Nakagawa et al., 2000), the mechanisms involved appear quite different and point to cell-specific actions of HES6 in muscle cells. Our results implicate HES6 as a key regulator of the muscle development program and suggest that MyoR is a downstream target of HES6.



Results

We serendipitously cloned a partial murine HES–related cDNA in a yeast two-hybrid screen using a bait derived from a tetratricopeptide repeat (TPR) (Lamb et al., 1995) domain–containing protein. The functional relevance of the HES protein–TPR domain interaction remains unclear and has not been explored further. Database searches identified expressed sequence tags that allowed cloning of a nearly full-length cDNA. While this work was in progress, the first characterization of a novel HES family member, HES6, was published (Bae et al., 2000; Koyano-Nakagawa et al., 2000; Pissara et al., 2000; Vasiliauskas and Stern, 2000). Sequence comparison revealed that the clone that we isolated is identical to the published mouse HES6 cDNA but lacks the first 16 amino acids. As described previously, HES6 contains the hallmark features of HES family members, including the Orange domain, a proline-rich region, the conserved proline residue within the basic region, the helix–loop–helix structure, and the COOH-terminal WRPW motif (Bae et al., 2000). A distinctive feature of HES6 is the shorter loop region, which lacks four or five residues when compared with other family members (Bae et al., 2000). The shorter loop prevents HES6 from binding the canonical HES DNA binding site, the N box (CACNAG) (Bae et al., 2000; Koyano-Nakagawa et al., 2000).

The WRPW motif at the extreme COOH-terminal of HES family members mediates interaction between HES proteins and TLE repressors, the mammalian homologues of the *Drosophila* Groucho gene product (Chen and Courey, 2000). We tested whether HES6 interacts with TLE1 using the yeast two-hybrid protein interaction assay. Western blot assays of extracts from yeast cells transfected with pAD-HES6 (GAL4 activation domain fused to HES6) or pAD-HES6-Δ (a deletion engineered to remove the WRPW COOH-terminal peptide from the HES6 sequence) confirmed that both fusion proteins are produced in yeast cells (Fig. 1 a; proteins migrating at ~32 kD). Yeast cells that were cotransfected with pBD-TLE1 (GAL4 DNA binding domain fused to TLE1) and pAD-HES6 grew on selection plates lacking Leu, Trp, and His to the same extent as cells transfected with the control plasmids, pBD-p53 and pAD-T (Fig. 1 d). The ability of the transformed cells to grow on His^- medium indicates that a transcriptionally competent GAL4 complex was reconstituted due to the interaction between TLE1 and

Figure 1. **HES6 interacts with TLE1 in yeast cells.** Yeast two-hybrid protein interaction assay. (a) Immunoblots of extracts from yeast cells transfected with pAD–HES6 (w.t.) or pAD–HES6-Δ (Δ) confirmed that both fusion proteins are produced in yeast cells. Left lane, molecular size markers in kiloDaltons. (b) Bait and target plasmids used to cotransfect yeast. BD, binding domain of GAL4; AD, activation domain of GAL4. The positive controls, pBD-p53 and pAD-T, were supplied with Stratagene's HybriZAP kit. (c) Growth on Leu^-, Trp^- plates. (d) Growth on Leu^-, Trp^-, and His^- plates. The ability of the transformed cells to grow on His^- medium indicates that a transcriptionally competent GAL4 complex was reconstituted due to the interaction between TLE1 and HES6. Note that the interaction requires the WRPW motif, as the HES6-Δ deletion mutant, deprived of this motif, did not interact with TLE1 to support growth on His^- plates.

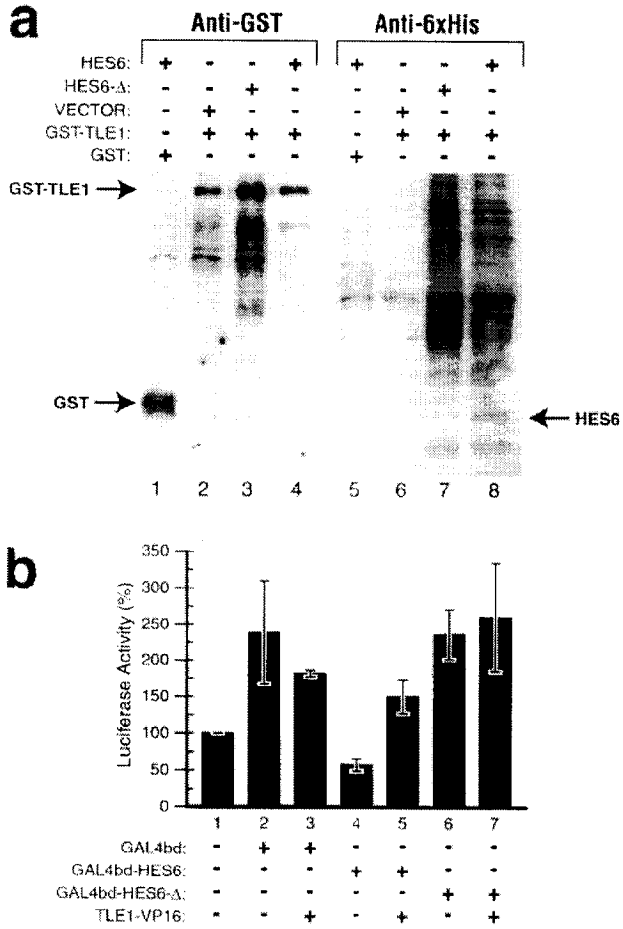


Figure 2. **HES6 binds TLE1 in mammalian cells.** (a) C2C12 myoblasts were cotransfected with expression vectors for GST epitope–tagged TLE1 and 6xHis epitope–tagged HES6. Cell extracts were precipitated with glutathione-Sepharose beads and analyzed by Western blotting using anti-GST (lanes 1–4) or anti-6xHis (lanes 5–8) antibodies. HES6, but not HES6-Δ, was coprecipitated with GST–TLE1. The empty GST expression vector (lanes 1 and 5) and empty HES6 expression vector, pEBVHis (lanes 2 and 6), served as negative controls for the specificity of the interaction. (b) Mammalian two-hybrid assay. 293 cells were cotransfected with the p5xGAL4UAS-SV40-luc reporter and pcDNA3-GAL4bd, pcDNA 3-GAL4bd–HES6, pcDNA3-GAL4bd–HES6-Δ, or pTLE1–VP16, alone or in combination, as indicated below the graph. Cells were collected 24 h after transfection and luciferase activity was assayed. Results are expressed as a percentage of expression relative to cells transfected with the reporter and empty vector alone. Means ± SD of three experiments are shown.

HES6. The interaction was confirmed in a positive filter assay for β-galactosidase activity (unpublished results). In contrast, cells cotransfected with pBD-TLE1 and pAD-HES6-Δ, while growing on Leu^-, Trp^- media (Fig. 1 c), could not grow on His^- plates (Fig. 1 d) and did not express β-gal (unpublished results). These results show that HES6 interacted with TLE1 in yeast cells through the WRPW motif.

To establish the physiological relevance of this interaction, we coprecipitated HES6 and TLE1 from transfected mammalian cells. C2C12 myoblasts were cotransfected with pGST-TLE1, a mammalian expression vector encoding a glutathione *S*-transferase (GST)–TLE1 fusion protein, and mammalian expression vectors for 6xHis epitope–tagged HES6 or HES6-Δ. Cell extracts were precipitated using glutathione-Sepharose beads and probed with anti-GST or anti-6xHis antibodies. Cells cotransfected with the empty pGST vector and HES6 served as controls for the specificity of the coprecipitation. Immunoblotting of transfected cell extracts confirmed that the epitope-tagged HES6 and HES6-Δ proteins were expressed at equivalent levels in transfected cells (unpublished results). GST and GST–TLE1 were expressed at similar levels and efficiently pulled down by the glutathione-Sepharose beads (Fig. 2 a, lanes 1–4). Probing the precipitates with the anti-6xHis antibody revealed that the epitope-tagged HES6 was specifically coprecipitated with GST–TLE1 (lane 8) and that the GST–TLE1–HES6 interaction required the WRPW sequence, since HES6-Δ was not coprecipitated with GST–TLE1 (lane 7). The binding of HES6 to GST–TLE1 involved the TLE1 moiety of the fusion protein, since expressing GST alone did not coprecipitate HES6 (lane 5).

We used a mammalian two-hybrid assay to confirm the coprecipitation results. Human 293 cells were transiently transfected with a reporter construct containing the luciferase gene under the control of the SV-40 promoter linked to five tandem GAL4 upstream activation sequence sites (5xGAL4UAS). This modified SV-40 promoter is basally active in mammalian cells (Grbavec et al., 1998). Cotransfection with a plasmid encoding the DNA binding domain of GAL4 (GAL4bd) resulted in a roughly 2.5-fold activation of transcription above basal level, as reported previously (Grbavec et al., 1999) (Fig. 2 b, compare lanes 1 and 2). In contrast, expression of a fusion protein of GAL4bd and HES6 (GAL4bd–HES6) had no transactivating effect; rather it resulted in a complete repression of GAL4bd-mediated activation and an ~50% repression of basal transcription (Fig. 2 b, lane 4). This result shows that HES6 mediates transcriptional repression when targeted to DNA (see also Fig. 4 below). Importantly, a fusion protein of GAL4bd and a truncated form of HES6 lacking the COOH-terminal WRPW (GAL4bd–HES6-Δ) motif was not able to repress basal transcription (Fig. 2 b, lane 6). GAL4bd–HES6 and GAL4bd–HES6-Δ were expressed at equal levels (unpublished results). These results suggest that the WRPW motif of HES6 is involved in its transcription repression ability by recruiting TLE corepressors. To test this possibility, cells were cotransfected with a plasmid encoding a fusion protein of TLE1 and the potent activation domain of the herpes simplex virion protein VP16. This manipulation was shown to convert TLE1 from a repressor to an activator (Wang et al., 2000). The expression of TLE1–VP16 had no significant effect on reporter gene expression in the presence of GAL4bd alone (Fig. 2 b, lane 3). In contrast, TLE1–VP16 blocked the ability of GAL4bd–HES6 to repress basal transcription and partly reduced activated transcription (Fig. 2 b, lane 5). This shows that TLE1–VP16 was recruited to the promoter site by interaction with HES6. This interaction required the WRPW motif of HES6 because TLE1–VP16 had no significant effect on GAL4bd–HES6-Δ (Fig. 2 b, lane 7). Together, these findings show that HES6 interacts with TLE proteins via its WRPW domain in mammalian cells and that this interaction is important for the ability of HES6 to mediate transcriptional repression.

The shorter loop region of the HES6 protein prevents it from binding N box–containing DNA (unpublished results)

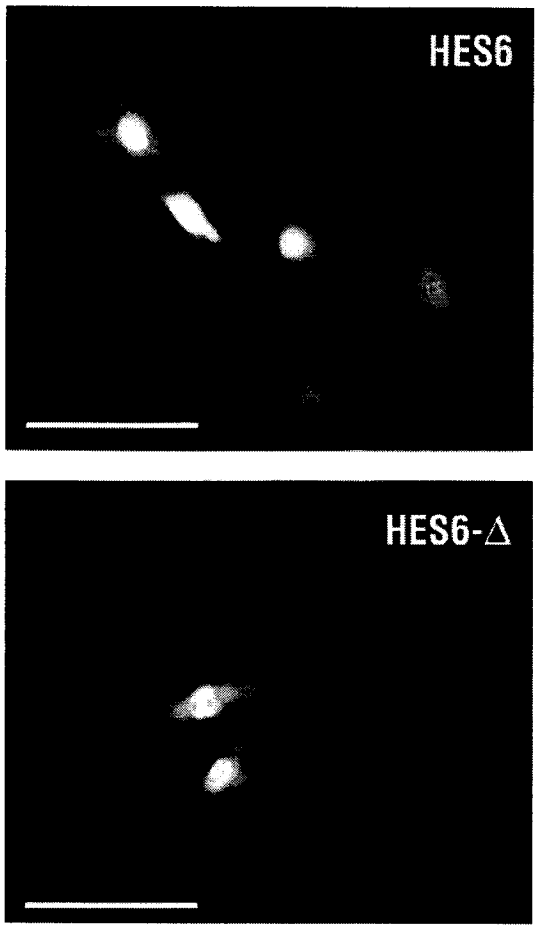


Figure 3. **HES6 and HES6-Δ localize to the nucleus in muscle cells.** C2C12 myoblasts were transiently transfected with epitope-tagged HES6 and HES6-Δ expression vectors and stained for immunofluorescence detection using antibodies directed against the epitope tag. Bar, 10 μm.

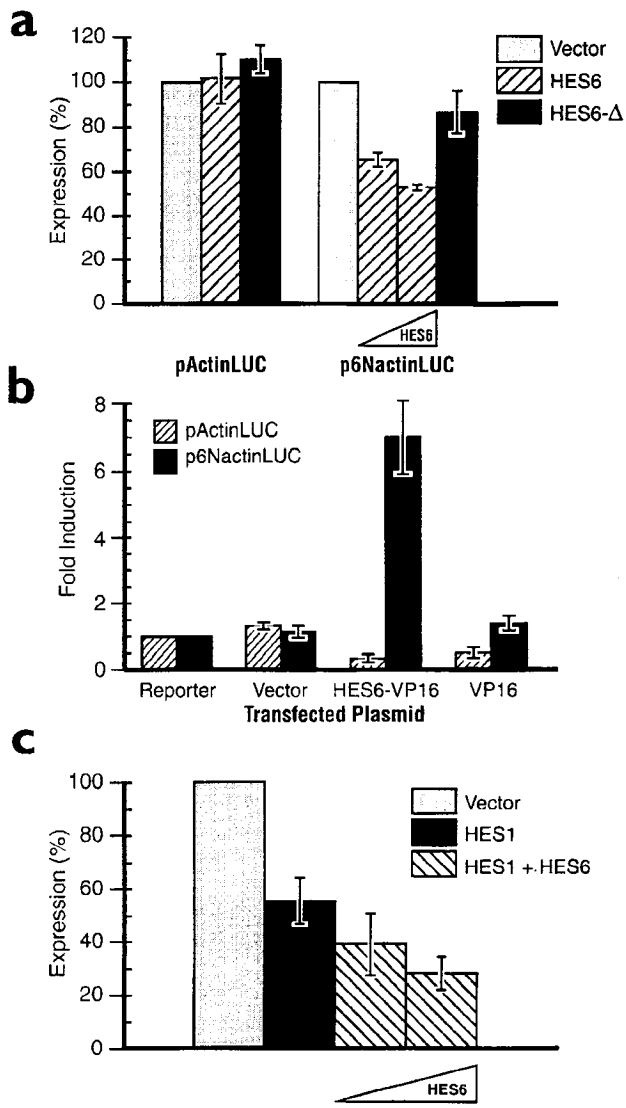


Figure 4. **HES6 binds the N box to repress transcription and does not antagonize HES1 in myoblasts.** Transient transfection of C2C12 myoblasts with HES6, HES6-Δ, HES6-VP16, and HES1 expression vectors, alone or in combination. (a) Transcriptional repression by HES6 in C2C12 cells. The control template (pActinLUC) did not contain N boxes, whereas the test template (p6NactinLUC) contained six copies of a canonical N box sequence. Results are expressed as a percentage of expression relative to cells transfected with the reporter and empty vector alone. (b) The HES6–VP16 activation domain fusion protein binds N boxes and activates the N box–containing reporter. Joining the VP16 activation domain to HES6 transformed HES6 from a repressor into an activator, and HES6–VP16 activated transcription specifically from the N box–containing promoter, whereas the full-length VP16 protein had no effect. Results are expressed as fold induction relative to cells transfected with the reporter alone. Vector, empty expression vector backbone. (c) HES6 cooperates with HES1 to maximally repress N box–dependent transcription in muscle cells. The reporter construct was p6NactinLUC. Results are expressed as a percentage of expression relative to cells transfected with the reporter and empty vector alone.

(Bae et al., 2000; Koyano-Nakagawa et al., 2000). However, HES6 was shown to antagonize the HES1-mediated repression of N box–containing promoters (Bae et al., 2000). We attempted to determine the influence of the WRPW sequence, and thus the binding of TLE factors, on N box–dependent transcriptional repression using expression vectors for HES6, HES6-Δ, and HES1. These experiments were performed in C2C12 myoblasts, which express TLE proteins (Grbavec et al., 1998). The HES6 and HES6-Δ proteins were expressed at similar levels and localized to the nucleus in transfected cells (Fig. 3). Surprisingly, in transiently transfected C2C12 myoblasts, HES6 suppressed transcription from reporter templates containing N boxes (Fig. 4 a). This effect was specific for the N box and required the WRPW COOH-terminal peptide, as HES6-Δ did not affect transcription from the N box–containing promoter (Fig. 4 a). The level of inhibition achieved by transfecting HES6 in myoblasts was similar to the inhibition observed when HES6 was tethered to DNA through the GAL4 DNA binding domain (Fig. 2 b, lane 4) and also to the level of inhibition generated by the transfection of similar amounts of HES1 in C2C12 cells (compare Fig. 4, a and c). We interpret these results to mean that in muscle cells, HES6 can dimerize with a bHLH partner to bind the N box, recruit TLE proteins, and repress transcription. To test this hypothesis, we engineered an HES6–VP16 fusion protein by joining the potent activation domain of the herpes simplex vir-

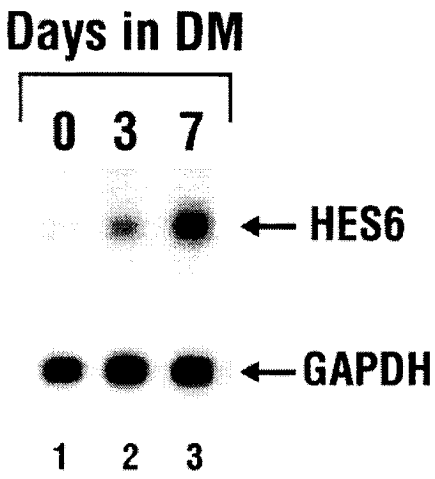


Figure 5. **Induction of HES6 mRNA expression during differentiation of C2C12 myoblasts.** The cells were grown to confluence in growth medium then switched to low serum DM. RNA was extracted after 0, 3, and 7 d of differentiation and analyzed by Northern blot assay using the HES6 cDNA. The membrane was then stripped and reprobed with the GAPDH cDNA to monitor for loading variations. Note the induction of HES6 expression during the differentiation of the C2C12 myoblasts into fused myotubes.

