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## MyoD Synergizes with the E-Protein HEB $\beta$ To Induce Myogenic Differentiation

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**The MyoD family of basic helix-loop-helix transcription factors function as heterodimers with members of the E-protein family to induce myogenic gene activation. The E-protein HEB is alternatively spliced to generate  $\alpha$  and  $\beta$  isoforms. While the function of these molecules has been studied in other cell types, questions persist regarding the molecular functions of HEB proteins in skeletal muscle. Our data demonstrate that HEB $\alpha$  expression remains unchanged in both myoblasts and myotubes, whereas HEB $\beta$  is upregulated during the early phases of terminal differentiation. Upon induction of differentiation, a MyoD-HEB $\beta$  complex bound the E1 E-box of the myogenin promoter leading to transcriptional activation. Importantly, forced expression of HEB $\beta$  with MyoD synergistically lead to precocious myogenin expression in proliferating myoblasts. However, after differentiation, HEB $\alpha$  and HEB $\beta$  synergized with myogenin, but not MyoD, to activate the myogenin promoter. Specific knockdown of HEB $\beta$  by small interfering RNA in myoblasts blocked differentiation and inhibited induction of myogenin transcription. Therefore, HEB $\alpha$  and HEB $\beta$  play novel and central roles in orchestrating the regulation of myogenic factor activity through myogenic differentiation.**

Basic helix-loop-helix (bHLH)-containing transcription factors play an important role in directing the development of a variety of cellular lineages by regulating expression of a restricted set of tissue-specific target genes (57). The myogenic regulatory factors (MRFs) are a group of muscle-specific bHLH transcription factors, consisting of MyoD, Myf5, myogenin, and MRF4, that control skeletal muscle development (41). Genetic and expression analyses indicate that Myf5 and MyoD act as determination factors that specify muscle progenitor cell identity. Although expression of MyoD or Myf5 commits a cell to the myogenic lineage, expression of MyoD is more effective at initiating differentiation. Myogenin is induced upon differentiation and plays an important role in activating the differentiation program. MRF4 appears to have a role as a determination factor in a subset of myocytes in the early somite and as a differentiation factor in later muscle fibers (24).

Transcription of muscle-specific genes is dependent upon dimer interactions between MRFs and the more ubiquitously expressed E-proteins, a process mediated by the helix-loop-helix motif. The E-protein family includes the E2A gene products (E12/E47), the E2-2 gene products (ITF-2A and 2B), and the HEB gene products (HEB $\alpha$  and HEB $\beta$ ). Dimerization is dependent upon the relative abundance of each transcription

factor and/or the presence of other factors that may potentiate or inhibit dimerization. Therefore, the ability of bHLH factors to homo- or heterodimerize in a variety of combinations raises the hypothesis that regulation of dimer formation is essential for control of muscle-specific gene expression.

The role of E2A in myogenesis was first postulated upon finding that MyoD was able to heterodimerize with E12 and E47 in vitro (34, 35). Moreover, cotransfection of MyoD and E47 is required for E-box transcriptional activity in COS cells (28). However, gene-targeting experiments have indicated that E12 and E47 are not essential for skeletal muscle formation or function (62). We previously demonstrated that C2C12 murine myoblasts and 10T1/2 fibroblasts, which are nonmyogenic yet amenable to myogenic conversion upon expression of MyoD, do not express E12 or E47 protein (43). Conway and colleagues confirmed this result by demonstrating that neither E12 nor E47 is expressed in C2C12 myoblasts or adult skeletal muscle tissue (9).

HEB protein, however, is expressed in 10T1/2 fibroblasts and C2C12 myoblasts (43). HEB is highly expressed in skeletal muscle as well as in the thymus and in B cells and is able to bind to E-boxes in a manner similar to other E-protein family members (21). During embryonic development, HEB is widely expressed in the limb buds, somites, and proliferating neuroblasts. Importantly, expression is highest during the initial stages of differentiation and decreases once cellular differentiation has been established (37). Mice lacking HEB die at 2 weeks of age; however, the characterization of muscle development has yet to be reported (61).

Alternative splicing of the HEB gene generates two isoforms termed HEB $\alpha$  and HEB $\beta$ . The HEB $\beta$  isoform is distinguished

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by insertion of a 24-amino-acid ankyrin-like motif, resulting from inclusion of a 72-bp alternate exon (27). HEB $\beta$  differs from HEB $\alpha$  in DNA binding affinity as well as homo- and heterodimerization properties. Prediction of the domain structure of HEB, based on studies of E12 and E47, reveals that this motif intersects the second putative activation domain (AD2) and may alter one face of the  $\alpha$ -helix present in this domain (18, 32, 33).

To determine if HEB plays a role in specifically regulating MRF transcriptional activity, we asked if HEB $\alpha$  and HEB $\beta$  show evidence of distinct transcriptional partner preferences or exhibit unique regulatory properties during skeletal muscle differentiation. Our experiments establish an essential and novel role for HEB $\beta$  in regulating the switch from *myogenin* repression to *myogenin* activation as an immediate-early step in the myogenic differentiation program. We demonstrate that HEB $\alpha$  and HEB $\beta$  specifically regulate MyoD transcriptional activity during early differentiation and myogenin activity later in differentiation. Furthermore, we show that HEB $\alpha$  and HEB $\beta$  function uniquely on different promoters and, more importantly, function distinctly on the same promoter. Importantly, we demonstrate that specific loss of HEB $\beta$  expression in myoblasts inhibited myogenin expression and fusion of myoblasts during differentiation. Together, the data establish a novel mechanism by which MRF/HEB complexes and isoform switching are critical to activating the terminally differentiated phenotype during skeletal muscle development.

#### MATERIALS AND METHODS

**Cell culture and transfections.** C3H10T1/2 fibroblasts (CCL-226; ATCC) and C2C12 myoblasts (CRL-1772; ATCC) were maintained in growth medium (GM) consisting of Dulbecco's modified eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gemini Bioscience), 0.1 units/ml of penicillin, and 0.1  $\mu$ g/ml of streptomycin (1 $\times$ ; Invitrogen). Transfections were performed using the calcium phosphate method as previously described (43). Briefly, 1 day prior to transfections, cells were plated at  $3.5 \times 10^4$  cells per 60-mm dish (Falcon) for 10T1/2 cells, or  $2.5 \times 10^4$  cells per 60-mm dish for C2C12 cells. Cells were incubated with the calcium phosphate precipitate for 16 h, washed twice with phosphate-buffered saline (PBS) and refed with GM. Twenty-four hours after refeeding, cells were either harvested (GM) or washed once with PBS and refed with differentiation medium (DM; DMEM plus 2% horse serum [Invitrogen] and penicillin/streptomycin) and cultured for an additional 48 h. Transfections were assayed for efficiency by inclusion of a plasmid expressing either  $\beta$ -galactosidase or green fluorescent protein.

Primary myoblasts were isolated as described previously (46) and maintained in Ham's F-10 medium (Invitrogen) supplemented with 20% FBS, 2 $\times$  penicillin/streptomycin, and 12.5 ng/ml basic fibroblast growth factor (Invitrogen). Primary myoblasts were induced to differentiate by washing the cells once with PBS and refeeding them with DMEM supplemented with 5% horse serum and 2 $\times$  penicillin/streptomycin.

**Plasmids.** Plasmids expressing MyoD, Myf5, or myogenin were constructed in pEMSV as previously described (13). Expression plasmids encoding HEB $\alpha$  and HEB $\beta$  were constructed by reverse transcription (RT)-PCR using RNA from differentiating primary myoblasts as a template and primers specific for the N and C termini of murine HEB: F1, 5'-ATGAATCCCCAGCAGCAGCGCAT G-3'; R1, 5'-CCATGGTCAGATGACCCATAGGGTTGGT-3'.

The PCR products were cloned into the EcoRI site of pEMSV. The plasmids encoding ITF-2A and ITF-2B were kind gifts from Ilona Skerjanc. The E12 expression plasmid was a kind gift from Lauren Snider, and the E47 expression plasmid was a kind gift from Cornelius Murre. The 4R-*Luc* plasmid was constructed by cloning the BamHI fragment from 4RtkCAT (55) into the BglII site of the pGL3 promoter reporter vector (Promega). The BamHI fragment from MLC-CAT (14) was also cloned into the BglII site of this vector to obtain the MLC-*Luc*. Myogenin-luciferase (*Mgn-Luc*) and the E-box mutants were constructed as previously described (5). Plasmids encoding deletion mutants of MyoD and the activated mutant of MEK1 were previously described (43). Vec-

tors lacking cDNA inserts or promoter elements were used as controls in the transfections.

