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.

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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 transcripts 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; Noziger 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/KFB2/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; Noziger 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 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.
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) 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).
HES6 induces myogenesis

(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-
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 containing heterodimers can cooperate with HES1 dimers to further inhibit transcription from N box–containing promoters in an additive manner.
HES6 induces myogenesis | Gao et al. 1167

as a dominant negative mutant to block endogenous HES6 function and prevent myogenesis. We also examined the behavior of cultures maintained in GM at confluence. Control and HES6-Δ-expressing cells retained their single-cell myoblast morphology when maintained in GM for 4 d postconfluence (Fig. 6, d and i), 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 confluence, HES6-expressing cells differentiated into myotubes under the same conditions (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 bHLH1 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 mediates 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; Vasiliev 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; Nozfiger 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 (Nozfiger 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 scere (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/T/myc-His; Invitrogen) using conventional methodology. The HES6Δ mutant lacking the COOH-terminal WRPY 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-hindIII 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/T/myc-His vector. Construct pTLE1–VP16 has been described previously (Wang et al., 2000) and was provided by Dr. D.K. Granner (Vanderbilt University, Nashville, TN).

Yeast two-hybrid protein interaction assay
Yeast cells were cotransfected with pGAT9–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 (Stratagen). Positive interaction was scored based on growth on media lacking tryptophan, leucine, and histidine, supplemented with 20 mM 3-amino triazole, and by testing for expression of the lacZ reporter gene as recommended (Stratagen). Western blot assays to confirm expression of the fusion proteins in yeast were performed using standard protocols (Ausbubel 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 pBEG–GST–TLE1 (McLarren et al., 2000) or pBEG–GST (Mizoguchi 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 (Ausbubel 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 1:2,000,000 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 p5xGAL4US–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 μg of GenePORTER reagent, 0.3 μg of reporter construct (pBACluc 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 μg of the internal control reporter, pCMV–RL (Promega). The total amount of DNA transfected was standardized to 3.0 μg by addition of empty pEBVHis vector. Cells were harvested 48 h after transfection 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 μg of pcmvRL internal control reporter (Promega), 0.2 μg of reporter construct (pBACluc or pActinLUC) (Sasai et al., 1992), and 1.5 μg of empty vector (pcDNA4/T/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 × 10⁶ 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 antimouse epitope antibody (Santa Cruz Biotechnology, Inc.; 1:200 dilution); rinsed, then treated with the secondary rhodamine-conjugated antimouse 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 200X.

RNA expression analysis
Total RNA was isolated using the RNeasy miniprep kit (Qiagen). Probes used were a mouse GAPDH cDNA fragment (Pieczaclcyk 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 32P-dCTP, and MyoR- or GAPDH-specific primers (sequences available on request) were added to a 20 µl 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 semiquanitative 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-HES6Δ. 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 1:50 dilution in PBS. After rinsing the primary antibody, the HRP-conjugated secondary antibody was added. After a 2 min incubation, the cells were rinsed in PBS, fixed in 3% paraformaldehyde in PBS for 40 min, rinsed in PBS, 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.

References
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