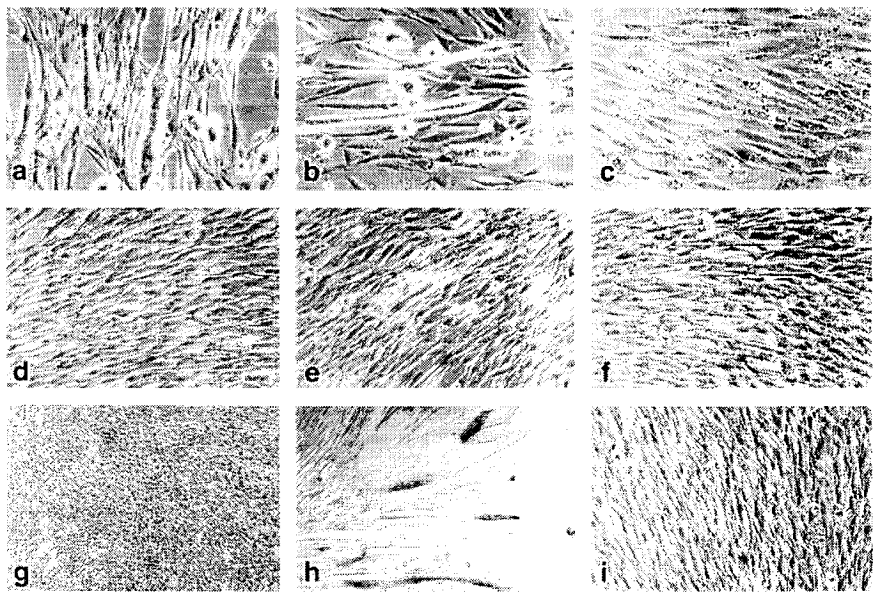
ion protein VP16 (amino acids 416–490) in frame to the COOH end of HES6-Δ. This manipulation transformed HES6 from a repressor into an activator, and HES6–VP16 activated transcription specifically from the N box–containing promoter, whereas the full-length VP16 protein had no effect (Fig. 4 b). These results confirm that HES6 binds to N boxes in myoblasts.

As reported previously in other cell types (Sasai et al., 1992; Bae et al., 2000), HES1 repressed transcription from the N box–containing reporter template in muscle cells (Fig. 4 c). When cotransfected with HES1 in myoblasts, HES6 did not antagonize HES1-mediated repression, but led to additive inhibition (Fig. 4 c). This suggests that HES6-containing heterodimers can cooperate with HES1 dimers to further inhibit transcription from N box–containing promoters in an additive manner.

The distinct repressor activity of HES6 in undifferentiated myoblasts (Fig. 4), as compared with its HES1 antagonist activity in other cell types (Bae et al., 2000), prompted us to examine the expression pattern of HES6 during myoblast differentiation and to investigate a putative role for HES6 in myogenesis. Fig. 5 shows that expression of the HES6 mRNA was induced when confluent cultures of C2C12 myoblasts were switched to low serum differentiation medium (DM). Expression was maximal after 7 d in DM (lane 3), when differentiated, fused myotubes became apparent in the cultures (unpublished results). Low levels of HES6 transcripts were detected in committed, undifferentiated myoblasts (lane 1). We used gain-of-function and dominant negative strategies to determine the role of HES6 in myogenic differentiation. Pools of stably transfected C2C12 cells expressing HES6 or HES6-Δ were isolated and cultured in high serum growth media (GM) or placed in DM, and their morphology and the expression of the muscle differentiation marker, myosin heavy chain (MHC), were examined. Cells transfected with the empty expression vector behaved as the parental cells and served as controls. Two independent pools of HES6- and HES6-Δ–expressing cells were isolated and exhibited similar characteristics, but only one set of results is presented here. The expression of the epitope-tagged transgenes, HES6 and HES6-Δ, was monitored by Western blot assay using the anti-6xHis antibody. The stable pools expressed comparable levels of HES6 and HES6-Δ (unpublished results).

When placed in DM, control and HES6-expressing cells differentiated to form fused myotubes (Fig. 6, a and b), and MHC protein expression was readily detected after 3 and 5 d in low serum (Fig. 7 a, lanes 2, 3, 5, and 6). In contrast, HES6-Δ–expressing cells never fused in DM (Fig. 6 c) and the undifferentiated myoblasts did not express MHC (Fig. 7 a, lanes 7–9). These observations suggest that HES6-Δ acted

Figure 6. **HES6 can modulate the myogenic differentiation program.** Stable pools of C2C12 myoblasts expressing HES6 (b, e, and h), HES6-Δ (c, f, and i), or the empty expression vector (a, d, and g) were induced to differentiate in DM (a, b, c) or maintained in GM for 4 (d–f) or 7 (g–i) d postconfluency. Note the absence of fused myotubes in HES6-Δ–expressing cells, even after 5 d in DM (c). At 4 d postconfluency in GM (d–f), HES6-expressing cells begin to differentiate (e). Differentiation of HES6-expressing cells in GM is more evident at 7 d, where several myotubes stain positively for MHC (h). Control (d and g) and HES6-Δ–expressing cells (f and i) never differentiate and don't stain for MHC (g and i) in GM.



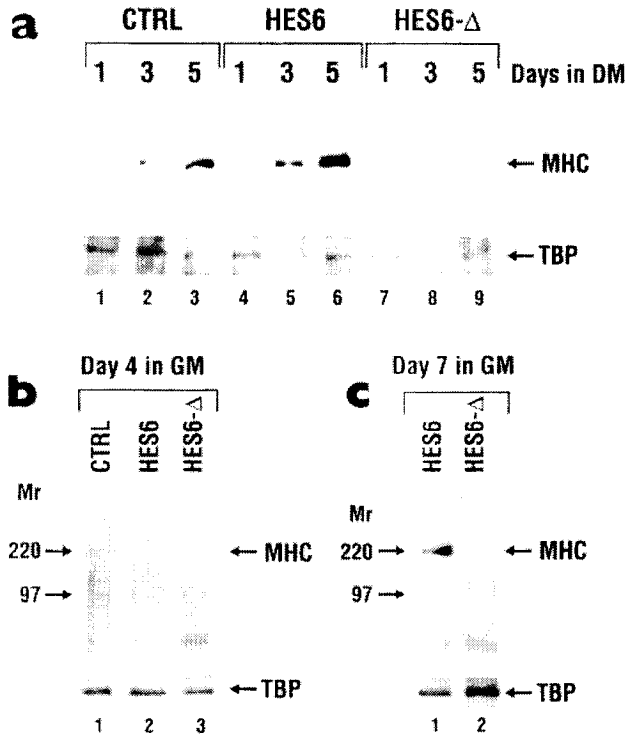


Figure 7. **Expression of MHC in HES6 and HES6-Δ stable transformants.** Whole cell extracts were prepared from the cultures described in the legend to Fig. 6 and assayed for MHC expression using Western blotting. (a) Control (ctrl) cells expressing the empty pEBVHis vector, HES6, and HES6-Δ transfected cells were placed in low serum DM for the indicated period of time. Note that HES6-Δ–expressing cells do not differentiate and do not express MHC. (b) The cells were maintained in GM for 4 d postconfluency. (c) The cells were kept in GM for 7 d after they reached confluence. Note that HES6-expressing cells differentiated and expressed MHC even when maintained in GM. In each panel, equal loading of sample wells was assessed by costaining with an anti-TATA binding protein (TBP) antibody.

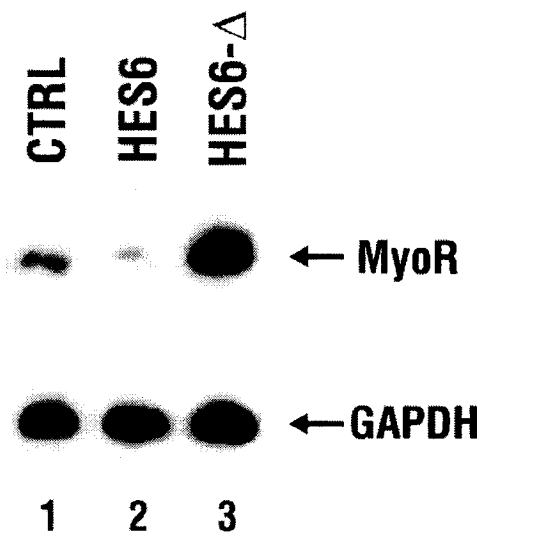


Figure 8. **MyoR is a putative downstream target of HES6.** MyoR expression was monitored by RT-PCR in confluent cultures of C2C12 cells expressing HES6, HES6-Δ, or transfected with the empty expression vector (CTRL). Under the conditions used, amplification was within the linear range of the reaction and GAPDH-specific primers yielded equivalent amounts of amplimers. Note the reduced expression of MyoR in HES6-expressing cells, and the dramatically increased expression in HES6-Δ–transfected cells.

as a dominant negative mutant to block endogenous HES6 function and prevent myogenesis. We also examined the behavior of cultures maintained in GM at confluency. Control and HES6-Δ–expressing cells retained their single-cell myoblast morphology when maintained in GM for 4 d postconfluency (Fig. 6, d and f), whereas some HES6-expressing cells began to fuse into myotubes under the same conditions (Fig. 6 e). MHC expression remained undetectable in all cultures during this period (Fig. 7 b). After 7 d in GM at confluency, HES6-expressing cells differentiated into myotubes (Fig. 6 h) expressing MHC (Figs. 6 h and 7 c, lane 1). Control cells (Fig. 6 g) and cells expressing HES6-Δ (Fig. 6 i) did not differentiate under the same conditions and did not turn on the expression of MHC (Fig. 6, g and i, respectively, and Fig. 7 c, lane 2). Together, the data from the dominant negative mutant expression and gain-of-function expression demonstrate that HES6 is a key regulator of the myogenic differentiation program.

Since HES6 acted as a transcriptional repressor in myoblasts, the induction of HES6 during C2C12 differentiation suggests that HES6 must repress the expression of an inhibitor of myogenesis in order for the muscle differentiation program to proceed. The kinetics of the induction of HES6 closely parallel the time-course of the downregulation of MyoR, which has recently been identified as such a repressor of myogenesis (Lu et al., 1999). The reciprocal expression patterns of HES6 and MyoR suggest that MyoR could be an HES6 target. We tested whether constitutive expression of HES6 or its dominant negative mutant HES6-Δ could modulate the expression pattern of MyoR in confluent cultures of C2C12 myoblasts. MyoR expression and the expression of the ubiquitous GAPDH gene were assessed using reverse transcriptase (RT)-PCR under linear amplification conditions, allowing accurate comparison of expression levels. MyoR expression was readily detectable in control cells (Fig. 8, lane 1). Constitutive expression of HES6 inhibited MyoR expression (lane 2), whereas blocking endogenous HES6 function with the HES6-Δ mutant dramatically augmented MyoR mRNA levels (lane 3). These data provide strong circumstantial evidence that HES6 could regulate MyoR expression.

Discussion

We have shown that HES6 binds TLE family members to repress transcription from an N box–containing promoter in muscle cells, and that it can cooperate with HES1 to achieve maximal transcriptional repression in these cells. These observations contrast with previous findings showing that HES6 antagonized HES1 repressor activity in fibroblasts and retinal explant cultures (Bae et al., 2000). The apparent muscle-specific repressor activity of HES6 prompted us to study the role of HES6 in myogenesis. Undifferentiated myoblasts express minimal levels of HES6 mRNA, and HES6 expression is induced in differentiated myotubes. Gain-of-

function and dominant negative mutations in cultured myoblasts revealed that HES6 is an important regulator of the muscle development program.

HES6 and transcription

Tethering HES6 to DNA by fusing it to the GAL4 DNA binding domain leads to transcriptional repression of GAL4 upstream activation sequence-containing reporter templates (Fig. 2 b). The repression required the WRPW tetrapeptide at the COOH terminus of HES6 and involved recruitment of the TLE1 corepressor. Can HES6 bind the canonical HES binding site, the N box, to repress transcription? Recent work has shown that the length of the loop region of bHLH molecules is critical for DNA binding activity and has identified a loop residue critical for DNA binding (Winston and Gottesfeld, 2000). Since the HES6 loop is 4 to 5 residues shorter than related family members, it was postulated and demonstrated that HES6 dimers could not bind to an N box sequence (Bae et al., 2000; Koyano-Nakagawa et al., 2000). We have confirmed these results in electrophoretic mobility shift assays (unpublished results). However, HES1–HES6 heterodimers were shown to bind DNA (Bae et al., 2000), demonstrating that HES6 can heterodimerize with other bHLH molecules to bind the N box. In fibroblasts and developing mouse retina, HES6 suppressed HES1 from repressing transcription (Bae et al., 2000). The proposed mechanism was that the structure of the HES1–HES6 heterodimer does not allow interaction with TLE corepressors, or that HES6 sequestered TLE molecules from HES1 (Bae et al., 2000). The interaction of HES6 with TLE molecules was not examined in that study. We have shown that HES6 can bind TLE corepressors in yeast and mammalian cells, and that this binding requires the WRPW COOH-terminal tetrapeptide.

In myoblasts, HES6 did not antagonize HES1, and coexpression of HES1 and HES6 led to additive repressor activity (Fig. 4). This could be due to a specific effect of the HES1–HES6 heterodimer in muscle cells, that could result from tissue-specific posttranslational modifications of one or both of the dimer partners. Alternatively, HES6 could dimerize with a muscle-specific bHLH partner to bind the N box, recruit TLE corepressors, and inhibit transcription. In this fashion, the HES6-containing heterodimers could cooperate with HES1 homodimers to fully repress N box–dependent transcription. We favor this interpretation since (a) we have observed that HES6 can repress transcription from N box–containing templates when transfected alone in myoblasts; (b) the HES6–VP16 activation domain fusion protein bound to N boxes to activate the N box–containing reporter (Fig. 4). What molecule dimerizes with HES6 in muscle cells? We were not able to detect interaction between HES6 and E proteins in EMSAs or pull-down assays (unpublished results), suggesting that ubiquitous bHLH proteins are not the partners involved in the repressor function of HES6 in myoblasts. Other HES family members appear mostly expressed in neural tissue (Lobe, 1997), although HES5 expression was detected in developing somites (Barrantes et al., 1999), suggesting that HES5 could dimerize with HES6 to repress transcription during muscle development. Recently, a new subclass of bHLH proteins, HRT1–3, was characterized and shown to be expressed in the developing heart and in the dermomyotome and sclerotome (Nakagawa et al., 1999). Thus, these Hairy-related transcriptional regulators represent putative dimerization partners for HES6 in developing muscle. It will prove interesting to determine whether a posttranslationally modified HES1–HES6 dimer or a distinct HES6-containing heterodimer mediate HES6-dependent repression in differentiating muscle cells.

HES6 and myogenesis

Forcing constitutive HES6 expression in myoblasts induced myotube fusion and expression of the muscle differentiation marker MHC (Figs. 6 and 7), even in cultures maintained in high serum concentration, where fusion is normally inhibited. Reciprocally, blocking the activity of endogenous HES6 by expressing a dominant negative mutant form of HES6 that lacks the corepressor-recruiting WRPW tetrapeptide inhibited differentiation when confluent cultures were placed in low serum media. These results show that HES6 plays an essential role during myoblast differentiation in culture. Is HES6 regulating myogenesis in vivo? A recent study describing the pattern of expression of HES6 in mouse embryos shows that HES6 is expressed during both myoblast commitment and differentiation, supporting a role for HES6 in the regulation of the muscle development program (Pissara et al., 2000; Vasiliauskas and Stern, 2000). We have also detected HES6 expression in adult muscle using Northern blot hybridization (unpublished results). The engineering of tissue-specific HES6 mouse mutants will help to formally prove the role of HES6 during muscle development.

Myogenic bHLH factors are expressed in proliferating, undifferentiated myoblasts, but they do not activate muscle differentiation until myoblasts exit the cell cycle. This suggests that inhibitors of the function of myogenic bHLH proteins are expressed in cycling myoblasts. Several such inhibitors have been identified, including c-Jun (Bengal et al., 1992), Id (Jen et al., 1992), Twist (Spicer et al., 1996), Mist-1 (Lemercier et al., 1998), and MyoR (Lu et al., 1999). Since HES6 acts as a transcriptional repressor in myoblasts, the induction of HES6 during C2C12 differentiation suggests that HES6 must repress the expression of an inhibitor of myogenesis in order for myotube differentiation to proceed. Amongst the various inhibitors of myogenesis listed above, MyoR appears like the best candidate target for HES6-mediated repression. Indeed, the kinetics of the downregulation of Id and Mist1 expression during myotube differentiation do not match the time-course of HES6 induction (Jen et al., 1992; Lemercier et al., 1998), whereas Twist is not expressed in growing myoblasts (Hebrok et al., 1994). Finally, these genes are strongly expressed in tissues showing high levels of HES6 transcripts postnatally (unpublished results), arguing against an inhibitory role of HES6 in the control of their expression. On the contrary, the kinetics of the induction of HES6 closely parallel the time-course of MyoR downregulation, suggesting that MyoR could be a downstream target of HES6. Indeed, modulating HES6 expression in myoblasts via gain-of-function or dominant negative mutations affected MyoR expression, strongly supporting a role for HES6 in the transcriptional control of MyoR

expression. The analysis of the MyoR promoter region could identify N boxes or other response elements through which HES6-containing dimers could regulate MyoR transcription in muscle cells.

To properly regulate cell fate choice and ensure normal organogenesis, several signaling pathways have been shown to inhibit skeletal myoblast differentiation by interfering with the expression or activity of myogenic bHLH proteins. Signaling through Notch inhibits myogenesis through an HES-1–independent pathway in tissue culture (Shawber et al., 1996; Rusconi and Corbin, 1998; Nofziger et al., 1999; Wilson-Rawls et al., 1999). Preliminary results using Northern blot analysis of RNA extracted from parental C2C12 cells and from C2C12 myoblasts transfected with constitutively active forms of Notch (Nofziger et al., 1999) show that activated Notch inhibited the induction of HES6 in myoblasts cultured in differentiation medium (G. Weinmaster and R. St-Arnaud, personal communication). Similarly, coculture of C2C12 cells with L cells expressing the Notch ligand Jagged1 (Lindsell et al., 1995) revealed that ligand activation of Notch inhibited HES6 induction (G. Weinmaster and R. St-Arnaud, personal communication). Although it will be important to confirm that this inhibition is not due to a general block in the myogenic program induced by Notch signaling, these results suggest that HES6 could represent a key target in Notch signaling during myogenesis.