**RNA isolation.** C2C12 myoblasts and 10T1/2 fibroblasts were grown to approximately 85 to 90% confluence in 60-mm dishes and either harvested as a GM sample or washed once with PBS and refed with DM. The cells were then harvested at various time points after being refed with DM. RNA was isolated according to manufacturer's instructions using the RNeasy mini kit (QIAGEN). The RNA was quantitated, and 5  $\mu$ g was run on a formaldehyde gel to ensure integrity.

**RT-PCR.** RT-PCR was carried out using the RNA PCR core kit (Applied Biosystems). Briefly, 1  $\mu$ g of RNA was reverse transcribed for 1 h at 42°C using random hexamers. One-eighth of the reaction mixture was used in a PCR (20 cycles) with primers specific for  $\beta$ -actin (forward, 5'-TGAGACCTCAACACC CCAG-3'; reverse, 5'-GAGCCAGAGCAGTAATCTCC-3') to ensure proper quantitation of the RNA. One-fourth of the reaction mixture was used in a PCR (30 cycles) with primers specific for HEB or E2A: HEB forward, 5'-GATCTC CTTCACCTCTCACAG-3'; HEB reverse, 5'-TAGTAGGCAGACTGGTAGA AG-3'. The primers for E2A were specific for the bHLH: E12 forward, 5'-CC AGACGAGCAGGAGCAGC-3'; E47 forward, 5'-AGTACAGATGAGGT GCTGTCC-3'; E2A reverse, 5'-ACCACGCCAGACACCTTCTCC-3'. The PCRs were optimized to ensure that amplification was within the linear range.

**Protein extraction.** Cells were washed twice with PBS, scraped with NP-40 lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1% NP-40, 1.5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 10 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM Na<sub>3</sub>VO<sub>4</sub>, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml pepstatin, and 1  $\mu$ g/ml leupeptin), and transferred to a 1.5-ml Eppendorf tube. The cells were incubated on ice for 30 to 45 min with periodic vortexing, and the lysates cleared by centrifugation. Protein concentration was determined using the modified Bradford assay (Bio-Rad) using bovine serum albumin as a standard.

**Immunoblotting and antibodies.** Western blot analysis was performed as described previously (43). The antibodies used were anti-HEB (A-20; Santa Cruz), anti-MyoD (C-20; Santa Cruz), anti-myogenin (F5D; Developmental Studies Hybridoma Bank), anti-Myf5 (C-20; Santa Cruz), anti-E2A (V-18; Santa Cruz), anti-tubulin (DM1A; Sigma), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ambion). For immunoblotting, all antibodies were used according to the manufacturer's instructions, and anti-myogenin was used at 1:10 (hybridoma supernatant). Anti-mouse and anti-rabbit secondary antibodies were used at 1:2,000 (Bio-Rad) and were detected using enhanced chemiluminescence (Amersham-Pharmacia).

**Chromatin immunoprecipitation assays.** For each immunoprecipitation, the protein and DNA in approximately  $1 \times 10^6$  C2C12 myoblasts/myotubes or  $1.5 \times 10^6$  transfected 10T1/2 fibroblasts were cross-linked by the addition of formaldehyde directly to the culture medium to a final concentration of 1% and incubated for 10 min at 37°C. The cells were washed twice with ice-cold PBS containing protease inhibitors (PBS+; 1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin A). The cells were lysed with 200  $\mu$ l of sodium dodecyl sulfate lysis buffer (Upstate), and the samples from the same cell type were pooled and redidged so each immunoprecipitation represented the same amount of starting material. The cross-linked protein-DNA complexes were isolated according to the manufacturer's instructions (chromatin immunoprecipitation [ChIP] assay kit; Upstate). For input DNA, 50  $\mu$ l of sample was removed prior to the immunoprecipitation (IP), the cross-links were reversed as instructed in the kit protocol, and the DNA was purified using the PCR purification kit (QIAGEN). The amount of input DNA was quantitated using the PicoGreen double-stranded DNA quantitation kit (Molecular Probes).

The antibodies used to immunoprecipitate the complexes were all supplied by Santa Cruz (anti-MyoD [C-20], anti-HEB [A-20], anti-Myf5 [C-20], and anti-myogenin [M-225]) and used at 1  $\mu$ g per IP. After the IP, the immunoprecipitated complexes were washed according to the manufacturer's protocol, the cross-links were reversed, and the DNA was purified.

Input DNA (8.2 ng) from 10T1/2 samples and 1.2 ng of input DNA from C2C12 samples were used in a 25-cycle PCR. The amount of immunoprecipitated DNA used for each PCR was determined based on the concentration of input DNA. PCRs were optimized to ensure that amplification was within the linear range, and the primers used were previously described (3).

**Reporter assays.** Transfected cells were washed twice with PBS and scraped with 1 $\times$  reporter lysis buffer (Promega). The cells were incubated on ice for 30 min with periodic vortexing, followed by one round of freeze-thaw lysis. The extracts were centrifuged and the supernatant transferred to a new tube. Luciferase activity was assayed using 10 to 15  $\mu$ l of extract and the luciferase assay system (Promega). The protein concentration was determined using the modified Bradford assay (Bio-Rad), and the relative light units (RLUs) from the luciferase assay were normalized using protein concentration. Relative activity was deter-

mined by setting the RLU for the reporter vector-alone sample as 1. Synergy is defined as

$$\text{synergy} = \frac{\text{RLUs(MRF + HEB)}}{\text{RLUs(MRF)} + \text{RLUs(HEB)}}$$

where the RLUs represent luciferase activity normalized to protein concentration.

**siRNA.** HEB target sequences were chosen from a group of possible sequences from the small interfering RNA (siRNA) target finder website (Ambion). The following DNA oligonucleotides were synthesized (Alpha DNA): no. 15, 5'-AAT GAC AGT CGA TTA GGA ACC CCTGTCTC-3' (antisense), 5'-AA GGT TCC TAA TCG ACT GTC A CCTGTCTC-3' (sense); no. 36, 5'-AAT CTC CTA GTT ACC CAT CTC CCTGTCTC-3' (antisense), 5'-AA GAG ATG GGT AAC TAG GAG A CCTGTCTC-3' (sense); no. 67, 5'-AAT GCA TCA ATT GGA AAC CTC CCTGTCTC-3' (antisense), 5'-AA GAG GTT TCC AAT TGA TGC A CCTGTCTC-3' (sense); no. 60 5'-AAC TTC ACG AGC ATT TGC AAG CCTGTCTC-3' (antisense), 5'-AA CTT GCA AAT GCT CGT GAA G CCTGTCTC-3' (sense). siRNA molecules were constructed using the *Silencer* siRNA construction kit (Ambion). GAPDH siRNA was synthesized using DNA oligonucleotides provided by Ambion and served as a positive control. The siRNA was labeled with Cy3 using the *Silencer* siRNA labeling kit (Ambion) to permit visualization of transfected cells.

C2C12 myoblasts were plated at  $1 \times 10^4$  cells per 35-mm plate. The following day, the cells were transfected with 18.75 pmol of siRNA using Oligofectamine (Invitrogen) and cultured for 2 days. The cells were washed once with PBS, refed with DM without penicillin/streptomycin, and cultured for 48 or 96 h.

**Immunocytochemistry.** Cells were fixed with 4% paraformaldehyde-PBS for 5 min at room temperature, and the fixation was stopped with 100 mM glycine-PBS for 5 min. The cells were washed three times with PBS and permeabilized in 0.3% Triton X-100-PBS for 5 min, followed by another three washes in PBS. The cells were blocked in 10% goat serum-PBS for 1 h, followed by incubation for 1 h with primary antibody (F5D [anti-myogenin] or MF20 [anti-myosin heavy chain], 1:20 in 10% goat serum-PBS; Developmental Studies Hybridoma Bank). The cells were then washed three times in PBS and incubated with fluorescein isothiocyanate-conjugated anti-mouse secondary (1:200 in 10% goat serum-PBS; Chemicon) for 1 h. The cells were washed three times in PBS, and nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI; 0.25 μg/ml in PBS) for 5 min. The cells were washed once in PBS and mounted with fluorescence mounting medium (DAKO).