Although the proposed mechanisms are significantly different, our results do support a role for HES6 in promoting instead of inhibiting differentiation, as was reported for neurogenesis (Bae et al., 2000; Koyano-Nakagawa et al., 2000). It will prove interesting to identify the partners, targets, and/or posttranslational modifications involved in the muscle-specific functions of HES6.

Materials and methods

Cloning of the HES6 cDNA and expression vectors

A partial mouse HES6 cDNA was isolated serendipitously in a yeast two-hybrid screen (see Results). Sequence comparison analysis identified overlapping expressed sequence tags that allowed cloning of a nearly full-length cDNA using PCR. The HES6 cDNA, lacking the first 16 amino acids, was then subcloned into a yeast two-hybrid assay vector (pGAD-GH; CLONTECH Laboratories, Inc.) or mammalian expression vectors (pEBVHis and pcDNA4/TO/myc-His; Invitrogen) using conventional methodology. The HES6-Δ mutant (lacking the COOH-terminal WRPW tetrapeptide) was engineered by PCR using the wild-type cDNA as template and subcloned into the same expression vectors. Plasmids pcDNA3–GAL4bd–HES6 and pcDNA3–GAL4bd–HES6-Δ were obtained by subcloning the appropriate PCR products into the filled-in BamHI site of pcDNA3–GAL4bd. The HES6–VP16 activation domain fusion protein was engineered by subcloning the PCR-amplified VP16 domain (amino acids 416–490) in frame downstream from HES6-Δ in the pcDNA4/TO/myc-His vector. Construct pTLE1–VP16 has been described previously (Wang et al., 2000) and was provided by Dr. D.K. Granner (Vanderbilt University School of Medicine, Nashville, TN)

Yeast two-hybrid protein interaction assay

Yeast cells were cotransfected with pGBT9–TLE1 (pBD–TLE1) (Grbavec and Stifani, 1996) and pGAD–GH–HES6 (pAD–HES6) or pGAD–GH–HES6-Δ (pAD–HES6-Δ) following a protocol derived from the HybriZAP two-hybrid instruction manual (Stratagene). Positive interaction was scored based on growth on media lacking tryptophan, leucine, and histidine, supplemented with 20 mM 3-aminotriazole, and by testing for expression of the lacZ reporter gene as recommended (Stratagene). Western blot assays to confirm expression of the fusion proteins in yeast were performed using standard protocols (Ausubel et al., 1993).

Coprecipitation assay

C2C12 myoblasts (Blau et al., 1985) were grown to 50–60% confluency in 100-mm tissue culture dishes and transfected using the GenePORTER transfection reagent (Gene Therapy Systems) according to the manufacturer's protocol. Cells were cotransfected with 3.0 μg of either pEBVHis–HES6, pEBVHis–HES6-Δ, or the empty pEBVHis vector (Invitrogen), together with 3.0 μg of pEBG–GST–TLE1 (McLarren et al., 2000) or pEBG–GST (Mizushima and Nagata, 1990). Cells were collected 24 h posttransfection, resuspended in homogenization buffer (25 mM Tris-HCl, pH 7.8, 200 mM NaCl, 0.5% Triton X-100), homogenized, and centrifuged (2 min at 6000 rpm). The cell extract supernatant was recovered and incubated with glutathione-Sepharose beads overnight at 4°C with gentle agitation. The beads were then collected by centrifugation, washed four times in homogenization buffer, and finally resuspended in SDS-PAGE buffer.

Immunoblotting

After electrophoresis, proteins were transferred to nitrocellulose membranes and probed by Western blotting using standard protocols (Ausubel et al., 1993). Detection was performed using the ECL Western blotting detection system (Amersham Pharmacia Biotech). Primary antibodies included anti-GST (1:3,000 dilution; Amersham Pharmacia Biotech), anti-6xHis (1:2,500 dilution; CedarLane Laboratories), anti-TBP (1:1,000 dilution; Upstate Biotechnology), and the MF20 anti-MHC hybridoma (1:500 dilution) (Bader et al., 1982). HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) were used at a 1:20,000 dilution.

Transient transfection assays

Human 293 cells were transiently transfected using the SuperFect reagent (QIAGEN) according to the manufacturer's instructions. Transfections included 1.0 μg of the luciferase reporter plasmid p5xGAL4UAS–SV-40-luc (Grbavec et al., 1998) and 1.0 μg each of pcDNA3–GAL4bd, pcDNA3–GAL4bd–HES6, pcDNA3–GAL4bd–HES6-Δ, or pTLE1–VP16 (Wang et al., 2000) alone or in combination. Total amount of DNA was normalized at 3.0 μg by addition of the empty pcDNA3 vector (Invitrogen) and included 0.25 μg of pCMV–β-galactosidase plasmid DNA to provide a means of normalizing the assays for transfection efficiency. Cells were collected 24 h after transfection. C2C12 myoblasts were grown in 6-well plates and transfected using 15 μl of GenePORTER reagent, 0.3 μg of reporter construct (p6NactinLUC or pActinLUC) (Sasai et al., 1992), 0.3–1.6 μg of pRc/CMV-HES1 (Grbavec and Stifani, 1996), pEBVHis-HES6, or pEBVHis-HES6-Δ, alone or in combination, and 50 pg of the internal control reporter, pcmvRL (Promega). The total amount of DNA transfected was standardized to 3.0 μg by addition of empty pEBVHis vector. Cells were harvested 48 h posttransfection and luciferase activity was assayed using the Dual Luciferase assay kit (Promega) in a Monolight 2010 (Analytical Luminescence Laboratory). The data from multiple experiments were pooled and the mean ± SEM were calculated. The final results are expressed as a percentage of expression relative to cells transfected with the reporter and empty vector alone. Transfections with the HES6–VP16 fusion protein were performed using 8.0 μl of FuGENE 6 reagent as per the manufacturer's recommended procedure (Roche Diagnostics). Total DNA was standardized to 2.0 μg and comprised 0.5 ng of pcmvRL internal control reporter (Promega), 0.2 μg of reporter construct (p6NactinLUC or pActinLUC) (Sasai et al., 1992), and 1.5 μg of empty vector (pcDNA4/TO/myc-His), HES6-VP16, or pMSVP16, a mammalian expression vector for the full length VP16 protein (Triezenberg et al., 1988). At the time of transfection, cells were switched to medium containing 2% horse serum and harvested 24 h later. Luciferase activity was measured as described above and results are expressed as mean fold induction ± SEM of three experiments performed in duplicates.

Immunocytochemistry

C2C12 cells were plated at 1.2×10^5 cells per 35-mm plate on gelatin-coated cover slips and transfected as described above with pcDNA4–HES6 or pcDNA4–HES6-Δ. The cells were rinsed in PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. After blocking with 1% BSA and 10% normal goat serum, the cells were incubated with an anti-myc epitope antibody (Santa Cruz Biotechnology, Inc.; 1:200 dilution), rinsed, then treated with the secondary rhodamine-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) at 1:200 in PBS containing 1% BSA. After washes, the coverslips were mounted in Vectashield (Vector Laboratories) and the cells visualized on a Leica microscope at 200×.

RNA expression analysis

Total RNA was isolated using the RNeasy miniprep kit (QIAGEN). Probes used were a mouse GAPDH cDNA fragment (Piechaczyk et al., 1984) for

assessing loading and a 1.2-kb HES6 fragment (bHLH domain to 3′-UTR). Northern hybridization was performed as per Amersham Pharmacia Biotech's Rapid-hyb instruction manual. For RT-PCR, first-strand cDNA synthesis was performed with SuperScript II reverse transcriptase (Canadian Life Technologies) followed by PCR amplification with 2 μl of the RT reaction, 0.1 μCi of ^{32}P-dCTP, and MyoR- or GAPDH-specific primers (sequences available on request). After a 2 min denaturation at 99°C, PCR conditions were set as follows: 94°C for 1 min, 60°C for 30 s, and 72°C for 1 min, for 23 cycles. Amplimers were separated on 6% polyacrylamide gels and exposed to film. Control reactions using 21 or 25 cycles (unpublished results) confirmed that amplification of both the GAPDH and MyoR fragments was within the linear range of the reaction, allowing semiquantitative comparison of expression levels from the RT-PCR data.

Myogenic conversion assay

C2C12 myoblasts were transfected as described above with 1 μg of pEBVHis, pEBVHis–HES6, or pEBVHis–HES6-Δ. Stable transfectants were isolated by pooling hygromycin-resistant cell colonies. Cells were maintained in GM (DME supplemented with 10% fetal bovine serum) until they reached confluency, then switched to DM (DME with 2% horse serum) to induce myotube fusion. In one experiment, confluent cultures were maintained in GM for up to 7 d. MHC expression was detected by Western blotting as described above or by direct staining of fixed cells. In brief, cells were rinsed in PBS, fixed in 3% paraformaldehyde in PBS for 40 min at room temperature, then blocked with 5% goat serum in PBS for 1 h. The blocking solution was removed, and the fixed cells were incubated for 1 h with the MF20 antibody (Bader et al., 1982) at 1:500 dilution in PBS. After rinsing the primary antibody, the HRP-conjugated secondary antibody (1:10,000) was added for 1 h. Excess antibody was washed away with PBS, and immunoreactive cells were stained with the VectaStain Elite ABC kit (Vector Laboratories) for 30 min.

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References

Anant, S., S. Roy, and K. VijayRaghavan. 1998. Twist and Notch negatively regulate adult muscle differentiation in *Drosophila*. *Development.* 125:1361–1369.

Artavanis-Tsakonas, S., K. Matsuno, and M.E. Fortini. 1995. Notch signaling. *Science.* 268:225–232.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1993. Current protocols in molecular biology. John Wiley and Sons, NY. 2232 pp.

Bader, D., T. Masaki, and D.A. Fischman. 1982. Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J. Cell Biol.* 95:763–770.

Bae, S.-K., Y. Bessho, M. Hojo, and R. Kageyama. 2000. The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation. *Development.* 127: 2933–2943.

Barrantes, I.B., A.J. Elia, K. Wunsch, M.H. De Angelis, T.W. Mak, J. Rossant, R.A. Conlon, A. Gossler, and J.L. de la Pompa. 1999. Interaction between Notch signalling and Lunatic fringe during somite boundary formation in the mouse. *Curr. Biol.* 9:470–480.

Benezra, R., R.L. Davis, D. Lockshon, D.L. Turner, and H. Weintraub. 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell.* 61:49–59.

Bengal, E., L. Ransone, R. Scharfmann, V.J. Dwarki, S.J. Tapscott, H. Weintraub, and I.M. Verma. 1992. Functional antagonism between c-Jun and MyoD proteins: a direct physical association. *Cell.* 68:507–519.

Blau, H.M., G.K. Pavlath, E.C. Hardeman, C.P. Chiu, L. Silberstein, S.G. Webster, S.C. Miller, and C. Webster. 1985. Plasticity of the differentiated state. *Science.* 230:758–766.

Chen, G., and A.J. Courey. 2000. Groucho/TLE family proteins and transcriptional repression. *Gene.* 249:1–16.

Grbavec, D., and S. Stifani. 1996. Molecular interaction between TLE1 and the carboxyl-terminal domain of HES-1 containing the WRPW motif. *Biochem. Biophys. Res. Commun.* 223:701–705.

Grbavec, D., R. Lo, Y. Liu, and S. Stifani. 1998. Transducin-like Enhancer of split 2, a mammalian homologue of *Drosophila* Groucho, acts as a transcriptional repressor, interacts with Hairy/Enhancer of split proteins, and is expressed during neuronal development. *Eur. J. Biochem.* 258:339–349.

Grbavec, D., R. Lo, Y. Liu, A. Greenfield, and S. Stifani. 1999. Groucho/transducin-like enhancer of split (TLE) family members interact with the yeast transcriptional co-repressor SSN6 and mammalian SSN6-related proteins: implications for evolutionary conservation of transcription repression mechanisms. *Biochem. J.* 337:13–17.

Hasty, P., A. Bradley, J.H. Morris, D.G. Edmondson, J.M. Venuti, E.N. Olson, and W.H. Klein. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature.* 364:501–506.

Hebrok, M., K. Wertz, and E.M. Fuchtbauer. 1994. M-twist is an inhibitor of muscle differentiation. *Dev. Biol.* 165:537–544.

Jarriault, S., O. Le Bail, E. Hirsinger, O. Pourquie, F. Logeat, C.F. Strong, C. Brou, N.G. Seidah, and A. Israel. 1998. Delta-1 activation of notch-1 signaling results in HES-1 transactivation. *Mol. Cell. Biol.* 18:7423–7431.

Jen, Y., H. Weintraub, and R. Benezra. 1992. Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins. *Genes Dev.* 6:1466–1479.

Kageyama, R., and S. Nakanishi. 1997. Helix-loop-helix factors in growth and differentiation of the vertebrate nervous system. *Curr. Opin. Genet. Dev.* 7:659–665.

Koyano-Nakagawa, N., J. Kim, D. Anderson, and C. Kintner. 2000. Hes6 acts in a positive feedback loop with the neurogenins to promote neuronal differentiation. *Development.* 127:4203–4216.

Lamb, J.R., S. Tugendreich, and P. Hieter. 1995. Tetratrico peptide repeat interactions: to TPR or not to TPR? *Trends Biochem. Sci.* 20:257–259.

Lemercier, C., R.Q. To, B.J. Swanson, G.E. Lyons, and S.F. Konieczny. 1997. Mist1: a novel basic helix-loop-helix transcription factor exhibits a developmentally regulated expression pattern. *Dev. Biol.* 182:101–113.

Lemercier, C., R.Q. To, R.A. Carrasco, and S.F. Konieczny. 1998. The basic helix-loop-helix transcription factor Mist1 functions as a transcriptional repressor of myoD. *EMBO J.* 17:1412–1422.

Lindsell, C.E., C.J. Shawber, J. Boulter, and G. Weinmaster. 1995. Jagged: a mammalian ligand that activates Notch1. *Cell.* 80:909–917.

Lobe, C.G. 1997. Expression of the helix-loop-helix factor, Hes3, during embryo development suggests a role in early midbrain-hindbrain patterning. *Mech. Dev.* 62:227–237.

Lu, F.M., and S.E. Lux. 1996. Constitutively active human Notch1 binds to the transcription factor CBF1 and stimulates transcription through a promoter containing a CBF1-responsive element. *Proc. Natl. Acad. Sci. USA.* 93: 5663–5667.

Lu, J., R. Webb, J.A. Richardson, and E.N. Olson. 1999. MyoR: a muscle-restricted basic helix-loop-helix transcription factor that antagonizes the actions of MyoD. *Proc. Natl. Acad. Sci. USA.* 96:552–557.

Massari, M.E., and C. Murre. 2000. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. *Mol. Cell. Biol.* 20:429–440.

McLarren, K.W., R. Lo, D. Grbavec, K. Thirunavukkarasu, G. Karsenty, and S. Stifani. 2000. The mammalian basic helix loop helix protein HES-1 binds to and modulates the transactivating function of the runt-related factor Cbfa1. *J. Biol. Chem.* 275:530–538.

Mizushima, S., and S. Nagata. 1990. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* 18:5322.

Molkentin, J.D., and E.N. Olson. 1996. Defining the regulatory networks for muscle development. *Curr. Opin. Genet. Dev.* 6:445–453.

Nabeshima, Y., K. Hanaoka, M. Hayasaka, E. Esumi, S. Li, and I. Nonaka. 1993. Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature.* 364:532–535.

Nakagawa, O., M. Nakagawa, J.A. Richardson, E.N. Olson, and D. Srivastava. 1999. HRT1, HRT2, and HRT3: A new subclass of bHLH transcription factors marking specific cardiac, somitic, and pharyngeal arch segments. *Dev. Biol.* 216:72–84.

Nofziger, D., A. Miyamoto, K.M. Lyons, and G. Weinmaster. 1999. Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts. *Development.* 126:1689–1702.

Ohtsuka, T., M. Ishibashi, G. Gradwohl, S. Nakanishi, F. Guillemot, and R. Kageyama. 1999. Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. *EMBO J.* 18:2196–2207.

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Hes6 Promotes Cortical Neurogenesis and Inhibits Hes1 Transcription Repression Activity by Multiple Mechanisms

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Hes1 is a mammalian basic helix-loop-helix transcriptional repressor that inhibits neuronal differentiation together with corepressors of the Groucho (Gro)/Transducin-like Enhancer of split (TLE) family. The interaction of Hes1 with Gro/TLE is mediated by a WRPW tetrapeptide present in all Hairy/Enhancer of split (Hes) family members. In contrast to Hes1, the related protein Hes6 promotes neuronal differentiation. Little is known about the molecular mechanisms that underlie the neurogenic activity of Hes6. It is shown here that Hes6 antagonizes Hes1 function by two mechanisms. Hes6 inhibits the interaction of Hes1 with its transcriptional corepressor Gro/TLE. Moreover, it promotes proteolytic degradation of Hes1. This effect is maximal when both Hes1 and Hes6 contain the WRPW motif and is reduced when Hes6 is mutated to eliminate a conserved site (Ser183) that can be phosphorylated by protein kinase CK2. Consistent with these findings, Hes6 inhibits Hes1-mediated transcriptional repression in cortical neural progenitor cells and promotes the differentiation of cortical neurons, a process that is normally inhibited by Hes1. Mutation of Ser183 impairs the neurogenic ability of Hes6. Taken together, these findings clarify the molecular events underlying the neurogenic function of Hes6 and suggest that this factor can antagonize Hes1 activity by multiple mechanisms.

In the developing mammalian central nervous system (CNS), differentiated neuronal and glial cells derive from multipotent neural progenitor cells located in the proliferative zone of the neural tube. The commitment of these progenitor cells to the neuronal lineage is regulated by the antagonistic activities of a number of positively and negatively acting transcription factors containing the basic helix-loop-helix (bHLH) DNA-binding and dimerization motif (reviewed in references 2 and 18). Neurogenic bHLH factors include several evolutionarily conserved molecules related to the proneural proteins Atonal and Achaete-Scute of *Drosophila* (8, 13, 21). They function by forming heterodimers with the ubiquitous bHLH protein E47. These dimers bind to DNA sequences commonly referred to as E boxes (CANNTG) and transactivate the expression of genes that promote the acquisition of the neuronal fate (17, 32).