**RESULTS**

**Induction of HEBβ during myogenic differentiation.** To examine the expression pattern of HEB and E2A during myogenic differentiation, Western blots were performed using extracts from C2C12 myoblasts, primary murine myoblasts, and 10T1/2 fibroblasts. C2C12 myoblasts and primary myoblasts were grown to 85 to 90% confluence and either harvested for protein isolation (GM; DMEM plus 10% FBS) or switched to differentiation medium (DM; DMEM plus 2% horse serum). The cells were harvested and the protein isolated daily over a 4-day period after stimulation with DM. Western blot analysis of protein extracts using an antibody that recognized both isoforms of HEB demonstrated that HEB was abundantly expressed in all three cell types (Fig. 1A). However, expression of HEB protein was almost undetectable by 4 days after the onset of differentiation in myoblasts, suggesting that stability of HEB protein, or translation of the message, is regulated during myogenic differentiation (Fig. 1A).

Western analysis revealed readily detectable E12 and E47 protein in tibialis anterior muscle (Fig. 1A, lane 1), transfected fibroblasts (Fig. 1A, lanes 2 and 3), and adult mouse mammary tissue (not shown). Strikingly, expression of E12 and E47 was below the limit of detection in C2C12 myoblasts, primary myoblasts, and 10T1/2 fibroblasts (Fig. 1A, lanes 4 to 14). Therefore, the low abundance of E12 and E47 and high expression of

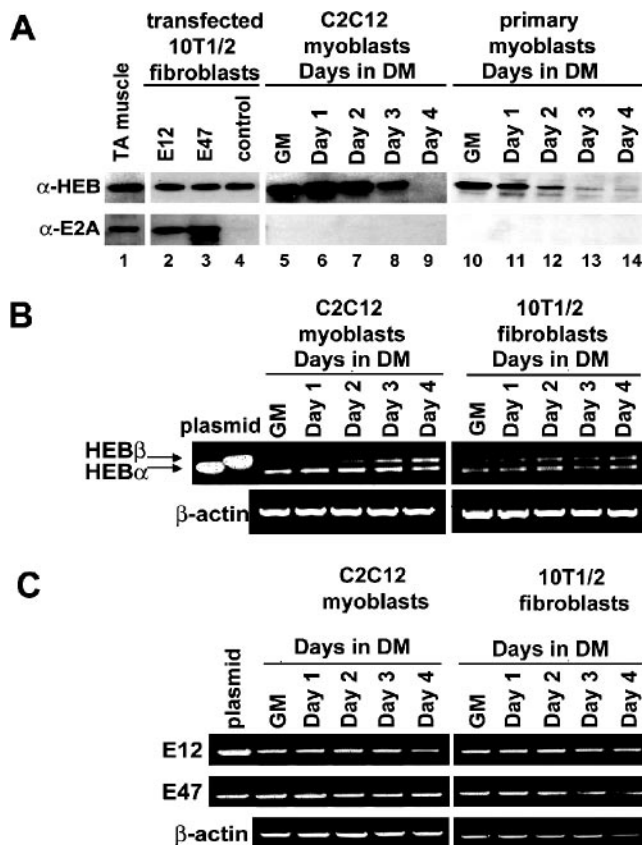


FIG. 1. HEB expression is highly regulated in proliferating and differentiating myoblasts. (A) Western analysis of HEB and E2A expression reveals abundant HEB protein but no detectable E2A protein in myoblasts and fibroblasts. (B) RNA was isolated from a differentiation time course of C2C12 myoblasts and 10T1/2 fibroblasts and analyzed by RT-PCR. *HEBα* was expressed under both growth and differentiation conditions in C2C12 cells, whereas *HEBβ* was induced upon differentiation. (C) RT-PCR detection of *E12* and *E47* mRNAs in C2C12 and 10T1/2 cells. α-HEB, anti-HEB; α-E2A, anti-E2A; TA, tibialis anterior muscle.

HEB proteins suggested that HEB is a significant transcriptional partner of the MyoD family of bHLH factors in myogenic cells.

To investigate the expression pattern of the two isoforms of HEB in myogenic differentiation, RT-PCR was performed using RNA isolated from a differentiation time course of C2C12 myoblasts and 10T1/2 fibroblasts (Fig. 1B). C2C12 myoblasts and 10T1/2 fibroblasts were grown to 85 to 90% confluence and either harvested for RNA isolation (GM; DMEM plus 10% FBS) or switched to differentiation medium (DM; DMEM plus 2% horse serum). The cells were harvested and the RNA isolated daily over a 4-day period after stimulation with DM.

To distinguish HEBα from HEBβ, PCR primers were designed that flanked the region surrounding the 72-bp ankyrin-like motif. RT-PCR analysis indicated that expression of HEBα mRNA remained constant throughout both proliferation and differentiation in C2C12 myoblasts (Fig. 1B) and primary myoblasts (not shown). By contrast, expression of HEBβ was induced upon differentiation with increased expression evident as



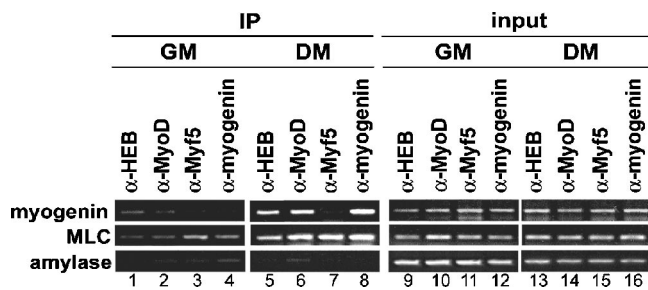


FIG. 2. HEB binds muscle-specific promoters. ChIP revealed that binding of HEB to the *myogenin* promoter and *MLC* enhancer increased markedly after differentiation. Amylase was amplified as a control for specificity, and input DNA was amplified with each primer set to ensure proper DNA quantitation.  $\alpha$ -HEB, anti-HEB;  $\alpha$ -MyoD, anti-MyoD;  $\alpha$ -Myf5, anti-Myf5;  $\alpha$ -myogenin, antimyogenin.

differentiation proceeded (Fig. 1B). Notably, 10T1/2 fibroblasts displayed no modulation in HEB isoform expression in high-versus low-mitogen conditions or upon expression of MyoD (Fig. 1B; data not shown). Furthermore, levels of E12 and E47 mRNAs, as well as ITF-2A and ITF-2B mRNAs, remained unaltered in myoblasts through differentiation or in 10T1/2 fibroblasts after serum withdrawal (Fig. 1C; data not shown). Thus, the absence of HEB $\beta$  in proliferating myoblasts and its upregulation during myogenic differentiation is consistent with a differentiation-specific role for HEB $\beta$ .

**HEB regulates binding of MyoD to muscle-specific promoters.** To determine if HEB is associated with promoters of myogenicity-specific genes, ChIP assays were performed using either proliferating or differentiating C2C12 myoblasts (Fig. 2). Prior to the immunoprecipitation, a small volume of the extract from each sample was removed, the cross-links reversed, and the DNA purified. This "input" DNA was carefully quantitated, and the amount of IP DNA used in each PCR was determined by normalizing it to the amount of input DNA for each immunoprecipitation. This was to ensure that the amount of DNA used in each IP PCR reflected the same amount of starting material. PCR amplification of the IP DNA was undertaken with primers specific for the *myogenin* promoter and the *MLC* enhancer; primers specific for the amylase promoter served as a negative control (Fig. 2, lanes 1 to 8). The input DNA was also amplified with the same primers to ensure accurate quantitation (Fig. 2, lanes 9 to 16).

In proliferating C2C12 myoblasts, both a HEB-specific antibody and a MyoD-specific antibody coimmunoprecipitated DNA corresponding to the *myogenin* promoter and the *MLC* enhancer but not the *amylase* promoter (Fig. 2, lanes 1 and 2). However, binding of MyoD and HEB to the *myogenin* and *MLC* enhancer promoter appeared minimal, even though both MyoD and HEB were expressed in proliferating myoblasts (data not shown). Myf5, on the other hand, is associated with the *MLC* enhancer during proliferation but is not associated with the *myogenin* promoter (Fig. 2, lane 3). A band corresponding to the *MLC* enhancer appears in the PCR amplification of the anti-myogenin ChIP DNA, but it is not enriched for relative to the amylase-negative control, indicating that myogenin is not associated with the enhancer in proliferation (Fig. 2, lane 4). This is consistent with myogenin expression

being restricted to differentiating C2C12 myoblasts (data not shown).

After 2 days in differentiation medium, MyoD, myogenin, and HEB were all associated with the *myogenin* promoter, yet Myf5 was conspicuously absent (Fig. 2A, lanes 5 to 8). In a similar manner, binding of MyoD, myogenin, and HEB to the *MLC* enhancer was enhanced during differentiation relative to proliferation (Fig. 2, lanes 5, 6, and 8). This was expected since both myogenin and *MLC* are expressed exclusively during differentiation. Interestingly, Myf5 remained associated with the *MLC* enhancer after 2 days in differentiation medium (Fig. 2, lane 7). Neither the *myogenin* promoter nor the *MLC* enhancer could be amplified from anti-E12/E47 ChIP DNA (data not shown). These findings indicate that HEB binds to myogenic E-boxes directly or indirectly in association with MyoD or myogenin.

**HEB $\alpha$  and HEB $\beta$  differentially synergize with MyoD and myogenin in a differentiation-specific manner.** To study the role of HEB in transcriptional activation, the mouse cDNAs for both HEB $\alpha$  and HEB $\beta$  were cloned by RT-PCR using mRNA isolated from differentiating wild-type primary myoblasts. The full-length cDNAs were cloned into the EMSV vector, the same vector from which the MRFs were expressed. MRF transcriptional activity was assessed using reporter vectors containing either a portion of the murine myogenin promoter (*Mgn-Luc*) or rat myosin light chain enhancer (*MLC-Luc*).

To determine the effect of each HEB isoform on each MRF, 10T1/2 fibroblasts were transfected with each reporter vector individually and one of *EMSV-MyoD*, *-Myf5*, *-myogenin*, or *EMSV* control in addition to *EMSV-HEB $\alpha$* , *EMSV-HEB $\beta$* , or *EMSV* alone. To ensure similar transfection efficiency, cells were transfected with a plasmid expressing *lacZ* under control of the simian virus 40 promoter in addition to each combination of plasmids indicated in Fig. 3. The cells were fixed, and examination of *lacZ* expression using 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) demonstrated that the transfection efficiency was similar in all transfection conditions (data not shown). Moreover, each relative activation value, or synergy value, represents the average of results from 9 or 12 samples from 3 or 4 different experiments, and the error bars represent the standard deviations. In proliferation conditions (GM), both HEB $\alpha$  and HEB $\beta$  increased the activity of MyoD on *MLC-Luc* but not Myf5 or myogenin activity (Fig. 3A, GM, Fold Activation panel). In differentiation conditions (DM), only the activity of myogenin was increased, and this was done specifically through HEB $\alpha$  (Fig. 3A, DM, Fold Activation panel).

A potential complication was that two transcription factors, both capable of binding to the E-box sequence, were being assayed. Therefore, to determine if the increase in activity was additive (each transcription factor acting separately to increase activity) or synergistic (the two factors acting cooperatively to increase activity), a value, designated as "synergy" was calculated (32). If the value for synergy was 1, or close to 1, then HEB and the MRF were deemed to be activating expression of the reporter independently of each other. If the value was significantly greater than 1, then the two transcription factors were considered to have functioned cooperatively to activate expression of the reporter (see Materials and Methods for

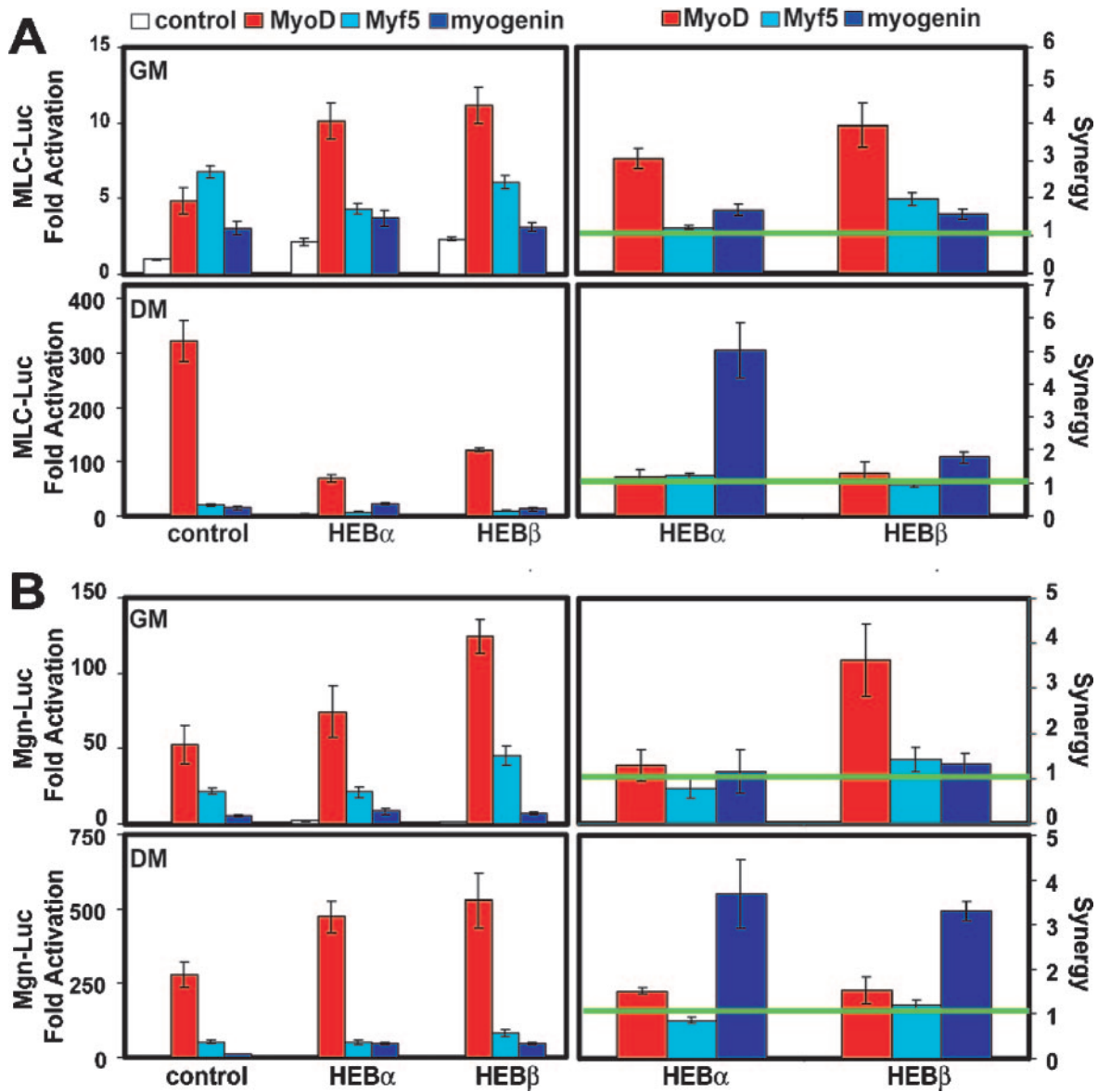


FIG. 3. HEB-dependent increase in MRF activity is synergistic and multifaceted. (A) 10T1/2 fibroblasts were transfected with the *MLC-Luc* reporter plasmid, plasmids expressing MyoD, Myf5, or myogenin, and either HEB $\alpha$  or HEB $\beta$  expression plasmids. The panels on the left represent relative activation. The activity of the *MLC-Luc* reporter plasmid alone was arbitrarily set at 1, and the activity in the presence of the effectors was determined relative to this value (fold activation). The bars represent the mean relative activation, and the error bars represent standard deviations ( $n = 9$ ). The panels on the right represent synergy (see Materials and Methods). The horizontal line represents the value at which the two effectors function independently or additively. The bars represent the mean synergy value, and the error bars represent standard deviations ( $n = 9$ ). (B) The same transfection as for panel A but with the *Mgn-Luc* reporter plasmid.

mathematical formula for synergy). The important distinction is that synergy quantitatively represents the ability of two transcription factors to increase transcriptional activation of a reporter as partners in a complex or dimer, whereas relative activation simply reflects the increase in activity resulting from the activity of transcription factors that may or may not function cooperatively.

Coexpression of HEB $\alpha$  and MyoD increased activity of the *MLC* enhancer more than threefold (synergy value) relative to the activity of the enhancer in the presence of MyoD and HEB $\alpha$  individually (Fig. 3A, GM, Synergy panel). HEB $\beta$  also

acted synergistically with MyoD in proliferation conditions to activate the *MLC* enhancer fourfold higher relative to each factor functioning individually (Fig. 3A, GM, Synergy panel). Although the cells were maintained in growth medium, they initiated differentiation, as indicated by expression of endogenous myogenin (Fig. 4A, lanes 4 to 6). This suggested that the increase in activity is representative of the initial stages of differentiation rather than a true proliferative response.