Antineurogenic bHLH factors include members of the Hairy/Enhancer of split (Hes) family (1, 26, 32). In contrast to proneural proteins, Hes factors like Hes1 and Hes5 mediate transcriptional repression and bind preferentially to DNA sequences referred to as N boxes (CACNAG) (32). They are thought to inhibit neuronal differentiation by antagonizing the neurogenic activity of the proneural proteins via multiple mechanisms, including direct involvement in the negative regulation of proneural gene expression (4, 20) and inhibition of the activity of E47-proneural protein heterodimers (1, 3, 32). Genetic perturbations that alter the normal balance of the activities of proneural and antineurogenic bHLH proteins have dramatic effects on CNS development in vivo, underscoring the importance of understanding how the functions of these factors are normally regulated (8, 16, 26, 36).

The *Hes1* gene is initially expressed in proliferating neural progenitor cells and becomes down-regulated during the progenitor-to-neuron transition (32). Persistent expression of *Hes1* inhibits neuronal development, whereas disruption of *Hes1* function results in the premature differentiation of neuronal cells and the up-regulation of proneural genes (15, 16, 36). These observations indicate that Hes1 acts in neural progenitor cells to control the timing of neuronal differentiation. Molecular mechanisms that contribute to the positive or negative regulation of Hes1 activity in neural progenitor cells are beginning to be elucidated. In particular, studies with both invertebrate and vertebrate species show that antineurogenic Hes proteins are coexpressed, and directly interact, with general transcriptional corepressors of the Groucho/Transducin-like Enhancer of split (Gro/TLE) family (7, 12, 24, 25, 29, 34, 40). This interaction is mediated by a WRPW tetrapeptide motif present at the carboxy termini of all Hes proteins (7, 11, 24). Mutations that disrupt the Hes-Gro/TLE interactions impair the ability of Hes proteins to mediate transcriptional repression (7, 24, 29). Moreover, Hes1 activates phosphorylation mechanisms that promote the transcription repression function of Gro/TLE (25). Together, these observations identify Gro/TLE proteins as positive regulators of Hes activity and suggest that Hes1 acts by recruiting hyperphosphorylated Gro/TLE to specific DNA sites where the latter mediate transcriptional repression (25).

Another protein that has recently been implicated in the regulation of Hes1 activity is the related Hes family member Hes6 (3, 19). The *Hes6* gene is expressed throughout the developing CNS, where it is found in both undifferentiated neural

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progenitors and differentiated neurons (3, 19, 30, 38). In contrast to Hes1, Hes6 acts as a positive regulator of neuronal differentiation in both murine retinal explants and *Xenopus* embryos (3, 19). Although little is known about the molecular mechanisms underlying the neurogenic ability of Hes6, a number of observations suggest that Hes6 may promote neurogenesis by antagonizing the function of Hes1. Studies with transfected nonneural cells show that Hes6 can heterodimerize with Hes1 and can inhibit the ability of Hes1 to both repress transcription from promoters containing N box sequences and suppress the activity of E47-proneural protein heterodimers (3). In addition, Hes6 does not require an intrinsic DNA-binding ability to promote neurogenesis, because mutation of the basic arm of its bHLH domain does not abolish its neurogenic ability in vivo (19). Together, these observations suggest that Hes6 may promote neuronal differentiation via DNA-binding-independent events that involve a negative regulation of Hes1 function in the CNS. Virtually nothing is known, however, about the molecular mechanisms underlying this inhibitory effect.

Here we describe experiments showing that Hes6 negatively regulates Hes1 activity by at least two mechanisms. Hes6 inhibits the interaction of Hes1 with Gro/TLE. In addition, it promotes proteolytic degradation of Hes1. This effect is maximal when both Hes1 and Hes6 contain the WRPW motif, and it is reduced by a point mutation (S183A) that removes a consensus site for phosphorylation by protein kinase CK2. In agreement with these findings, Hes6 inhibits Hes1-mediated transcriptional repression in cortical neural progenitor cells and promotes their neuronal differentiation. Moreover, the S183A mutation attenuates Hes6 phosphorylation by protein kinase CK2 and impairs the ability of Hes6 to promote neuronal differentiation. Taken together, these findings identify novel mechanisms through which Hes6 may act as a negative regulator of Hes1 activity and a positive regulator of neuronal differentiation.

MATERIALS AND METHODS

Plasmids. PCR was used to amplify the sequences encoding full-length Hes6 (oligonucleotide primers Hes6-1 [5′-GACCATGGCTCCGTCCCA] and Hes6-2 [5′-TCACCAAGGCCTCCACACACTC]) or Hes6ΔWRPW (oligonucleotide primers Hes6-1 and Hes6-3 [5′-TCACACACTCTGAGCCCGGCGAGC]) with the full-length *Hes6* cDNA Image clone W66929 as the template (5). The sequence encoding a truncated form of Hes6 lacking the first 13 amino acids [Hes6(14-224)] was also amplified by PCR (oligonucleotide primers Hes6-4 [5′-TCAGGAGGATGAGGACCGCTGGGAA] and Hes6-2); Hes6 and Hes6(14-224) behaved equally in our studies. PCR products were subcloned into the pcDNA3-GAL4bd vector digested with *Bam*HI (followed by filling in with Klenow DNA polymerase) or into the pCMV2-HA plasmid digested with *Eco*RV or *Sma*I. The pCMV2-HA-Hes6(S183A) plasmid was obtained by first generating the sequence encoding the indicated point mutation by using a PCR-based strategy (the mutated oligonucleotide primers were Hes6-5F [5′-GACCT GTGTGCTGACCTAGAGGAGAT] and Hes6-5R [5′-TCTAGGTCAGCACA CAGGTCGT]), followed by subcloning into pBluescript plasmid and DNA sequencing. The verified mutant sequence was then subcloned into pCMV2-HA-Hes6 digested with *Sma*I, replacing the wild-type sequence. Constructs for the bacterial expression of fusion proteins of glutathione *S*-transferase (GST) and Hes6 or Hes6(S183A) were obtained by digesting pCMV2-HA-Hes6 or pCMV2-HA-Hes6(S183A) with *Bgl*II and *Bam*HI, followed by subcloning into pGEX1 digested with *Bam*HI. The pGEX1-Hes1 DNA has been described previously (23). Constructs pEBG-Hes6 and pEBG-Hes6ΔWRPW were generated by digesting pcDNA3-GAL4bd-Hes6 or pcDNA3-GAL4bd-Hes6ΔWRPW, respectively, with *Eco*RI, followed by filling in with Klenow DNA polymerase and subcloning into the filled-in *Cla*I site of pEBG to generate plasmids for the expression of fusion proteins of GST and Hes6 or Hes6ΔWRPW in mammalian cells. Plasmid pCMV2-FLAG-Hes1ΔWRPW:Gro/TLE1 was generated by first subcloning the region encoding Hes1ΔWRPW (obtained by PCR amplification with primers Hes1-1 [5′-AATGCCAGCTGATATAATGGAG] and Hes1-2 [5′-ACATGGAGTCCGCAGTGAGCGA]) into pCMV2-FLAG digested with *Eco*RV. This was followed by in-frame ligation of the sequence encoding Gro/TLE1 (also obtained by PCR with primers Gro/TLE1-1 [5′-GGATGTTCCCG CAGAGCCGG] and Gro/TLE1-2 [5′-TCAGTAGATGACTTCATAGAC]) into an *Xba*I site located downstream of the last codon of *Hes1*. Ligation products were analyzed and confirmed by sequencing. Plasmids pCMV2-FLAG-Hes1, pCMV2-FLAG-Hes1ΔWRPW, pEBG-Hes1, and pEBG-Hes1ΔWRPW have been described previously (12, 23, 24). Plasmids pFOX-Luc1, pFOX-ngn3p-Luc1 (containing a portion of the *neurogenin3* [*ngn3*] promoter extending ~2.6 kbp upstream of the transcription start site) and pFOX-ΔN-box-ngn3p-Luc1 (containing a mutated version of the ~2.6-kbp *ngn3* promoter lacking the Hes1-binding sites located within 200 bp proximal to the transcription start site) have been described previously (20).

Transient transfections, protein-protein interaction assays, and Western blot analysis. Human 293A cells were cultured and transfected by using the SuperFect reagent (Qiagen) as described previously (23–25). When appropriate, transfected cells were incubated for 6 h in the presence of 10 μM MG132 (Calbiochem) prior to cell lysis. Treatment of cell lysates with calf intestinal phosphatase was performed as described previously (14). To examine the effect of Hes6 on Hes1 stability, cells were transfected for 36 h with pCMV2-FLAG-Hes1/Hes1ΔWRPW (50 ng/transfection) in the absence or presence of Hes6, Hes6ΔWRPW, or Hes6(S183A) expression plasmids (200 to 800 ng/transfection). To examine the effect of Hes6 on the Hes1-Gro/TLE interaction, cells were transfected for 24 h with Hes1 or Hes1ΔWRPW expression plasmids (100 to 200 ng/transfection) in the absence or presence of Hes6 or Hes6ΔWRPW expression plasmids (100 to 200 ng/transfection). Cell lysates were prepared, and GST coprecipitation (23, 24), immunoprecipitation (14, 40), and Western blotting (6, 25, 28) studies were performed as described previously. The antibodies used were panTLE (6, 28, 34), anti-GST and anti-GAL4bd (Santa Cruz Biotechnology), antihemagglutinin (anti-HA) (Roche), or anti-FLAG (Sigma).

In vitro phosphorylation of bacterially purified Hes proteins. Fusion proteins of GST and Hes6 or Hes6(S183A) were purified from bacteria as described previously (12, 23). Roughly 50 ng of each fusion protein was resuspended in buffer A (50 mM Tris-HCl [pH 7.6], 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Triton X-100, 200 μm ATP) containing 200 μCi of [γ-^{32}P]ATP per ml in the presence of 0.5 U of purified protein kinase CK2 (New England Biolabs) per μl for 15 min at 30°C. Reactions were terminated by the addition of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and incubation at 65°C for 5 min. After gel electrophoresis, proteins were transferred to nitrocellulose and exposed to film. After autoradiography, membranes were subjected to Western blotting with anti-GST antibodies.

Telencephalic neural progenitor cell cultures. Primary neural progenitor cell cultures were established from dorsal telencephalic cortices dissected from embryonic day 13.5 (E13.5) mouse embryos as described previously (10, 33). Cells were seeded into either four-well chamber slides (Nalge Nunc International) for immunocytochemical studies or six-well dishes (BD Labware) for transcription assays. All chambers and dishes were coated with 0.1% poly-D-lysine and 0.2% laminin (BD Biosciences). Cells were cultured in Neurobasal medium supplemented with 1% N2, 2% B27, 0.5 mM glutamine, 1% penicillin-streptomycin (Invitrogen), and 40 ng of fibroblast growth factor 2 (Collaborative Research) per ml.

Transient-transfection and transcription studies with neural progenitor cells. Approximately 1.5×10^6 cells/ml were seeded at the start of the experiments. After 24 h in vitro (day 1), when ~90% of the cultured cells were mitotic (10, 25, 35), transfections were performed by mixing the appropriate combinations of plasmids (the total amount of DNA was adjusted to 2.0 μg/well in each transfection) with OptiMEM medium (Invitrogen). An equal volume of OptiMEM medium was mixed separately with Lipofectamine 2000 reagent (Invitrogen) (1.25 μl/μg of DNA) and then combined with the DNA mixture and incubated for 20 min. The DNA–Lipofectamine 2000 mix was then added dropwise to each well. In each case, a pRSV-β-galactosidase DNA was cotransfected to provide a means of normalizing the assays for transfection efficiency. Twenty-four hours after transfection, cells were harvested and luciferase and β-galactosidase activities were determined as described previously (23–25). Results were expressed as mean values ± standard deviations (SD).

Immunocytochemical analysis of differentiating neural progenitor cells. Approximately 4×10^5 cells/ml were seeded at the start of the experiments. After 48 h in vitro, cells were transfected as described above by using plasmids encod-

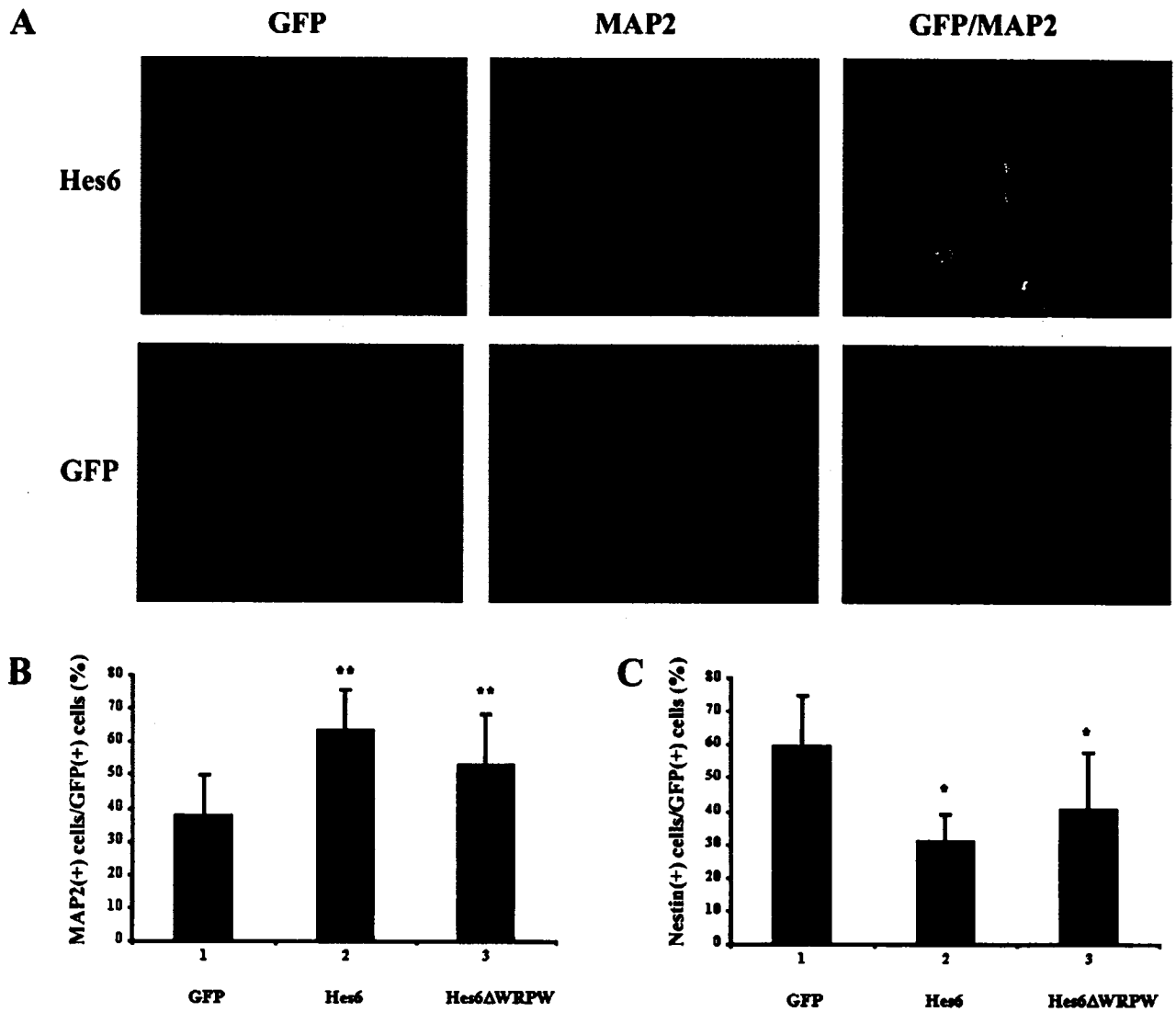

FIG. 1. Promotion of cortical neurogenesis by Hes6. (A) Primary cultures of E13.5 mouse embryonic cortical neural progenitor cells were transfected with plasmids encoding either GFP alone (bottom panels) or a combination of GFP and Hes6 (top panels). Forty-eight hours later, cells were fixed and subjected to double-labeling analysis of the expression of GFP (left panels) or MAP2 (middle panels). The combined GFP and MAP2 staining is shown in the right panels. (B and C) Quantitation of the percentage of GFP-MAP2-double-positive cells (B) or of cells in similar double-labeling experiments conducted in parallel with antibodies against nestin. Results are shown as the means ± SD ($n = 5$). *, $P < 0.01$; **, $P < 0.001$.

ing either enhanced green fluorescent protein (GFP) alone (0.2 μg/well) or combinations of GFP and Hes6, Hes6ΔWRPW, or Hes6(S183A) (0.5 μg of Hes6 plasmid per well). The total amount of DNA was adjusted to 1.0 μg. Cells were allowed to differentiate until day 4 to 5 in vitro, when they were fixed and subjected to double-label immunocytochemical analysis of the expression of GFP, nestin (a marker of undifferentiated neural progenitor cells), or MAP2 or NeuN (markers of differentiated neurons) as described (33, 35). Antinestin (BD PharMingen), anti-MAP2 (Sigma), or anti-NeuN (Chemicon) antibodies were used. Digital image acquisition and analysis were performed with the Northern Eclipse software (Empix Inc.). Results were expressed as mean values ± SD.