This also suggests that the increase in activity may also be due to HEB in complex with myogenin. However, neither HEB $\alpha$  nor HEB $\beta$  synergistically activated the *MLC* enhancer

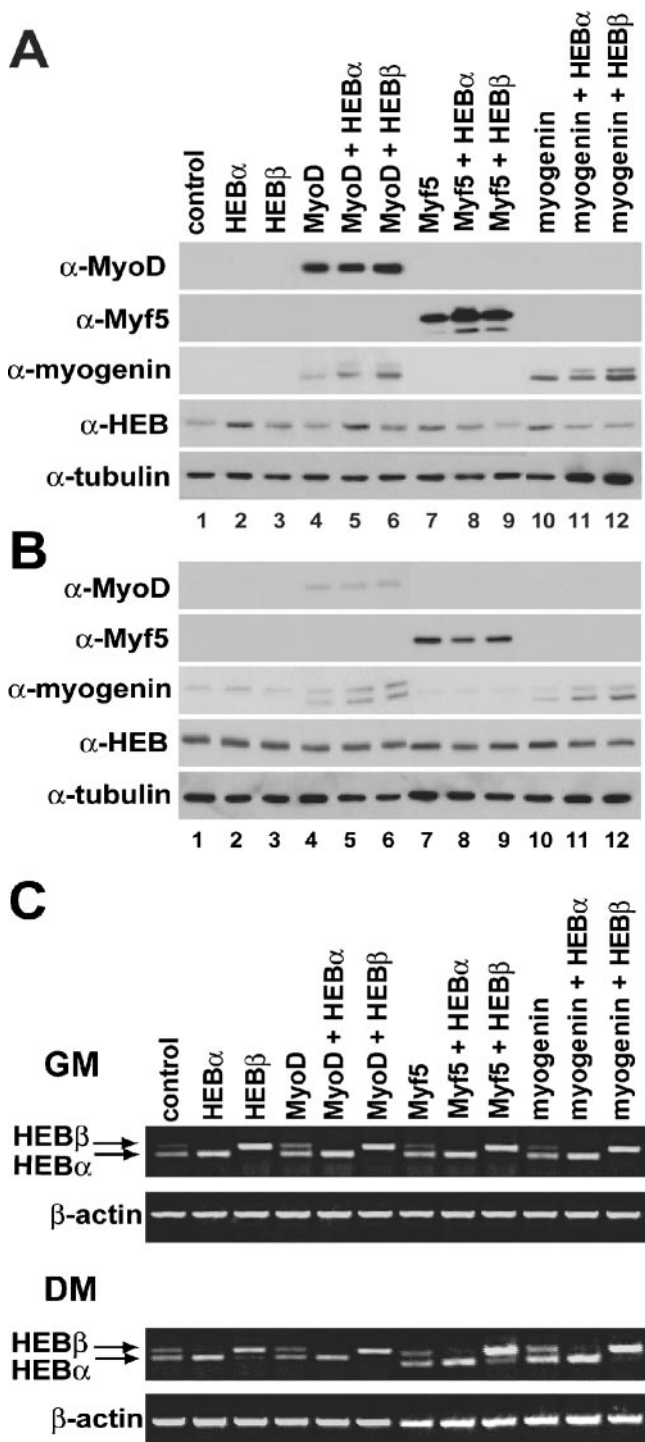


FIG. 4. HEB $\beta$  enhances MyoD-mediated expression of endogenous myogenin. (A) 10T1/2 fibroblasts were transfected as indicated and maintained in growth medium. Protein was isolated, and 25  $\mu$ g was analyzed by Western blotting with the indicated antibodies. The expression of tubulin was monitored as a control for protein quantitation. (B) The same transfection and Western blots as for panel A, but protein was isolated from cells induced to differentiate for 2 days after transfection. (C) The same transfection as for panels A and B, but RNA was isolated and analyzed by RT-PCR. Transfection of HEB $\alpha$  specifically increases levels of HEB $\alpha$ , and transfection of HEB $\beta$  specifically increases levels of HEB $\beta$ .  $\alpha$ -MyoD, anti-MyoD;  $\alpha$ -Myf5, anti-Myf5;  $\alpha$ -myogenin, antimyogenin;  $\alpha$ -HEB, anti-HEB;  $\alpha$ -tubulin, antitubulin.

in the presence of myogenin in GM (Fig. 3A, GM, Synergy panel). Moreover, after 2 days in differentiation medium, HEB $\alpha$  specifically synergized with myogenin and not MyoD to activate the *MLC* enhancer (Fig. 3A, DM, Synergy panel). This corresponded with the results observed in C2C12 myoblasts in which only HEB $\alpha$  activated the *MLC* enhancer (data not shown).

The activity of the *myogenin* promoter (*Mgn-Luc*) was regulated differently. HEB $\beta$  specifically and synergistically activated the *myogenin* promoter in association with MyoD during the initial stage of differentiation (Fig. 3B, GM, Synergy panel). HEB $\alpha$  was unable to synergize with any of the MRFs to activate *Mgn-Luc*. However, in later differentiation, both HEB $\alpha$  and HEB $\beta$  synergized with myogenin and not MyoD to activate the *myogenin* promoter (Fig. 3B, DM, Synergy panel). Given that HEB $\beta$  expression was restricted to differentiating myoblasts (Fig. 1B), these data strongly suggest that HEB $\beta$  plays a role in the initiation of myogenic differentiation by regulating expression of myogenin.

To further validate the data obtained using reporter vector assays, we exogenously expressed the MRFs in 10T1/2 cells either alone or with HEB $\alpha$  or HEB $\beta$ . In agreement with the reporter vector assay results, HEB $\beta$  was able to cooperate with MyoD to enhance expression of endogenous myogenin to a greater extent than HEB $\alpha$  (Fig. 4A, lanes 4 to 6). This was not the result of increased MyoD or Myf5 expression, as MyoD levels remained constant and Myf5 was undetectable in 10T1/2 cells transfected with MyoD. In addition, transfection with Myf5 did not induce expression of myogenin in GM, even when coexpressed with HEB isoforms (Fig. 5A, lanes 7 to 9). This is consistent with the ChIP data, in which Myf5 did not bind the *myogenin* promoter in vivo (Fig. 2A).

Consistent with the transfection data, myogenin-mediated expression of myogenin in serum-starved 10T1/2 fibroblasts was enhanced in the presence of exogenous HEB $\alpha$  and HEB $\beta$  (Fig. 5B, lanes 4 to 6 and 10 to 12). This was not the result of myogenin-induced expression of endogenous MyoD, as MyoD was undetectable by Western blotting (Fig. 4B, lanes 10 to 12). In addition, it was not the result of a HEB-mediated increase in the activity of the *MSV* long terminal repeat, since the expression of *EMSV-MyoD* and *EMSV-Myf5* were not altered in the presence of either HEB $\alpha$  or HEB $\beta$ .

Surprisingly, the level of HEB protein remained constant even after transfection with plasmids encoding HEB $\alpha$  or HEB $\beta$ . Given that the antibody recognizes both isoforms of HEB, we sought to determine if the level of each isoform was altered after transfection. 10T1/2 cells were transfected as indicated on Fig. 4C, and RNA was isolated from cells maintained in GM or induced to differentiate for 2 days (in DM) (Fig. 4C). RT-PCR analysis demonstrated that exogenous expression of HEB $\alpha$  resulted in a predominance of the HEB $\alpha$  isoform and exogenous expression of HEB $\beta$  resulted in a predominance of the HEB $\beta$  isoform in all transfection conditions. Taken together, these data reveal a hitherto unknown role for HEB $\beta$  in directing transcription during initiation of myogenic differentiation.