RESULTS

Promotion of cortical neurogenesis by Hes6. Hes6 was shown to promote neuronal differentiation in *Xenopus* embryos and mouse retinal explants (3, 19). To determine whether Hes6 might also promote the development of cortical neurons, we transfected exogenous Hes6 in primary cultures of neural progenitor cells isolated from the dorsal telencephalons of E13.5 mouse embryos. These cortical progenitors endogenously express Hes6 (reference 3 and data not shown), as well as Hes1 and Gro/TLE (6, 32, 40). Enhanced GFP was coexpressed to mark the transfected cells. Exogenous Hes6 expression led to a significant increase in the number of differentiated neurons compared to that with GFP alone, as revealed by immunocytochemistry with antibodies against the neuron-specific protein MAP2 (Fig. 1A and B [cf. bars 1 and 2]). This increase was correlated with a decrease in the number of un-

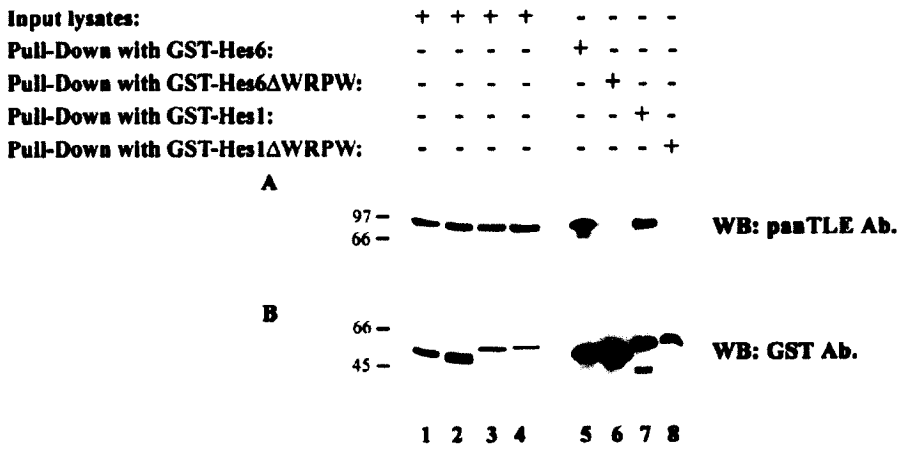

FIG. 2. Interaction of Hes6 and Hes1 with Gro/TLE. 293A cells were transfected with plasmids encoding the indicated GST fusion proteins. Cell lysates were collected, and the fusion proteins were isolated on glutathione-Sepharose beads. The precipitated material (Pull-Down) was subjected to SDS-PAGE (lanes 5 to 8) on a 10% gel, together with 1/10 of each input lysate collected prior to incubation with glutathione-Sepharose beads (lanes 1 to 4). This was followed by Western blotting (WB) with either antibodies (Ab.) that recognize all Gro/TLE proteins (panTLE) (A) or anti-GST antibodies (B). Positions of size standards are indicated in kilodaltons.

differentiated neural progenitor cells expressing the protein nestin (Fig. 1C, cf. bars 1 and 2). These results thus show that Hes6 promotes cortical neuronal differentiation. Since previous studies have shown that the neurogenic ability of *Xenopus* Hes6 does not require its carboxy-terminal WRPW motif involved in Gro/TLE binding (19), we next examined whether Hes6ΔWRPW, a truncated form lacking this motif, would also promote cortical neuronal differentiation. Exogenous Hes6ΔWRPW also caused an increase in the number of differentiated neurons, although less effectively than Hes6 (Fig. 1B, cf. bars 1 to 3). Hes6 and Hes6ΔWRPW were expressed at equivalent levels (see Fig. 3A). Together, these findings strongly suggest that Hes6 promotes the differentiation of cortical progenitor cells into postmitotic neurons. They further suggest that its WRPW motif is not required for, but contributes to, a maximal neurogenic effect. This is consistent with the finding that although both Hes6 and Hes6ΔWRPW can promote neurogenesis in *Xenopus* embryos, the former elicited a more robust neurogenic effect than the latter (19).

Comparison of the interaction of Hes6 or Hes1 with Gro/TLE. To elucidate the molecular mechanisms underlying the neurogenic activity of Hes6, we tested whether this function might involve an inhibition of the antineurogenic activity of Hes1. Both Hes1 and Hes6 bind to Gro/TLE (9, 12, 24, 25) and are coexpressed with the latter in a number of tissues (3, 6, 9, 12, 32, 34, 39). In particular, *Hes1* and *Hes6* are coexpressed in neural progenitor cells but not in differentiated neurons, where *Hes6* continues to be expressed while *Hes1* is down-regulated (3, 19, 32). This suggested that Hes6 might act as a negative regulator of Hes1 activity in neural progenitors by competing with Hes1 for binding to Gro/TLE, thus titrating away the corepressor function that Gro/TLE provides to Hes1. To examine this possibility, we first tested whether Hes6 had a higher affinity than Hes1 for Gro/TLE. 293A cells that express endogenous Gro/TLE (Fig. 2A, lanes 1 to 4) were transfected with plasmids encoding either GST-Hes6 or GST-Hes1. The precipitation of equivalent amounts of these fusion proteins (Fig. 2B, cf. lanes 5 and 7) resulted in the coprecipitation of equivalent amounts of endogenous Gro/TLE (Fig. 2A, cf. lanes 5 and 7). In contrast, expression of fusion proteins of GST and truncated forms of Hes6 or Hes1 lacking the WRPW motif (Fig. 2B, lanes 6 and 8) did not result in the coprecipitation of Gro/TLE (Fig. 2A, lanes 6 and 8), consistent with the demonstrated requirement for this motif for Gro/TLE binding (24). These findings show that Hes1 and Hes6 interact with Gro/TLE with similar affinities when they are expressed at equivalent levels.

Effect of Hes6 on the interaction of Hes1 with Gro/TLE. To directly test whether Hes6 might compete with Hes1 for Gro/TLE binding, we performed Hes1-Gro/TLE coimmunoprecipitation studies in the absence or presence of Hes6. 293A cells were transfected with FLAG epitope-tagged Hes1, followed by immunoprecipitation with anti-FLAG antibodies. In the absence of HA-Hes6 (Fig. 3A, lane 1), Gro/TLE coimmunoprecipitated efficiently with Hes1 (Fig. 3E, lane 1). When Hes6 was coexpressed with Hes1 (Fig. 3A, lane 2), we observed a significant decrease in the amount of Gro/TLE that coimmunoprecipitated with Hes1 (Fig. 3E, cf. lanes 1 and 2). Under these conditions (see Materials and Methods), Hes6 expression did not cause a significant decrease in the level of transfected Hes1 (Fig. 3B and D, cf. lanes 1 and 2) and had no negative effect on the expression of endogenous Gro/TLE (Fig. 3C), suggesting that the decreased Gro/TLE coimmunoprecipitation was not simply the result of decreased levels of these proteins. In this and succeeding figures, the relative intensities of the Hes1 and Hes6 immunoreactive bands do not reflect the actual relative amounts of these factors, because different antibodies were used for each protein and blots were not developed for equal lengths of time. To test whether the reduction in Hes1-Gro/TLE coimmunoprecipitation resulted from a titration effect mediated by Hes6 homodimers, the same assays were performed with Hes6ΔWRPW (Fig. 3A, lane 3). This protein also caused a decrease in Gro/TLE coimmunoprecipitation with Hes1, although this reduction was not as robust as

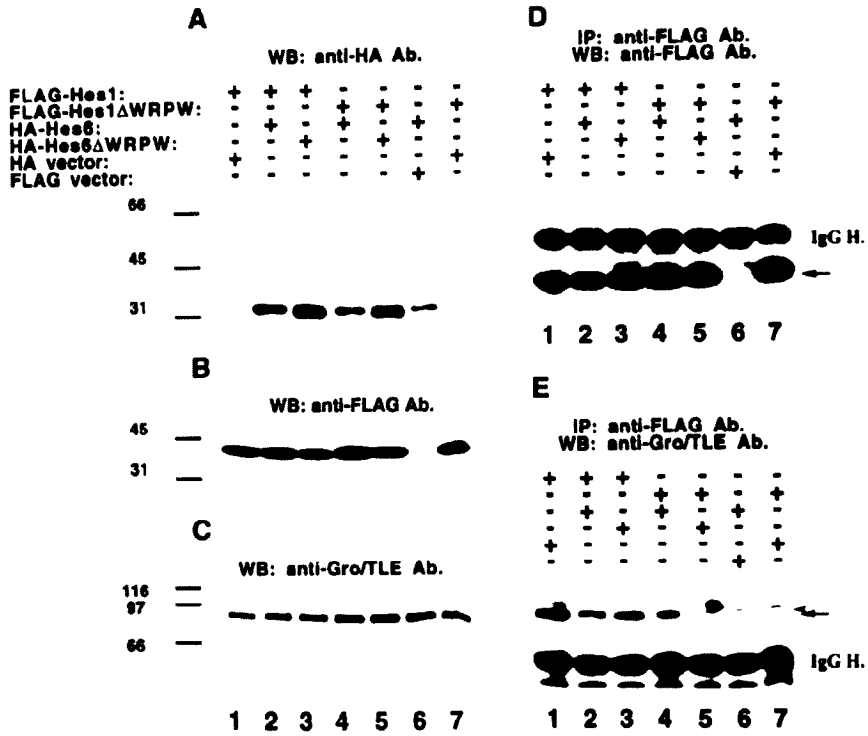

FIG. 3. Inhibition of the coimmunoprecipitation of Gro/TLE with Hes1 by Hes6 and Hes6ΔWRPW. 293A cells were transfected with plasmids encoding Hes1 or Hes1ΔWRPW in the absence or presence of HA-Hes6 or HA-Hes6ΔWRPW, as described in Materials and Methods. One-tenth of each cell lysate was subjected to SDS-PAGE (A to C) and the remaining lysates were subjected to immunoprecipitation (IP) with anti-FLAG antibodies (D and E). Samples were analyzed by Western blotting (WB) with anti-HA (A), anti-FLAG (B and D), or anti-Gro/TLE (C and E) antibodies (Ab.). The arrow in panel D points to the position of migration of Hes1. The arrow in panel E points to the position of migration of Gro/TLE, and the arrowhead indicates a nonspecific band. IgG H., immunoglobulin G heavy chains. Positions of size standards are indicated in kilodaltons.

with Hes6 (Fig. 3E, cf. lanes 1 and 3). Coexpression of Hes6ΔWRPW did not affect the levels of Hes1 or Gro/TLE (Fig. 3B and C). Similar studies were performed with fusion proteins of Hes6 and the DNA-binding domain of GAL4 (GAL4bd). Expression of increasing amounts of GAL4bd-Hes6 (Fig. 4A, lanes 2 and 3) led to a significant inhibition of the coimmunoprecipitation of Gro/TLE with Hes1 (Fig. 4D, cf. lanes 1 to 3) without significantly affecting the expression of either Hes1 (Fig. 4B, lanes 1 to 3) or Gro/TLE (Fig. 4C, lanes 1 to 3). GAL4bd-Hes6ΔWRPW had a similar effect, although it was somewhat less effective than GAL4bd-Hes6 (Fig. 4D, lanes 4 and 5).

To extend these observations, cells were transfected with Hes1ΔWRPW, followed by immunoprecipitation with anti-FLAG antibodies. As expected, in the absence of cotransfected Hes6, Gro/TLE did not coimmunoprecipitate with Hes1ΔWRPW (Fig. 3E, lane 7). In contrast, Gro/TLE coimmunoprecipitated with Hes1ΔWRPW when the latter was cotransfected with Hes6 (Fig. 3E, lane 4) but not with Hes6ΔWRPW (Fig. 3E, lane 5). As previously reported (3), the Hes1 and Hes6 proteins heterodimerized with each other under the experimental conditions used for these assays (data not shown). Expression of Hes6 alone followed by immunoprecipitation with anti-FLAG antibodies did not result in Gro/TLE coprecipitation (Fig. 3E, lane 6). The expression of Hes1ΔWRPW was not affected by Hes6 expression (Fig. 3B and D). Taken together, these findings demonstrate that Hes6 can antagonize the interaction of Hes1 with Gro/TLE. The WRPW motif of Hes6 is not necessary for this effect, suggesting that this is not solely the result of a competition by Hes6 homodimers for Gro/TLE binding.

Effect of Hes6 on stability of Hes1. During the course of our transfection experiments, we noted that under appropriate conditions (see Materials and Methods), the coexpression of increasing levels of Hes6 caused a gradual decrease of FLAG-Hes1 immunoreactivity (Fig. 5A and B, cf. lanes 1 to 3). A similar effect was observed when Hes6ΔWRPW was expressed (Fig. 5A and B, cf. lanes 1, 4, and 5), although this truncated form appeared to cause a smaller decrease in Hes1 levels compared with Hes6. The expression of Hes1ΔWRPW was also reduced in the presence of Hes6, but not as significantly as in the case of Hes1 (Fig. 5A, cf. lanes 1 to 3 and 6 to 8). In contrast, Hes6ΔWRPW had no significant effect on Hes1ΔWRPW levels (Fig. 5A, cf. lanes 6, 9, and 10). These findings were specific, because the levels of endogenous Gro/TLE were not affected by either Hes6 or Hes6ΔWRPW (Fig. 5C). To corroborate these results and exclude any effects due to the presence of the HA epitope on Hes6, similar studies were performed with GAL4bd-Hes6. Expression of both GAL4bd-Hes6 and GAL4bd-Hes6ΔWRPW also caused a de-

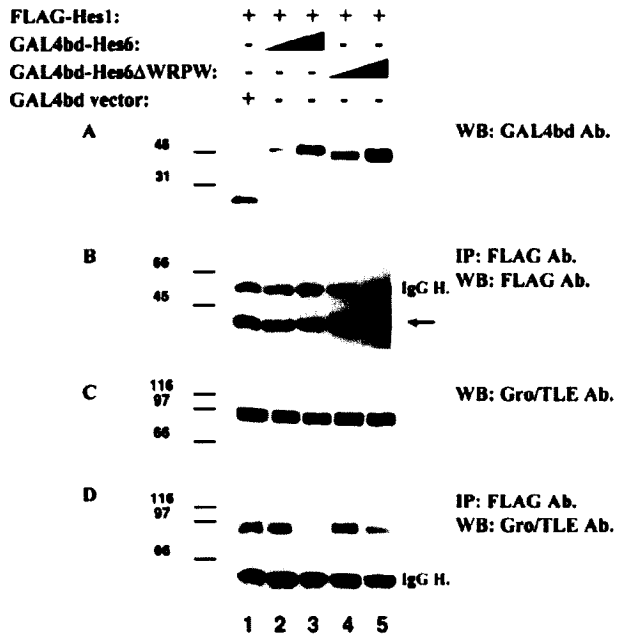

FIG. 4. Inhibition of the coimmunoprecipitation of Gro/TLE with Hes1 by Hes6 and Hes6ΔWRPW. 293A cells were transfected with plasmids encoding the indicated combinations of proteins, as described in Materials and Methods. Cell lysates were collected and subjected to Western blotting (WB) with anti-GAL4bd (A) or anti-Gro/TLE (C) antibodies (Ab.) or immunoprecipitation (IP) with anti-FLAG antibodies followed by Western blotting with anti-FLAG (B) or anti-Gro/TLE (D) antibodies. The arrow in panel B points to the position of migration of Hes1. IgG H., immunoglobulin G heavy chains. Positions of size standards are indicated in kilodaltons.

crease in Hes1 immunoreactivity compared to the expression of GAL4bd alone (data not shown). These combined observations suggest that Hes6 promotes mechanisms that negatively regulate the stability of Hes1.

To elucidate these mechanisms further, we tested whether the stability of Hes6 and/or Hes1 might be increased by inhibition of the 26S proteasome. Unexpectedly, we observed a decrease in both HA-Hes6 and GAL4bd-Hes6 immunoreactivity when cells were treated with the protease inhibitor MG132 (Fig. 6A and B, cf. lanes 2 and 3). The proteasome inhibitor lactacystin also caused a similar decrease in Hes6 immunoreactivity (data not shown). This effect was specific, because it was not observed when Hes6ΔWRPW was tested (Fig. 6A and B, cf. lanes 4 and 5). Moreover, MG132 had no effects on the expression of either Hes1 (Fig. 6C, lanes 1 and 2) or Gro/TLE (Fig. 6C, lanes 3 and 4). We also observed that the decrease in full-length HA-Hes6 or GAL4bd-Hes6 was not correlated with the appearance of smaller immunoreactive species. In particular, we did not observe bands migrating near or above the position where GAL4bd migrates (~19 kDa) (Fig. 4A), suggesting that MG132 treatment caused extensive degradation of the Hes6 proteins. These combined findings show that Hes6 is susceptible to proteolytic mechanisms that can be mimicked or activated (rather than suppressed) by treatment with MG132. These mechanisms depend on the presence of the WRPW motif, perhaps because Hes6 is more prone to degradation when it is competent to associate with Gro/TLE or because the WRPW motif unmasks sites that are involved in degradation pathways.

These observations raised the possibility that the susceptibility of Hes6 to proteolytic degradation might be correlated with its negative effect on the stability of Hes1. To test this, Hes1 was expressed in the absence or presence of increasingly high levels of Hes6. We found that the gradual decrease in Hes1 stability induced by transfecting increasing amounts of

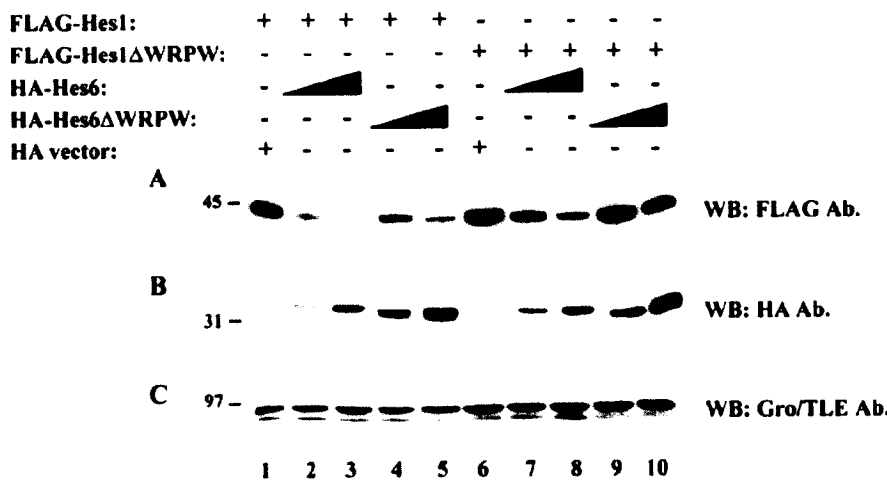

FIG. 5. Effect of Hes6 expression on Hes1 stability. 293A cells were transfected with either FLAG-Hes1 or FLAG-Hes1ΔWRPW (50 ng/transfection), as indicated, in the absence (lanes 1 and 6) (HA vector) or presence of increasing amounts of HA-Hes6 or HA-Hes6ΔWRPW (200 ng/transfection in lanes 2, 4, 7, and 9 or 600 ng/transfection in lanes 3, 5, 8, and 10). Cell lysates were subjected to SDS-PAGE on an 11% gel, followed by sequential Western blotting (WB) with either anti-FLAG (A), anti-HA (B), or anti-Gro/TLE (C) antibodies (Ab.). Positions of size standards are indicated in kilodaltons.