**Binding to the E1 E-box in the *myogenin* promoter by MyoD-HEB $\beta$  regulates induction of transcription during differentiation.** The observation that HEB $\alpha$  and HEB $\beta$  are able to function in unique ways on the *myogenin* promoter suggested



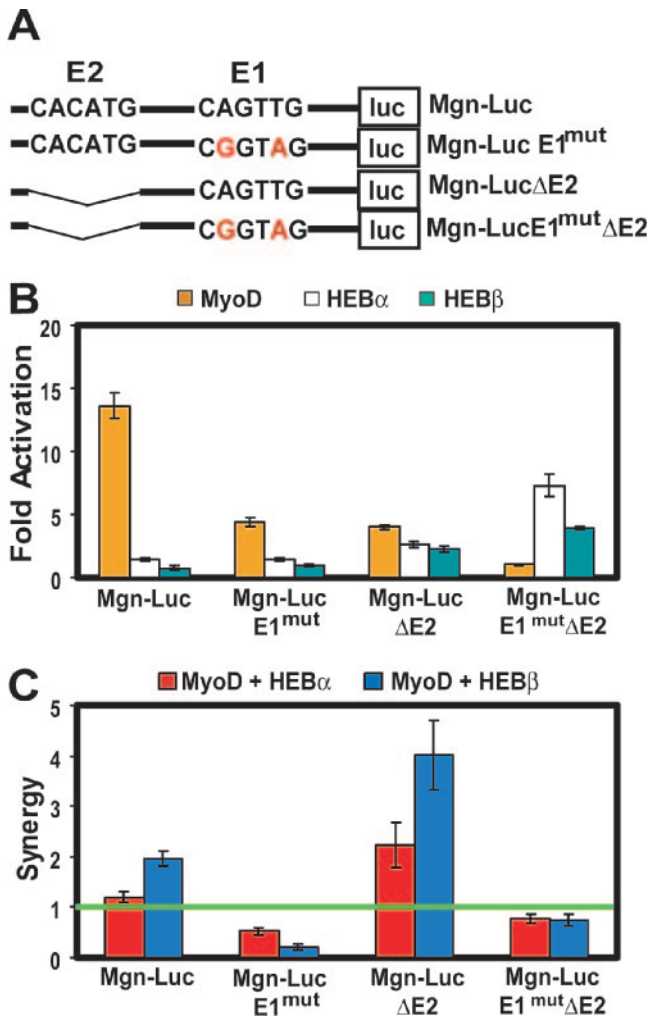


FIG. 5. MyoD activity on the myogenin promoter is E1 E-box specific. (A) Schematic diagram of the E-boxes in the *Mgn-Luc* reporter vector and the mutant reporter vectors in which the E1 E-box has been mutated (*Mgn-LucE1<sup>mut</sup>*), the E2 E-box has been deleted (*Mgn-Luc $\Delta$ E2*), or both (*Mgn-LucE1<sup>mut</sup> $\Delta$ E2*). (B) Activity of *Mgn-Luc* in transfected 10T1/2 fibroblasts. Cells were transfected with either EMSV control, MyoD, HEB $\alpha$ , or HEB $\beta$  in addition to each of the reporter plasmids indicated. The cells were maintained in growth medium, harvested, and assayed for luciferase activity. The bars represent the mean activation relative to the control, and the error bars represent standard deviations ( $n = 3$ ). (C) Synergy between MyoD and HEB isoforms in the activation of *Mgn-Luc* in transfected 10T1/2 fibroblasts. Cells were transfected with combinations of MyoD and HEB $\alpha$  or MyoD and HEB $\beta$ . The horizontal line represents the value at which the two effectors function in an additive manner. The bars represent the mean synergy values, and the error bars represent standard deviations ( $n = 3$ ).

the possibility that specific complexes are being recruited to discrete E-boxes. The 224-bp minimal *myogenin* promoter required to recapitulate myogenin expression in vivo contains two consensus E-box sequences, E1 (CAGTTG) and E2 (CACATG) (Fig. 5A). To determine which complex is regulated by HEB, reporter vectors, in which the E1 and E2 E-boxes are selectively mutated, were employed.

Mutation of the E1 E-box (*Mgn-LucE1<sup>mut</sup>*) or deletion of the E2 box (*Mgn-Luc $\Delta$ E2*) decreased MyoD-mediated activa-

tion of the *myogenin* promoter during early differentiation (Fig. 5B). Moreover, the double mutant reporter vector (*Mgn-LucE1<sup>mut</sup> $\Delta$ E2*) was completely unresponsive to MyoD, demonstrating the importance of these two canonical E-boxes in MyoD-mediated activation of *Mgn-Luc*. By contrast, the ability of HEB $\alpha$  and HEB $\beta$  to activate *Mgn-Luc* transcription in the absence of MyoD was unaffected by mutation of E1 and was increased upon deletion of E2. Furthermore, HEB $\beta$  and, more strikingly, HEB $\alpha$  displayed dramatically increased activity on the double E-box mutant reporter vector (*Mgn-LucE1<sup>mut</sup> $\Delta$ E2*). Interestingly, each canonical E-box is flanked by a non-canonical E-box. Importantly, Berkes and colleagues demonstrate that in vitro-translated MyoD and E12 are able to bind to the noncanonical E-boxes (5). This suggests that HEB $\alpha$  and HEB $\beta$  are able to bind to the noncanonical E-boxes in the myogenin promoter. Moreover, the data also indicate that the canonical E-boxes, E1 and E2, function in two ways—activating *myogenin* transcription in the presence of MyoD and precluding activation of the *myogenin* promoter in the absence of MyoD.

As previously described, synergy reflects the ability of two transcription factors to function cooperatively as a complex. A synergy value of 1 indicates that the two factors function independently, and a value greater than 1 is indicative of cooperation. However, a value less than 1 reflects active repression of the complex. This may involve repression of a complex that is bound to DNA or inhibition of DNA binding. Simply inhibiting the formation of the active complex without affecting DNA binding of each individual factor would result in independent activity (synergy = 1).

In the absence of the E1 E-box, HEB $\alpha$  and HEB $\beta$  functioned as repressors of MyoD activity on the *myogenin* promoter, as evidenced by synergy values of less than 1 (Fig. 5C). Importantly, MyoD-HEB $\beta$  synergy was more dramatically reduced than MyoD-HEB $\alpha$  synergy. In contrast, deletion of the E2 E-box resulted in a robust twofold increase in synergy between MyoD and HEB $\beta$  (Fig. 5C). Both HEB $\alpha$  and HEB $\beta$  were unable to function synergistically with MyoD to activate *Mgn-LucE1<sup>mut</sup> $\Delta$ E2* (Fig. 5C). We interpret these data to suggest that, during initiation of differentiation, a transcriptionally active MyoD-HEB $\beta$  complex binds the E1 E-box to stimulate *myogenin* transcription. Furthermore, the increase in synergy in the absence of the E2 E-box suggests that an unidentified complex, bound to the E2 E-box, negatively regulates the activity of the E1 E-box.

**MEK1 specifically inhibits the MyoD-HEB $\alpha$  complex.** There are a number of defined functional domains within MyoD, including a DNA binding domain (amino acids 102 to 162), a transcriptional activation domain (amino acids 3 to 56), and chromatin remodeling domains (amino acids 63 to 99 and 218 to 269) (12, 15, 49). Deletion of amino acids 3 to 56 (MyoD $\Delta$ 3-56) or 63 to 99 (MyoD $\Delta$ 63-99) abolishes transcriptional activity on a transiently transfected reporter vector and negatively impacts expression of a particular subset of MyoD-regulated genes (5, 49, 56; data not shown). Importantly, mutation of amino acids 96 and 98 (MyoD-W96A/C98A) or serine 253 (MyoD-S253P) alters the expression of a subset of genes distinct from that of MyoD $\Delta$ 3-56 (5). Specifically, full activation of myogenin expression requires these amino acids (Trp96, Cys98, and Ser253), yet these amino acids are not required for



expression of muscle creatine kinase, a gene induced later in myogenic differentiation (5). To investigate if specific regions within either the N or C terminus are required for HEB-mediated synergistic activation of MyoD, deletion mutants of MyoD were cotransfected with either HEB $\alpha$  or HEB $\beta$  and *Mgn-Luc*.

Strikingly, the transcriptional activity of MyoD $\Delta$ 63-99 was not increased by coexpression of HEB $\alpha$  or HEB $\beta$  and was unable to be synergistically activated by either isoform of HEB (Fig. 6A). This domain is implicated in maintaining stable binding of MyoD to the *myogenin* promoter by mediating the formation of a complex with the homeobox-containing protein Pbx1 (5). Our data suggest that amino acids 63 to 99 are also required for HEB $\beta$  to synergize with MyoD and activate the *myogenin* promoter. Conversely, the activity of the *myogenin* promoter in the presence of MyoD $\Delta$ 3-56 and HEB $\beta$  is 10-fold higher than in the presence of MyoD $\Delta$ 3-56 alone. Moreover, MyoD lacking amino acids 3 to 56 (MyoD $\Delta$ 3-56) was fourfold more responsive to HEB $\beta$  than wild-type MyoD, indicating that this region is responsible for negatively regulating the synergistic activity of the MyoD-HEB $\beta$  complex (Fig. 6A).

We have previously demonstrated that MyoD lacking amino acids 3 to 56 (MyoD $\Delta$ 3-56) is unresponsive to MEK1-mediated repression of MyoD activity, whereas deletion of amino acids 170 to 209 (MyoD $\Delta$ 170-209) has no effect (43). Moreover, expression of MyoD lacking amino acids 3 to 56 inhibits formation of a MyoD-MEK1 and HEB-MEK1 complex. However, the antibody that recognizes HEB is unable to distinguish between  $\alpha$  and  $\beta$  isoforms. If deletion of amino acids 3 to 56 increases MyoD-HEB $\beta$  synergy and this region is important for MEK1-mediated repression of MyoD activity, then does MEK1 inhibit MyoD-HEB $\beta$  synergy?

An activated form of MEK1 (ActMEK1) was cotransfected with MyoD, MyoD $\Delta$ 3-56, or MyoD $\Delta$ 170-209 in addition to HEB $\alpha$  or HEB $\beta$  and *Mgn-Luc* (Fig. 6B and C). ActMEK1 dramatically reduced synergy of the MyoD-HEB $\alpha$  complex to a value less than 1, yet it had no effect on a complex containing MyoD $\Delta$ 3-56 and a modest effect on a complex containing MyoD $\Delta$ 170-209 (Fig. 6C). This indicates that ActMEK1 actively represses the MyoD-HEB $\alpha$  complex, and this repression requires amino acids 3 to 56. Activated MEK1 also reduced synergy of the MyoD-HEB $\beta$  complex, but it did so to a value close to 1, indicating that the two transcription factors were functioning independently in the presence of activated MEK1 (Fig. 6D). Although it is possible that MEK1 inhibits formation of an active complex containing MyoD and HEB $\beta$ , the data also suggest that activated MEK1 represses a MyoD-HEB $\alpha$  complex specifically. This is consistent with the fact that activated MEK1 is present during myoblast proliferation, at which point HEB $\alpha$ , but not HEB $\beta$ , is expressed (43) (Fig. 1).

**Knockdown of HEB $\beta$  inhibits differentiation.** Our experiments indicate that, during initiation of myogenic differentiation, MyoD and HEB $\beta$  synergistically activate *myogenin* transcription. These data therefore suggest that induction of HEB $\beta$  in myoblasts acts as a switch that permits progression through the immediate early differentiation program. To investigate the effect of loss of HEB expression on myogenic differentiation, C2C12 myoblasts were transfected with siRNAs designed specifically for HEB (no. 15, 36, 67, and 60; see Materials and Methods for specific sequences) and assessed for myogenin

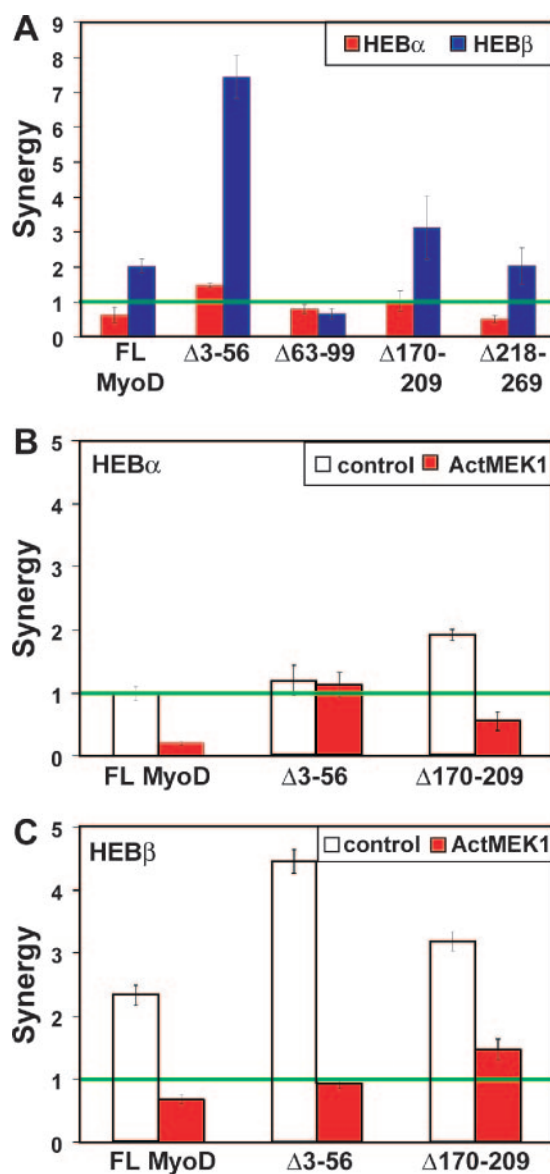


FIG. 6. MEK1 specifically inhibits the MyoD-HEB $\alpha$  complex. (A) 10T1/2 fibroblasts were transfected with the *Mgn-Luc* reporter vector, plasmids expressing either full-length (FL) MyoD or deletion mutants of MyoD in addition to HEB $\alpha$  or HEB $\beta$ . The bars represent the mean synergy values, and the error bars represent standard errors of the means ( $n = 8$ ). The green horizontal line represents the value at which the two effectors function independently or additively. (B) The same transfection as for panel A but including either empty vector or plasmid expressing activated MEK1. The bars represent the mean synergy values, and the error bars represent standard errors of the means ( $n = 8$ ). The green horizontal line represents the value at which MyoD and HEB $\alpha$  function independently or additively. (C) The same transfection as for panel B but with HEB $\beta$  instead of HEB $\alpha$ .

expression 48 h after stimulation with differentiation medium (Fig. 7). Typically, transient transfection resulted in about 10 to 12% of cells transfected, as judged by Cy3-labeled siRNA uptake.

Transfection of GAPDH-specific siRNA effectively inhibited expression of GAPDH protein as demonstrated by Western blot analysis (Fig. 7A). This indicated that a dramatically