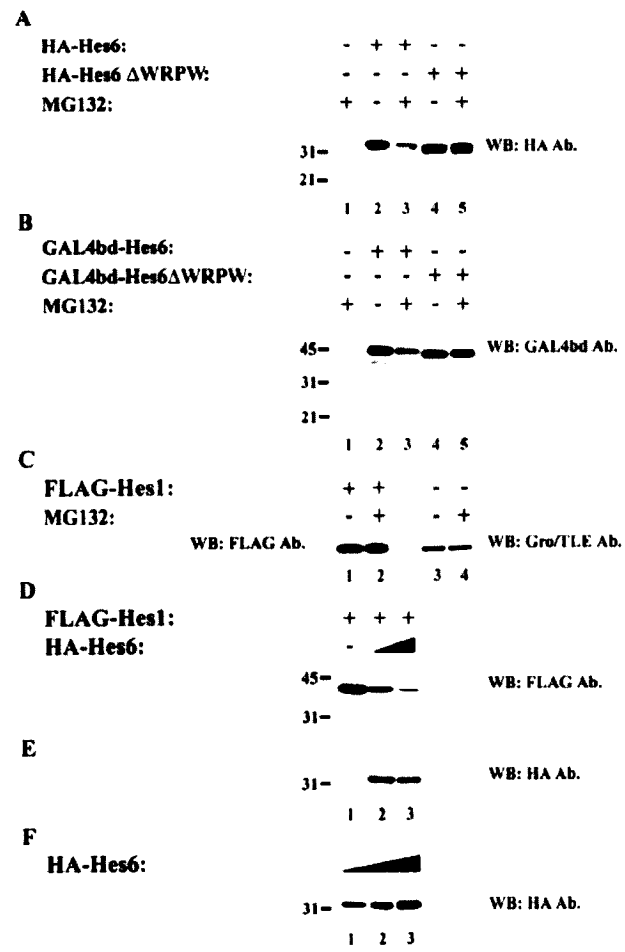

FIG. 6. Analysis of Hes6 stability. (A to C) 293A cells were transfected with the indicated combinations of proteins and then incubated in the absence or presence of MG132 as indicated, followed by cell lysis and Western blotting (WB) analysis. The levels of both HA-Hes6 (panel A, lanes 2 and 3) and GAL4bd-Hes6 (panel B, lanes 2 and 3) were reduced in the presence of MG132. In contrast, the levels of Hes6ΔWRPW (panel A, lanes 4 and 5), GAL4bd-Hes6ΔWRPW (panel B, lanes 4 and 5), Hes1 (panel C, lanes 1 and 2), and Gro/TLE (panel C, lanes 3 and 4) were not affected. Ab., antibodies. (D and E) 293A cells were transfected with increasing amounts of HA-Hes6 expression plasmid (400 ng/transfection in lane 2 or 800 ng/transfection in lane 3) in the presence of a constant amount of Hes1 (50 ng/transfection), followed by Western blotting with either anti-FLAG (D) or anti-HA (E) antibodies. (F) Cells were transfected with HA-Hes6 expression plasmid at 200 (lane 1), 400 (lane 2), or 800 (lane 3) ng/transfection in the absence of Hes1, followed by Western blotting with anti-HA antibodies. Positions of size standards are indicated in kilodaltons.

Hes6 DNA (Fig. 6D) was not correlated with a gradual increase in Hes6 immunoreactivity (Fig. 6E). In contrast, when Hes6 was transfected in the absence of Hes1, we observed the expected correlation between larger amounts of DNA and increasing protein levels (Fig. 6F). Taken together, these results show that Hes6 promotes degradation of Hes1 in a dose-dependent manner. They suggest further that Hes6 may become increasingly unstable when it is bound to Hes1. This in turn raises the possibility that Hes1 becomes targeted for degradation due to its association with Hes6. This process is maximally effective when both Hes1 and Hes6 contain the WRPW motif involved in Gro/TLE binding.

Inhibition of Hes1-mediated transcriptional repression by Hes6 in telencephalic neural progenitor cells. The previous results show that Hes6 can negatively regulate both the stability of Hes1 and its interaction with Gro/TLE. Since these effects are predicted to impair Hes1-mediated transcriptional repression, we next tested the possibility that Hes6 might suppress the ability of Hes1 to act as a transcriptional repressor in a cellular context where these proteins are normally coexpressed. Primary cultures of cortical neural progenitor cells were established and transfected with a reporter plasmid containing the luciferase gene under the control of the *ngn3* promoter. Hes1 has been shown previously to specifically bind to this promoter and repress its activity (20). We found that the *ngn3* promoter drove strong expression of the reporter gene in transfected neural progenitors and that Hes1 significantly suppressed transcription from this promoter (Fig. 7A, cf. bars 1 and 2). When increasing amounts of Hes6 were cotransfected, Hes1-mediated repression was progressively reduced (Fig. 7A, cf. bars 2 to 4). Expression of Hes6ΔWRPW also resulted in an inhibition of Hes1-mediated repression (Fig. 7A, bars 5 and 6). Control experiments showed that neither Hes6 nor Hes6ΔWRPW had an activating effect on the *ngn3* promoter when transfected in the absence of Hes1 (Fig. 7A, bars 12 and 13). Moreover, no significant effects were observed when the *ngn3* promoter was mutated to delete the Hes1-binding sites present within its proximal region (20) (Fig. 7B). These results show that Hes6 has the ability to inhibit transcription repression mediated by Hes1 in neural progenitor cells.

We then investigated whether this inhibitory effect was the result of either a promotion of Hes1 degradation or the prevention of Hes1-Gro/TLE complex formation (or a combination of both). We hypothesized that transcriptional repression mediated by a chimeric protein in which Hes1 was constitutively associated with Gro/TLE might be suppressed by Hes6 if that involved a proteolysis of Hes1 but not if it required an inhibition of Hes1-Gro/TLE interaction. A fusion protein (Hes1ΔWRPW:Gro/TLE1) in which the WRPW motif of Hes1 was removed and the entire sequence of Gro/TLE1 was subcloned in its place was engineered. This chimeric protein repressed transcription driven by the *ngn3* promoter in neural progenitor cells, and its repressive activity was comparable to that of Hes1 (Fig. 7A, cf. bars 2 and 7). We found that cotransfection of increasing amounts of Hes6 had a derepression effect on Hes1ΔWRPW:Gro/TLE1, although this was somewhat weaker than its inhibitory effect on Hes1 (Fig. 7A, cf. bars 2 to 4 and 7 to 9). These findings indicate that Hes6 can antagonize Hes1 transcriptional repression activity even when Hes1 is constitutively bound to Gro/TLE, strongly suggesting that an inhibition of the Hes1-Gro/TLE interaction is not the only mechanism utilized by Hes6 to suppress Hes1. In turn, this implicates mechanisms involving the promotion of Hes1 degradation in this event. Importantly, although Hes6ΔWRPW had an inhibitory effect on Hes1 (Fig. 7A, cf. bars 2, 5, and 6), it had no significant effect on Hes1ΔWRPW:Gro/TLE1 (Fig. 7A, cf. bars 7, 10, and 11).

In agreement with these findings, examination of the expres-

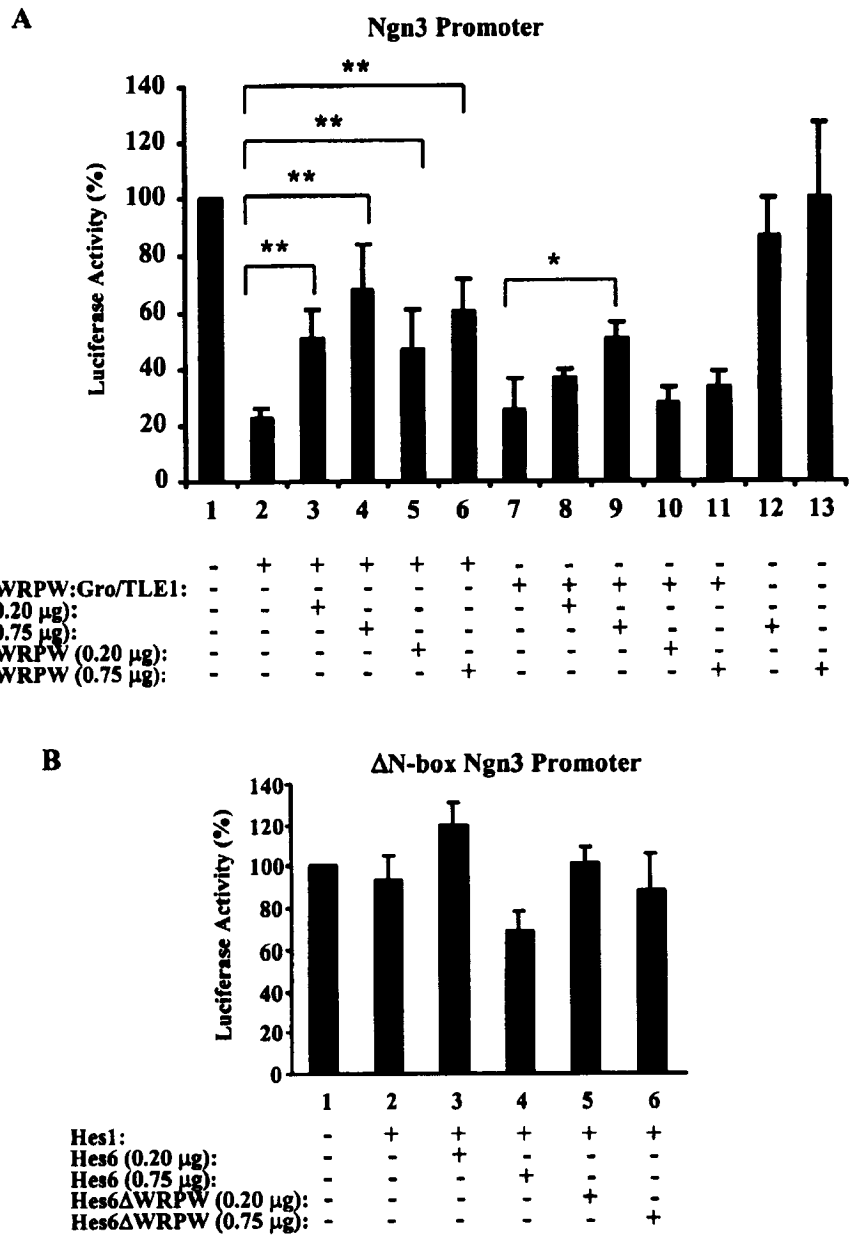

FIG. 7. Inhibition of Hes1-mediated transcriptional repression by Hes6 and Hes6ΔWRPW. Primary cultures of neural progenitor cells isolated from the dorsal telencephalons of E13.5 mouse embryos were transfected with either the pFOX-ngn3p-Luc1 (A) or the pFOX-ΔN-box-ngn3p-Luc1 (B) reporter construct, as indicated, in the absence or presence of Hes1 or Hes1ΔWRPW:Gro/TLE1 and the indicated amounts (per transfection) of either HA-Hes6 or HA-Hes6ΔWRPW. The activity of the reporter gene in the absence of any expression plasmid was considered to be 100%. Luciferase activities were expressed as the means ± SD from at least five independent experiments performed in duplicate. *, $P < 0.001$; **, $P < 0.0001$.

sion of Hes1ΔWRPW:Gro/TLE1 by using antibodies against the amino-terminal FLAG epitope showed that Hes6 caused a significant reduction in immunoreactivity compared to controls (Fig. 8A, cf. lanes 2 and 3), indicating that Hes6 promotes degradation of Hes1ΔWRPW:Gro/TLE1. Both GAL4bd-Hes6 and HA-Hes6 had the same effect on the expression of Hes1ΔWRPW:Gro/TLE1 (data not shown). In contrast, Hes6ΔWRPW did not affect the expression of this fusion protein (Fig. 8A and B, cf. lanes 2 and 4), consistent with the lack of a negative effect of Hes6ΔWRPW on the transcription repression ability of Hes1ΔWRPW:Gro/TLE1 described above. Reprobing with anti-Gro/TLE antibodies directed against the carboxy-terminal domain of this fusion protein confirmed that Hes6ΔWRPW did not decrease the expression of Hes1ΔWRPW:Gro/TLE1 like Hes6 did (Fig. 8C, cf. lanes 2 to 4). Moreover, using these antibodies, we noticed that coexpression of Hes6

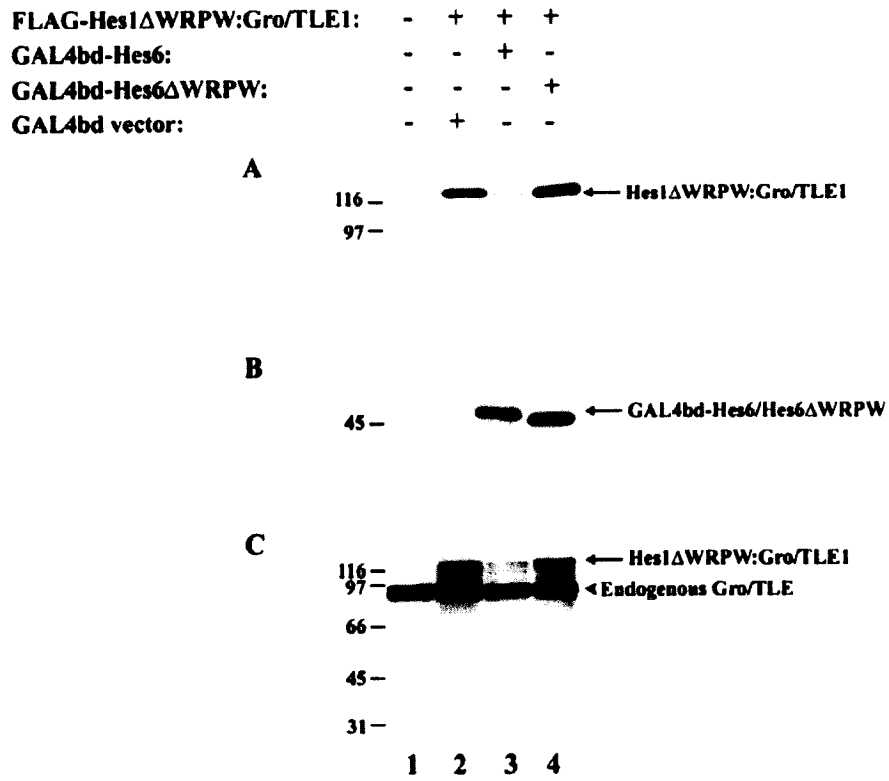

FIG. 8. Effect of Hes6 on expression of Hes1ΔWRPW:Gro/TLE1. 293A cells were transfected with plasmids encoding the indicated combinations of proteins, followed by preparation of cell lysates and Western blotting with either anti-FLAG (A), anti-GAL4bd (B), or anti-Gro/TLE (C) antibodies. Positions of size standards are indicated in kilodaltons.

was not correlated with detectable immunoreactive species migrating between endogenous Gro/TLEs (Fig. 8C) and full-length Hes1ΔWRPW:Gro/TLE1 (Fig. 8C) or lower forms of smaller size. These observations suggest that Hes6 expression caused a general proteolysis of the Hes1ΔWRPW:Gro/TLE1 fusion protein and not solely a confined degradation of its amino-terminal portion. Taken together, these findings show that Hes6 inhibits Hes1-mediated transcriptional repression in neural progenitor cells and strongly suggest that the promotion of Hes1 proteolysis by Hes6 is important for this inhibitory effect.

Involvement of Ser183 in the ability of Hes6 to promote Hes1 degradation and neuronal differentiation. Previous studies (37) have shown that the *Drosophila* Hes family members Enhancer of split m5, m7, and m8 contain an evolutionarily conserved sequence motif characterized by a carboxy-terminal consensus site for phosphorylation by protein kinase CK2, defined as (S/T)(D/E)X(D/E), preceded at a short distance by the sequence SP(A/V)SS. This sequence, hereafter referred to as the SPXSS-SDXE motif is located within a region with a high PEST score (37). PEST-rich sequences behave as *cis*-acting signals that regulate protein turnover and have been suggested to be activated via phosphorylation (27, 31). The *Drosophila* m5, m7, and m8 proteins were shown to associate with and be phosphorylated by protein kinase CK2 at their conserved SPXSS-SDXE sequences. This phosphorylation is believed to activate their PEST domains and result in decreased stability (37).

Using the program PESTfind (http://at.embnet.org/embnet/tools/bio/PESTfind), we identified a conserved potential PEST sequence at the carboxy termini of mouse and human Hes6 proteins (Fig. 9A) (PEST score, +13.02; PEST scores of greater than +5 are considered significant). This region contains a conserved sequence similar to the SPXSS-SDXE motif found in the PEST domain of *Drosophila* m5, m7, and m8 (Fig. 9A). This raised the possibility that Hes6 might be phosphorylated by protein kinase CK2 and that this event may regulate its stability through modulation of PEST sequence activity. To test this, we first determined whether Hes6 is a phosphorylated protein. Lysates from cells transfected with Hes6 were incubated in the absence or presence of calf intestinal phosphatase, followed by gel electrophoresis. After this treatment, Hes6 exhibited a faster electrophoretic mobility, indicating that it is a phosphorylated protein (Fig. 9B, cf. lanes 1 and 2). In addition, purified protein kinase CK2 directly phosphorylated a fusion protein of GST and Hes6 isolated from bacteria (Fig. 9C and D, lanes 2). Importantly, an S183A mutation within the SPXSS-SDXE motif significantly attenuated phosphorylation of Hes6 by protein kinase CK2 even when Hes6(S183A) was present at higher levels than wild-type Hes6 (Fig. 9C and D, cf. lanes 2 and 4). Hes1, which does not contain an SPXSS-SDXE motif, was not phosphorylated by protein kinase CK2 (Fig. 9E,

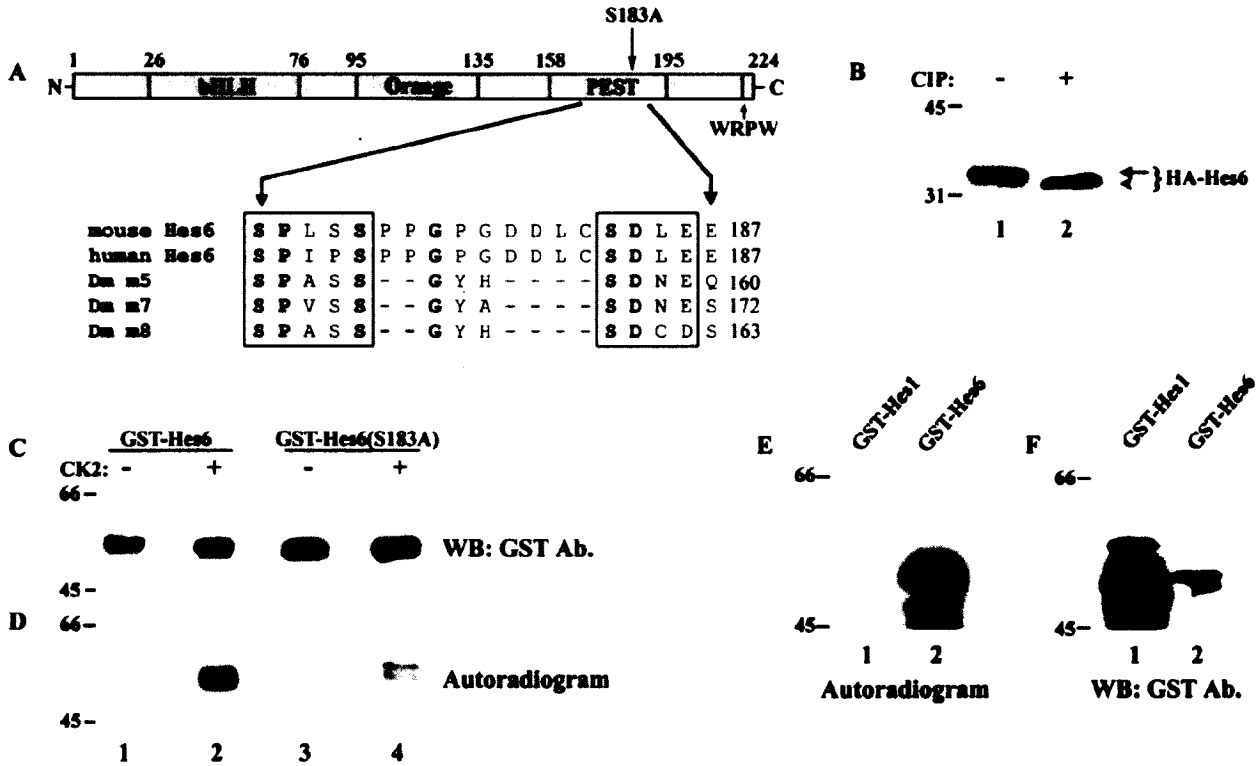

FIG. 9. Analysis of Hes6 phosphorylation. (A) Schematic representation of the domain structure of Hes6. Indicated are the bHLH domain, the Orange domain predicted to form helices 3 and 4, the PEST region containing the SPXSS-SDXE motif and its resident Ser183, and the WRPW tetrapeptide. Shown in detail are the sequences of the SPXSS-SDXE elements from mouse and human Hes6 (3) and Drosophila Enhancer of split m5, m7, and m8 (37). Invariant residues are indicate in boldface. (B) 293A cells were transfected with HA-Hes6, and cell lysates were incubated in the absence or presence of calf intestinal phosphatase (CIP), followed by Western blotting with anti-HA antibodies. (C and D) The indicated GST fusion proteins were purified and subjected to in vitro phosphorylation in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of purified protein kinase CK2, followed by autoradiography (D) and Western blotting (WB) with anti-GST antibodies (Ab.) (C). (E and F) The indicated GST fusion proteins were purified and subjected to in vitro phosphorylation in the presence of purified protein kinase CK2, followed by autoradiography (E) and Western blotting with anti-GST antibodies (F). Positions of size standards are indicated in kilodaltons in panels B to F.

lane 1) even when expressed at significantly higher levels than Hes6 (Fig. 9F, cf. lanes 1 and 2). Taken together, these findings identify Hes6 as a specific target of protein kinase CK2 and strongly suggest that this kinase can phosphorylate Hes6 at Ser183.

Based on these observations, we tested whether Ser183 might be important for the ability of Hes6 to cause a reduced stability of Hes1. 293A cells were transfected with Hes1 alone or in the presence of Hes6 or Hes6(S183A). Hes6 caused a dramatic decrease in Hes1 expression, whereas Hes6(S183A) had a weaker, although still detectable, effect (Fig. 10A and B, cf. lanes 1 to 3). These findings suggest that phosphorylation of Ser183 plays a positive role in the ability of Hes6 to promote Hes1 degradation. In turn, this raised the possibility that Hes6(S183A) might have a weaker neurogenic activity than wild-type Hes6 due to its reduced ability to decrease Hes1 stability. To examine this possibility, cortical progenitor cells were transfected with Hes6 or Hes6(S183A), and the transfected cells were examined for the expression of markers of either proliferating cells (the Ki67 protein) or differentiated neurons (the NeuN protein), as described previously (33). We found that exogenous Hes6 led to the differentiation of supernumerary neurons (Fig. 10C, cf. bars 1 and 2) and a decrease in undifferentiated progenitors (Fig. 10D, cf. bars 1 and 2). In contrast, Hes6(S183A) did not promote similar effects (Fig. 10C and D, bars 3). Taken together, these findings identify an important role for Ser183 in the neurogenic activity of Hes6 and show a correlation between phosphorylation of this residue by protein kinase CK2 and the ability of Hes6 to negatively regulate Hes1 functions and promote neuronal development.

DISCUSSION

Involvement of Hes6 in neuronal differentiation. Previous studies with mouse and *Xenopus* have revealed that *Hes6* expression is correlated with the transition of neural progenitor cells to the neuronal fate (3, 19, 30, 38). In *Xenopus*, *Hes6* activation follows the expression of neuronal determination genes such as *ngn* family members and overlaps with neuronal differentiation genes such as *NeuroD* (19). In mice, *Hes6* expression was detected in both the proliferative zone containing neural progenitor cells and areas containing postmitotic neurons (3). Taken together with the demonstration that *Xenopus Hes6* expression is not activated by the Notch signaling path-

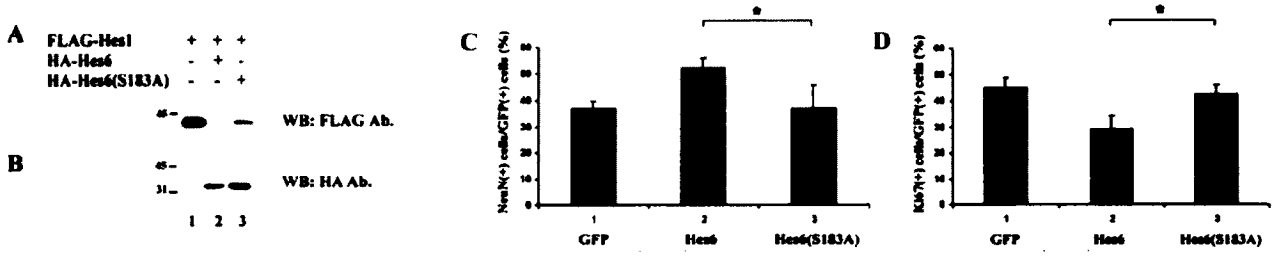

FIG. 10. Effects of S183A mutation on Hes6 functions. (A and B) 293A cells were transfected with FLAG-Hes1 (50 ng/transfection) in the absence (lane 1) or presence of either HA-Hes6 (lane 2) or HA-Hes6(S183A) (lane 3) (600 ng/transfection). Cell lysates were subjected to SDS-PAGE, followed by Western blotting (WB) with either anti-FLAG (A) or anti-HA (B) antibodies (Ab.). Shown is a representative example of results from four separate experiments that gave the same results. Positions of size standards are indicated in kilodaltons. (C and D) E13.5 mouse embryonic cortical progenitor cells were transfected with plasmids encoding either GFP alone or a combination of GFP and Hes6 or GFP and Hes6(S183A). Forty-eight hours later, cells were fixed and subjected to double-labeling analysis of the expression of GFP, NeuN, or Ki67. Shown is the quantitation of the percentage of GFP-NeuN (C)- or GFP-Ki67 (D)-double-positive cells. Results are shown as the means ± SD (n = 4). *, $P < 0.01$.

way, which plays an antineurogenic role, but rather appears to be driven by neurogenic bHLH proteins (19), these observations first suggested an involvement of Hes6 in mechanisms that positively regulate neurogenesis. This possibility was confirmed by ectopic expression studies with *Xenopus* embryos and murine retinal explants that revealed that Hes6 promotes neuronal differentiation (19). Importantly, those studies also suggested that Hes6 may act primarily by promoting the differentiation of progenitors that already express proneural proteins, perhaps by antagonizing the functions of inhibitors of the latter. By removing this inhibition, Hes6 may allow proneural proteins to perform their neurogenic functions more effectively, leading to enhanced neuronal differentiation. In an effort to clarify how Hes6 may antagonize inhibitory activities that negatively regulate proneural protein functions, we have focused on the Hes1 protein, a well-characterized member of a family of bHLH proteins that act as inhibitors of proneural proteins in both invertebrates and vertebrates (18). In particular, Hes1 inhibits transcription from proneural gene promoters (4, 20) and the expression of proneural genes is prematurely activated in *Hes1* nullizygous mice (16), suggesting that Hes1 acts as a negative regulator of proneural proteins in vivo. *Hes1* and *Hes6* are coexpressed in differentiating neural progenitor cells (3, 19, 32), and they can heterodimerize in transfected cells and in vitro (3). Moreover, Hes6 was shown to reduce the ability of Hes1 to repress transcription from an artificial promoter in NIH 3T3 cells (3). These observations raised the possibility that Hes6 acts as a negative regulator of the antineurogenic activity of Hes1. However, they did not clarify the molecular mechanisms that underlie this function. To address this important question, we have performed a combination of molecular and cellular investigations that have characterized two complementary mechanisms that Hes6 may utilize to negatively regulate Hes1 activity and positively regulate neuronal differentiation.

Inhibition of Hes1-Gro/TLE interaction by Hes6. Our studies have shown that the interaction of Hes1 with its transcriptional corepressor Gro/TLE is reduced when Hes6 is coexpressed at levels that do not have a significant effect on the stability of either Hes1 or Gro/TLE. This effect is unlikely to result solely from a competition for Gro/TLE between Hes6 and Hes1 homodimers, because a truncated form of Hes6 that is unable to bind to Gro/TLE also inhibits the interaction of Hes1 with the latter. Our finding that Hes1ΔWRPW-Hes6 heterodimers, which have only one WRPW motif, appear to interact with Gro/TLE like Hes1-Hes6 heterodimers, which have both WRPW motifs, suggests instead that Hes1-Hes6 heterodimers interact more poorly with Gro/TLE than homodimers of either protein. Reasons for this reduced affinity may include the fact that the folding of these heterodimers may not allow a proper alignment of the WRPW motifs of Hes1 and Hes6. Gro/TLE proteins exist as tetramers, so the correct alignment of WRPW motifs may be critical for the establishment of a strong interaction between Hes factors and Gro/TLE. A weaker association may be caused by differential posttranslational modifications, including phosphorylation of Ser183 of Hes6 (see below). Alternatively, other cofactors that may interact selectively with either Hes1 or Hes6 may not allow a strong interaction between Gro/TLE and Hes1-Hes6 heterodimers. In either case, the formation of Hes1-Hes6 heterodimers that interact poorly with Gro/TLE is likely to prevent or reduce the interaction of Hes1 homodimers with Gro/TLE, thereby depriving Hes1 of its critical transcriptional corepressor and negatively regulating its functions. As discussed below, this situation may lead, under conditions of increasing Hes6 expression, to an additional mechanism of Hes1 suppression, namely, the targeting of Hes1-Hes6 dimers for proteolytic degradation.

Regulation of Hes1 stability by Hes6. Our investigations have shown for the first time that expression of increasing amounts of Hes6 causes a gradual decrease of Hes1 stability resulting in a loss of full-length protein. This finding raises the interesting possibility that Hes6 may act as a negative regulator of Hes1 activity by regulating the stability of the latter. Such a situation may occur, for instance, in determined neural progenitor cells, in which increased proneural protein activity may promote an up-regulation of Hes6 expression. In turn, Hes6 may cause inactivation of Hes1 by affecting its turnover, thereby contributing to the mechanisms that will drive those progenitors into the neuronal lineage. Such a situation might explain not only the ability of Hes6 to suppress Hes1-mediated repression but also the previous observation that Hes6 can also suppress the ability of Hes1 to inhibit the activity of E2A-proneural protein heterodimers (3). Inhibition of proneural

protein activity by Hes1 is thought to involve the formation of heterodimers between Hes1 and ubiquitous bHLH proteins such as E47, thus titrating away the latter from the proneural proteins. Proteolytic degradation of Hes1 would therefore be expected to prevent these interactions and inhibit this effect.

We have shown that Hes6 is intrinsically susceptible to proteolytic degradation events that can be uncovered by exposure to the protease inhibitor MG132. The mechanisms underlying the effect of MG132 on Hes6 are still unclear and likely involve indirect effects resulting from either the MG132-mediated activation of genes that encode factors that may destabilize Hes6, the inhibition of proteolytic pathways that may normally degrade factors that reduce Hes6 stability, or the inhibition of pathways leading to the expression of factors that render Hes6 more stable. Regardless of the exact nature of the events induced by MG132, the observation that Hes6 is prone to proteolytic degradation is in agreement with the presence of an evolutionarily conserved PEST domain containing an SPXSS-SDXE subdomain that includes a resident consensus protein kinase CK2 phosphorylation site at Ser183. The presence of PEST domains is characteristic of proteins that undergo increased turnover, and phosphorylation of PEST sequences by protein kinase CK2 was shown to negatively affect intrinsic protein stability (22, 27, 31). The *Drosophila* Hes family members Enhancer of split m5, m7, and m8 share with Hes6 a similar SPXSS-SDXE motif within a carboxy-terminal region characterized by a high PEST score. They were shown to bind directly to protein kinase CK2 and to be phosphorylated by this kinase at their conserved SDXE site. This phosphorylation is believed to decrease their stability (37). In agreement with those results, we have shown that Hes6, but not Hes1, is phosphorylated by protein kinase CK2 at Ser183 within the SDXE motif, suggesting a previously unrecognized relatedness of Hes6 to the m5-m7-m8 subgroup of *Drosophila* Enhancer of split proteins.

Our studies have also shown that maximal Hes6-mediated degradation of Hes1 is correlated with a decreased stability of Hes6 itself. This observation suggests that the formation of Hes1-Hes6 heterodimers may increase the intrinsic susceptibility of Hes6 to degradation, causing the recruitment of Hes1 into the same proteolytic mechanisms. Although the molecular events underlying such a process remain to be fully elucidated, our investigations have revealed important roles for both the protein kinase CK2 phosphorylation site at Ser183 of Hes6 and the WRPW motif. We have shown that mutation of Ser183 into Ala attenuates, albeit does not eliminate, the destabilizing effect of Hes6 on Hes1. This finding suggests that the SPXSS-SDXE motif of Hes6 and its resident Ser183 may contribute to the mechanisms that activate the PEST domain of Hes6. Heterodimerization with Hes1 may render this region more accessible to such mechanisms, thereby promoting the degradation of Hes6 and Hes1. Alternatively, the phosphorylation of Ser183 may cause a misalignment of the WRPW motifs of Hes1 and Hes6 when these factors heterodimerize, leading to a conformation that results in suboptimal Gro/TLE binding compared to homodimers of either protein. This may lead to the formation of misfolded Hes1-Hes6-Gro/TLE ternary complexes that may be recognized as defective and targeted for removal via proteolytic degradation. Mutation of Ser183 may allow Hes1-Hes6 dimers to interact better with Gro/TLE, resulting in the formation of properly folded complexes with increased stability.

The possibility that enhanced proteolysis of Hes1 and Hes6 may be caused by their association into incorrectly folded complexes is also suggested by our observation that the formation of Hes1-Hes6 heterodimers does not appear to be sufficient to activate proteolytic degradation of these proteins by itself, because removal of the WRPW motif from either Hes1, Hes6, or both progressively attenuates Hes1 degradation promoted by Hes6. Moreover, Hes6ΔWRPW had no detectable effect on the stability of the chimeric protein Hes1ΔWRPW:Gro/TLE1, in contrast to the significant degradation induced by full-length Hes6. In addition, heterodimers of Hes1 and Hes6 do not efficiently coimmunoprecipitate with Gro/TLE, regardless of whether they contain one or two WRPW motifs, suggesting that they may not be able to form stable complexes. Since removal of the WRPW motif does not impair the ability of Hes1 and Hes6 to heterodimerize (data not shown), these observations suggest that heterodimers of Hes1 and Hes6 may be more susceptible to degradation if they are associated with Gro/TLE through their WRPW motifs. Heterodimers lacking this motif, and thus unable to interact with Gro/TLE, may be able to fold more properly and avoid extensive degradation. Based on these combined observations, we propose that Hes1-Hes6 heterodimers are prone to increased degradation when they form complexes with Gro/TLE. This situation may be due to specific structural features of these proteins that may not allow the formation of properly folded complexes with Gro/TLE, in turn resulting in the activation of proteolytic mechanisms involving Ser183 of Hes6. Conversely, it may be phosphorylation of Ser183 that causes a misfolding of the carboxy termini of these heterodimers and an impaired ability to bind to Gro/TLE, resulting in degradation as a secondary effect to remove the misfolded complexes. Future studies will be aimed at distinguishing between these possibilities. In either case, it appears that Ser183 plays an important role in Hes6 functions, as further indicated by the inability of Hes6(S183A) to promote neuronal differentiation (see below for further details).

We recognize that other mechanisms are also possible. For instance, the WRPW motif of Hes6 may promote the instability of Hes1-Hes6 heterodimers in a Gro/TLE-independent manner, possibly by acting as a binding site for proteins other than Gro/TLE, resulting in the direct or indirect recruitment of proteolytic enzymes. However, it remains to be determined whether the WRPW motif mediates interactions with proteins other than Gro/TLE. In addition, we cannot rule out the possibility that the destabilizing effect of Hes6 on Hes1 is the result of the activity of Hes6 as a transcriptional repressor. Hes6 may directly suppress the expression of factors that promote the stability of Hes1. This seems unlikely, however, because Hes6ΔWRPW, which cannot recruit the Gro/TLE corepressor and was shown to be unable to mediate transcriptional repression when fused to GAL4bd (9), also promotes Hes1 degradation. In addition, the in vivo neurogenic activity of Hes6 does not appear to be DNA-binding dependent, arguing against mechanisms that are based solely on direct transcriptional functions (19). It remains possible, though, that Hes6 mediates as-yet-uncharacterized transcriptional mechanisms that may affect Hes1 expression in a dose-dependent manner.

Characterization of the molecular mechanisms underlying the suppression of Hes1-mediated transcriptional repression by Hes6. To begin to elucidate whether different mechanisms of Hes1 inhibition are used by Hes6 in combination (to achieve maximal effects) or separately (perhaps depending on particular cellular and/or developmental conditions), we have examined the effect of Hes6 on the ability of Hes1 to mediate transcriptional repression in cortical progenitor cells, where these proteins are coexpressed. We have found that Hes6 suppresses Hes1-mediated repression. Both Hes6 and Hes6ΔWRPW have a similar inhibitory effect. This observation does not suggest that the suppression of Hes1 activity derives from the Hes6-mediated repression of a gene(s) encoding a positive regulator(s) of Hes1, because previous studies have shown that Hes6 requires its WRPW motif to repress transcription when targeted to DNA as a fusion protein with GAL4bd (9). Moreover, this finding also argues against a mechanism involving solely a competition for Gro/TLE between Hes1 and Hes6 homodimers. To determine whether Hes1 suppression was the result of the inhibition of the interaction of Hes1 with Gro/TLE or the promotion of Hes1 proteolysis (or a combination of both), we have examined the effect of Hes6 on a chimeric protein in which Hes1 is constitutively bound to Gro/TLE. This fusion protein represses transcription in cortical progenitor cells like full-length Hes1, and its repressive ability should not be affected by conditions that would otherwise inhibit Gro/TLE recruitment. Our investigations have revealed that Hes6 still has an inhibitory effect on Hes1ΔWRPW:Gro/TLE1, although this is weaker than its effect on Hes1. These findings thus suggest that the promotion of Hes1 degradation plays an important role in the inhibitory effect of Hes6 on Hes1-mediated repression. In agreement with this possibility, we have found that Hes6ΔWRPW, which does not promote a significant proteolysis of Hes1ΔWRPW:Gro/TLE1, does not have a negative effect on repression mediated by the latter. Together, these findings clarify mechanisms that underlie the ability of Hes6 to act as a negative regulator of Hes1 in cortical neural progenitor cells.

Promotion of cortical neurogenesis by Hes6. To determine if Hes6 is involved in the regulation of neuronal differentiation in the mammalian forebrain, we have examined the consequence of exogenous Hes6 expression in primary cultures of cortical neural progenitor cells. In our studies, Hes6 induced a decrease in the number of undifferentiated progenitor cells and an increase in the number of differentiated neurons arising from these progenitors, showing that Hes6 promotes cortical neuronal differentiation. This effect is likely the result of the recruitment of supernumerary progenitors into the neuronal lineage. Because neural progenitor cells of the dorsal telencephalon express proneural proteins such as Ngn1 and -2, our results are consistent with previous studies on *Xenopus* suggesting that Hes6 promotes the neuronal differentiation of Ngn-expressing neural progenitor cells (19). Based on these results and our demonstration that Hes6 efficiently suppresses Hes1-mediated transcriptional repression in cortical progenitors, we propose that the inhibition of Hes1 activity is at least one of the mechanisms utilized by Hes6 to promote neuronal differentiation. In possible agreement with this, we have found that the mutated protein Hes6(S183A) had an attenuated negative effect on the stability of Hes1 compared to wild-type Hes6 and did not promote neuronal differentiation. These observations suggest a correlation between a reduced ability to promote Hes1 degradation and reduced Hes6 neurogenic activity. We found that Hes6(S183A) was able to cause a detectable decrease of Hes6 stability in 293A cells but failed to promote the neuronal differentiation of cortical progenitors. This situation may reflect that the observed residual levels of Hes1 may be sufficient to inhibit neuronal differentiation or that Hes6(S183A) may have a weaker effect on Hes1 in neural progenitors compared to 293A cells. It is entirely possible, however, that additional mechanisms involving Ser183 may be important for the neurogenic activity of Hes6. Further elucidation of the mechanisms underlying Hes6 activity will clarify important events regulating vertebrate neurogenesis.

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REFERENCES

1. **Akazawa, C., Y. Sasai, S. Nakanishi, and R. Kageyama.** 1992. Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system. J. Biol. Chem. **267**:21879–21885.
2. **Anderson, D. J.** 2001. Stem cells and pattern formation in the nervous system: the possible versus the actual. Neuron **30**:19–35.
3. **Bae, S. K., Y. Bessho, M. Hojo, and R. Kageyama.** 2000. The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation. Development **127**:2933–2943.
4. **Chen, H. A., A. Thiagalingam, H. Chopra, M. W. Borges, J. N. Feder, B. D. Nelkin, S. B. Baylin, and D. W. Ball.** 1997. Conservation of the Drosophila lateral inhibition pathway in human lung cancer: a hairy-related protein (HES-1) directly represses achaete-scute homolog-1 expression. Proc. Natl. Acad. Sci. USA **94**:5355–5360.
5. **Cossins, J., A. E. Vernon, Y. Zhang, A. Philpott, and P. H. Jones.** 2002. Hes6 regulates myogenic differentiation. Development **129**:2195–2207.
6. **Dehni, G., Y. Liu, J. Husain, and S. Stifani.** 1995. *TLE* expression correlates with mouse embryonic segmentation, neurogenesis, and epithelial determination. Mech. Dev. **53**:369–381.
7. **Fisher, A. L., S. Ohsako, and M. Caudy.** 1996. The WRPW motif of the Hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. Mol. Cell. Biol. **16**:2670–2677.
8. **Fode, C., Gradwohl, X. Morin, A. Dierich, M. LeMeur, C. Goridis, and F. Guillemot.** 1998. The bHLH protein NEUROGENIN2 is a determination factor for epibranchial placode-derived sensory neurons. Neuron **20**:483–494.
9. **Gao, X., T. Chandra, M. O. Gratton, I. Quelo, J. Prud'homme, S. Stifani, and R. St.-Arnaud.** 2001. HES6 acts as a transcriptional repressor in myoblasts and can induce the myogenic differentiation program. J. Cell Biol. **154**:1161–1171.
10. **Ghosh, A., and M. E. Greenberg.** 1995. Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. Neuron **15**:89–103.
11. **Grbavec, D., and S. Stifani.** 1996. Molecular interaction between TLE1 and the carboxyl-terminal domain of HES-1 containing the WRPW motif. Biochem. Biophys. Res. Commun. **223**:701–705.
12. **Grbavec, D., R. Lo, Y. Liu, and S. Stifani.** 1998. Transducin-like Enhancer of split 2, a mammalian homologue of Drosophila Groucho, acts as a transcriptional repressor, interacts with Hairy/Enhancer of split proteins, and is expressed during neuronal development. Eur. J. Biochem. **258**:339–349.
13. **Guillemot, F., L. C. Lo, J. E. Johnson, A. Auerbach, D. J. Anderson, and A. L. Joyner.** 1993. Mammalian *achaete-scute* homolog 1 is required for the early development of olfactory and autonomic neurons. Cell **75**:463–476.
14. **Husain, J., R. Lo, D. Grbavec, and S. Stifani.** 1996. Affinity for the nuclear compartment and expression during cell differentiation implicate phosphorylated Groucho/TLE1 forms of higher molecular mass in nuclear functions. Biochem. J. **317**:523–531.

15. **Ishibashi, M., K. Moriyoshi, Y. Sasai, K. Shiota, S. Nakanishi, and R. Kageyama.** 1994. Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. EMBO J. **13**:1799–1805.
16. **Ishibashi, M., S. L. Ang, K. Shiota, S. Nakanishi, R. Kageyama, and F. Guillemot.** 1995. 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. Genes Dev. **9**:3136–3148.
17. **Johnson, J. E., S. J. Birren, T. Saito, and D. J. Anderson.** 1992. DNA binding and transcriptional regulatory activity of mammalian achaete-scute homologous (MASH) proteins revealed by interaction with a muscle specific enhancer. Proc. Natl. Acad. Sci. USA **89**:3596–3600.
18. **Kageyama, R., and S. Nakanishi.** 1997. Helix-loop-helix factors in growth and differentiation of the vertebrate nervous system. Curr. Opin. Genet. Dev. **7**:659–665.
19. **Koyano-Nakagawa, N., J. Kim, D. Anderson, and C. Kintner.** 2000. Hes6 acts in a positive feedback loop with the neurogenins to promote neuronal differentiation. Development **127**:4203–4216.
20. **Lee, J. C., S. B. Smith, H. Watada, J. Lin, D. Scheel, J. Wang, R. G. Mirmira, and M. S. German.** 2001. Regulation of the pancreatic pro-endocrine gene Neurogenin3. Diabetes **50**:928–936.
21. **Lee, J. E., S. M. Hollenberg, L. Snider, D. L. Turner, N. Lipnick, and H. Weintraub.** 1995. Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science **268**:836–844.
22. **Liu, Z. P., R. L. Galindo, and S. A. Wasserman.** 1997. A role for CKII phosphorylation of the cactus PEST domain in dorsoventral patterning of the Drosophila embryo. Genes Dev. **11**:3413–3422.
23. **McLarren, K. W., R. Lo, D. Grbavec, K. Thirunavukkarasu, G. Karsenty, and S. Stifani.** 2000. The mammalian basic helix loop helix protein HES-1 binds to and modulates the transactivating function of the Runt-related factor Cbfa1. J. Biol. Chem. **275**:530–538.
24. **McLarren, K. W., F. Theriault, and S. Stifani.** 2001. Association with the nuclear matrix and interaction with Groucho and RUNX proteins regulate the transcription repression ability of the basic helix loop helix factor Hes1. J. Biol. Chem. **276**:1578–1584.
25. **Nuthall, H., J. Husain, K. W. McLarren, and S. Stifani.** 2002. Role for Hes1-induced phosphorylation in Groucho-mediated transcriptional repression. Mol. Cell. Biol. **22**:389–399.
26. **Ohtsuka, T., M. Ishibashi, G. Gradwhohl, S. Nakanishi, F. Guillemot., and R. Kageyama.** 1999. *Hes1* and *Hes5* as Notch effectors in mammalian neuronal differentiation. EMBO J. **18**:2196–2207.
27. **Packman, L. C., K. Kubota, J. Parker, and N. J. Gay.** 1997. Casein kinase II phosphorylates Ser468 in the PEST domain of the Drosophila IkappaB homologue cactus. FEBS Lett. **400**:45–50.
28. **Palaparti, A., A. Baratz, and S. Stifani.** 1997. The Groucho/Transducin-like Enhancer of split transcriptional repressors interact with the genetically defined amino-terminal silencing domain of histone H3. J. Biol. Chem. **272**:26604–26610.
29. **Paroush, Z., R. L. Finley, T. Kidd, S. M. Wainwright, P. W. Ingham, R. Brent, and D. Ish-Horowicz.** 1994. Groucho is required for Drosophila neurogeneis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. Cell **79**:805–815.
30. **Pissarra, L., D. Henrique, and A. Duarte.** 2000. Expression of hes6, a new member of the Hairy/Enhancer of split family, in mouse development. Mech. Dev. **95**:275–278.
31. **Rechsteiner, M., and S. W. Rogers.** 1996. PEST sequences and regulation by proteolysis. Trends Biochem. Sci. **21**:267–271.
32. **Sasai, Y., R. Kageyama, Y. Tagawa, R. Shigemoto, and S. Nakanishi.** 1992. Two mammalian helix-loop-helix factors structurally related to Drosophila *hairy* and *Enhancer of split*. Genes Dev. **5**:2620–2634.
33. **Seidah, N., S. Benjannet, L. Wickham, J. Marcinkiewicz, S. Belanger Jasmin, S. Stifani, A. Basak, A. Prat, and M. Chretien.** 2003. The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. Proc. Natl. Acad. Sci. USA **100**:928–933.
34. **Stifani, S., C. M. Blaumueller, N. J. Redhead, R. E. Hill, and S. Artavanis-Tsakonas.** 1992. Human homologs of a Drosophila *Enhancer of split* gene product define a novel family of nuclear proteins. Nat. Genet. **2**:119–127.
35. **Toma, J. G., H. El-Bizri, F. Barnabe-Heider, R. Aloyz, and F. D. Miller.** 2000. Evidence that helix-loop-helix proteins collaborate with retinoblastoma tumor suppressor protein to regulate cortical neurogenesis. J. Neurosci. **20**: 7648–7656.
36. **Tomita, K., M. Ishibashi, K. Nakahara, S. L. Ang, S. Nakanishi, M. Asashima, and R. Kageyama.** 1996. Mammalian *hairy* and *Enhancer of split* homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron **16**:723–734.
37. **Trott, R. L., K. Madhavi, Z. Paroush, and A. P. Bidwai.** 2001. *Drosophila melanogaster* casein kinase II interacts with and phosphorylates the basic helix-loop-helix proteins m5, m7, and m8 derived from the *Enhancer of split* complex. J. Biol. Chem. **276**:2159–2167.
38. **Vasiliauskas, D., and C. Stern.** 2000. Expression of mouse HES-6, a new member of the Hairy/Enhancer of split family of bHLH transcription factors. Mech. Dev. **98**:133–137.
39. **Yao, J., Y. Liu, R. Lo, I. Tretjakoff, A. Peterson, and S. Stifani.** 2000. Disrupted development of the cerebral hemispheres in transgenic mice expressing the mammalian Groucho homologue Transducin-like Enhancer of split 1 in postmitotic neurons. Mech. Dev. **93**:105–115.
40. **Yao, J., E. Lai, and S. Stifani.** 2001. The winged-helix protein brain factor 1 interacts with Groucho and Hes proteins to repress transcription. Mol. Cell. Biol. **21**:1962–1972.

Piechaczyk, M., J.M. Blanchard, L. Marty, C. Dani, F. Panabieres, S. El Sabouty, P. Fort, and P. Jeanteur. 1984. Post-transcriptional regulation of glyceraldehyde-3-phosphate-dehydrogenase gene expression in rat tissues. *Nucleic Acids Res.* 12:6951–6963.

Pissara, L., D. Henrique, and A. Duarte. 2000. Expression of *hes6*, a new member of the Hairy/Enhancer-of-split family, in mouse development. *Mech. Dev.* 95:275–278.

Quong, M.W., M.E. Massari, R. Zwart, and C. Murre. 1993. A new transcriptional-activation motif restricted to a class of helix-loop-helix proteins is functionally conserved in both yeast and mammalian cells. *Mol. Cell. Biol.* 13:792–800.

Rawls, A., M.R. Valdez, W. Zhang, J. Richardson, W.H. Klein, and E.N. Olson. 1998. Overlapping functions of the myogenic bHLH genes MRF4 and MyoD revealed in double mutant mice. *Development.* 125:2349–2358.

Rudnicki, M.A., P.N. Schnegelsberg, R.H. Stead, T. Braun, H.H. Arnold, and R. Jaenisch. 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell.* 75:1351–1359.

Rusconi, J.C., and V. Corbin. 1998. Evidence for a novel Notch pathway required for muscle precursor selection in *Drosophila*. *Mech. Dev.* 79:39–50.

Sasai, Y., R. Kageyama, Y. Tagawa, R. Shigemoto, and S. Nakanishi. 1992. Two mammalian helix-loop-helix factors structurally related to *Drosophila* hairy and Enhancer of split. *Genes Dev.* 6:2620–2634.

Schwarz, J.J., T. Chakraborty, J. Martin, J.M. Zhou, and E.N. Olson. 1992. The basic region of myogenin cooperates with two transcription activation domains to induce muscle-specific transcription. *Mol. Cell. Biol.* 12:266–275.

Shawber, C., D. Nofziger, J.J. Hsieh, C. Lindsell, O. Bogler, D. Hayward, and G. Weinmaster. 1996. Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway. *Development.* 122:3765–3773.

Spicer, D.B., J. Rhee, W.L. Cheung, and A.B. Lassar. 1996. Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. *Science.* 272:1476–1480.

Tamura, K., Y. Taniguchi, S. Minoguchi, T. Sakai, T. Tun, T. Furukawa, and T. Honjo. 1995. Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-Jk/Su(H). *Curr. Biol.* 5:1416–1423.

Tietze, K., N. Oellers, and E. Knust. 1992. Enhancer of split-D. A dominant mutation of *Drosophila*, and its use in the study of functional domains of a helix-loop-helix protein. *Proc. Natl. Acad. Sci. USA.* 89:6152–6156.

Triezenberg, S.J., R.C. Kingsbury, and S.L. McKnight. 1988. Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. *Genes Dev.* 2:718–729.

Vasiliauskas, D., and C.D. Stern. 2000. Expression of mouse HES-6, a new member of the Hairy/Enhancer of split family of bHLH transcription factors. *Mech. Dev.* 98:133–137.

Wang, J.C., M. Waltner-Law, K. Yamada, H. Osawa, S. Stifani, and D.K. Granner. 2000. Transducin-like enhancer of split proteins, the human homologs of *Drosophila* groucho, interact with hepatic nuclear factor 3beta. *J. Biol. Chem.* 275:18418–18423.

Weintraub, H., V.J. Dwarki, I. Verma, R. Davis, S. Hollenberg, L. Snider, A. Lassar, and S.J. Tapscott. 1991. Muscle-specific transcriptional activation by MyoD. *Genes Dev.* 5:1377–1386.

Wilson-Rawls, J., J.D. Molkentin, B.L. Black, and E.N. Olson. 1999. Activated notch inhibits myogenic activity of the MADS-Box transcription factor myocyte enhancer factor 2C. *Mol. Cell. Biol.* 19:2853–2862.

Winston, R.L., and J.M. Gottesfeld. 2000. Rapid identification of key amino-acid-DNA contacts through combinatorial peptide synthesis. *Chem. Biol.* 7:245–251.

Yun, K., and B. Wold. 1996. Skeletal muscle determination and differentiation: story of a core regulatory network and its context. *Curr. Opin. Cell Biol.* 8:877–889.