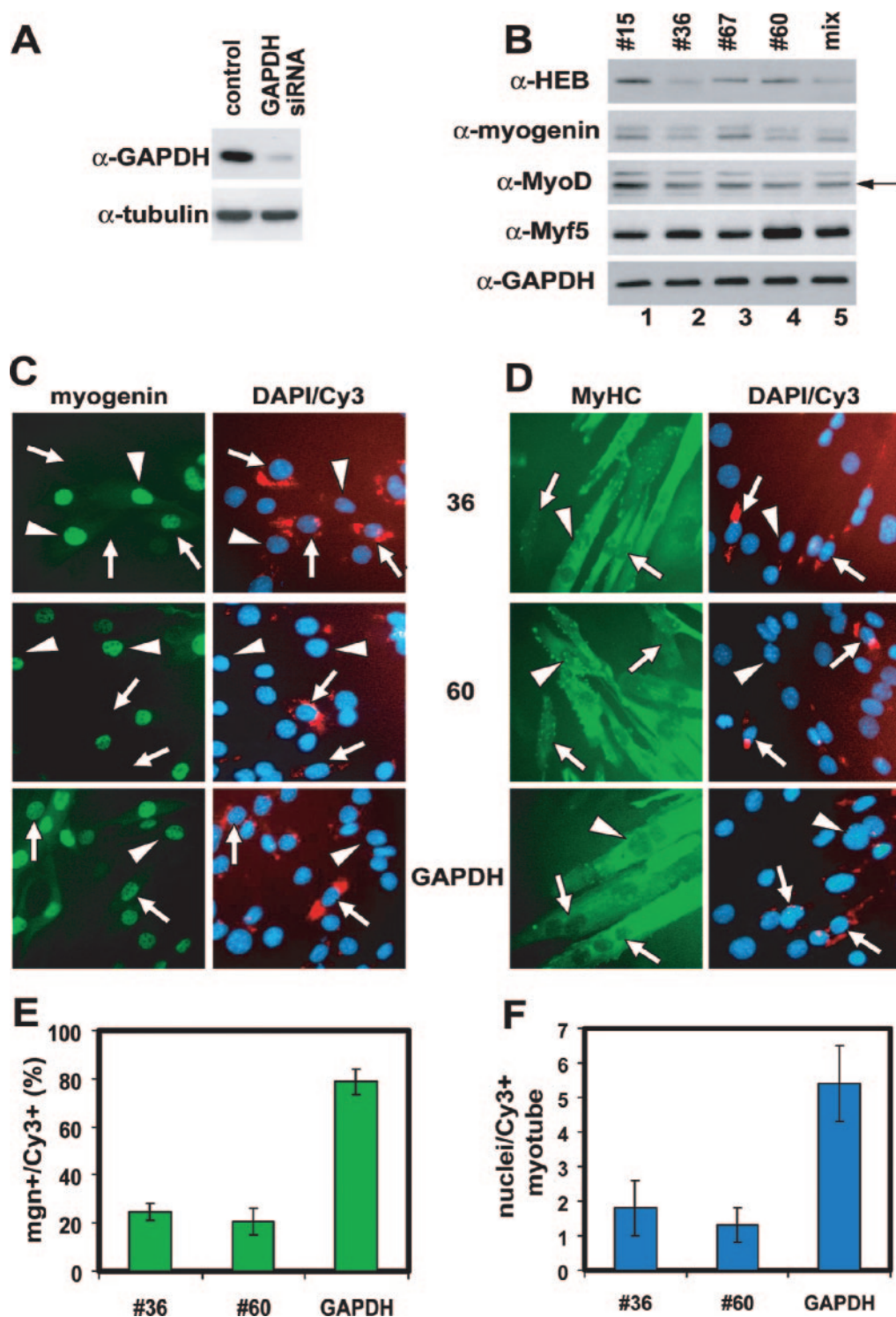


FIG. 7. Knockdown of HEB expression inhibits induction of myogenin and differentiation. (A) Western analysis of C2C12 myoblasts transfected with GAPDH siRNA. Note the reduced levels of GAPDH protein only in cells transfected with siRNA specific for GAPDH. The expression of tubulin was monitored as a control for protein quantitation. (B) Western analysis of C2C12 cells transfected with HEB-specific siRNAs and then induced to differentiate for 48 h. Note the reduced level of HEB and myogenin protein present in cells transfected with siRNAs specific for HEB $\alpha$ / $\beta$  (no. 35, mix) and HEB $\beta$  (no. 60). HEB siRNA no. 15 did not reduce HEB expression. (C and D) Immunofluorescent detection of myogenin and MyHC in siRNA-transfected C2C12 cells. Cells were fixed either after 48 h of differentiation for detection of myogenin (C) or after 96 h for detection of MyHC (D). Nuclei were counterstained with DAPI. Note the inhibition in myogenin induction (C) and delayed formation of multinucleated myotubes (D) after HEB $\alpha$ / $\beta$  (no. 36) and HEB $\beta$  (no. 60) induced knockdown of HEB expression. (E) Quantitation of the results in panel C. The bars represent the average percentages of cells positive for both myogenin and siRNA (Cy3) per field of view (magnification,  $\times 10$ ). The error bars represent standard deviations ( $n = 4$  fields). (F) Quantitation of the results in panel D. The bars represent the average number of nuclei per siRNA-positive myotubes, and the error bars represent standard deviations ( $n = 25$  myotubes).  $\alpha$ -HEB, anti-HEB;  $\alpha$ -myogenin, antimyogenin;  $\alpha$ -MyoD, anti-MyoD;  $\alpha$ -Myf5, anti-Myf5;  $\alpha$ -GAPDH, anti-GAPDH.

higher percentage of cells was transfected than indicated by Cy3 staining. This is likely due to insufficient brightness of the fluorescent marker at a low siRNA concentration. Transfecting a higher concentration of Cy3-labeled GAPDH- or HEB-specific siRNA produced over 90% Cy3-positive cells; however, a number of the cells quickly detached from the plate and appeared to undergo apoptosis (data not shown).

Transfection of C2C12 myoblasts with pan-HEB-specific siRNA no. 36 either alone or in combination with the other HEB-specific siRNAs (mix) resulted in a high-level inhibition of HEB expression given the overall apparent transfection efficiency, again suggesting a higher percentage of cells transfected than indicated by Cy3 fluorescence (Fig. 7B, lanes 2 and 5). Importantly, siRNA no. 36 knockdown of HEB $\alpha$ / $\beta$  expression resulted in markedly reduced myogenin expression despite a similar level of MyoD expression compared to siRNA no. 67 (Fig. 7B, compare lanes 2 and 3). Importantly, siRNA no. 60, designed to specifically bind HEB $\beta$  and not HEB $\alpha$ , inhibited myogenin expression comparable to siRNA no. 36, consistent with the notion that MyoD and HEB $\beta$  are required for initiation of differentiation (Fig. 7B, compare lanes 2 and 4). Interestingly, siRNA no. 60 also induced an increase in Myf5 expression.

Although a dramatically higher percentage of cells was transfected than indicated by Cy3 fluorescence, cells positive for Cy3 can be considered definitively transfected with siRNA. The apparent transfection efficiency for each siRNA, while the cells were still in GM, was approximately equal, with 9.8% of cells transfected with GAPDH siRNA, 12.8% with siRNA no. 36, and 12.4% with siRNA no. 60. As demonstrated in Fig. 7C, C2C12 cells transfected with high levels of siRNA no. 36 and no. 60 exhibited an inability to upregulate endogenous myogenin (Fig. 7C) relative to some of the potentially untransfected cells (Fig. 7C). By contrast, cells transfected with GAPDH siRNA expressed normal levels of myogenin (Fig. 7C). Myogenin was expressed in 78.7% cells transfected with GAPDH siRNA but was detected in only 24.6% of cells transfected with HEB siRNA no. 36 and 20.5% of cells transfected with HEB $\beta$ -specific siRNA no. 60 (Fig. 7E).

During the process of myogenic differentiation, myoblasts must exit from the cell cycle and regulate expression of a large number of genes, including transcription factors, such as myogenin, and structural proteins, such as myosin heavy chain (MyHC) and myosin light chain (MLC). Moreover, the differentiating myoblasts must fuse to form multinucleated myotubes. After 4 days in DM, GAPDH siRNA-transfected and untransfected C2C12 cells displayed normal differentiation kinetics, as evidenced by the presence of multiple nuclei and MyHC expression (Fig. 7D). By contrast, cells transfected with HEB-specific siRNAs displayed an overall delay in differentiation as judged by fusion index (Fig. 7D, arrows). For example, GAPDH siRNA-transfected cells contained on average 5.4 nuclei per myotube, whereas cells transfected with HEB siRNA no. 36 exhibited an average of 1.8 nuclei per myotube (Fig. 7F). Moreover, those transfected with HEB $\beta$ -specific siRNA no. 60 displayed an average of 1.3 nuclei per myotube. Together, these data confirm that HEB $\beta$  is required for the switch from myoblast proliferation to myogenic differentiation.

## DISCUSSION

Initiation of myogenic differentiation is characterized by cell cycle withdrawal, stimulation of MyoD transcriptional activity, and induction of myogenin expression. Cell cycle stimulatory molecules, such as E2F1, inhibit expression of myogenin and prevent myogenic differentiation (11, 16, 20, 47, 52, 53). Similarly, mitogenic factors, such as fibroblast growth factor, transforming growth factor  $\beta$ , and MEK1, also negatively regulate expression of myogenin (29, 43). Conversely, expression of myogenin is positively regulated by insulin-like growth factor 1 (IGF-1), specifically through activation of phosphatidylinositol 3-kinase (10, 58, 59). The MEF2 family of transcription factors also stimulate myogenin expression and myogenic differentiation, and are thought to participate in the formation of a complex with the MRFs and their E-protein heterodimerization partners (6, 7, 36, 39, 40, 44, 48, 51, 60).

However, this simplistic model does not account for the possibility that each myogenic gene, or group of genes, may be regulated distinctively. Recent studies have shown that MyoD directs multiple subprograms of gene expression, each of which is uniquely regulated (3). For example, activation of a subset of late-activated MyoD target genes requires p38 mitogen-activate protein kinase, whereas expression of an early target, myogenin, requires formation of a MyoD-Pbx1 complex (5, 42). Genes expressed early in differentiation, such as myogenin, are induced primarily by MyoD (8). In contrast, MyoD initiates regional histone modification at late-expressed targets, such as MyHC and MLC; however, full expression requires myogenin transcriptional activity.

E12 and E47 have been proposed to function as the E-protein heterodimerization partners for the MRFs, thereby enhancing activation of muscle-specific genes (28). Our experiments surprisingly demonstrated that while C2C12 myoblasts and primary myoblasts expressed E12/E47 mRNA, the level of E2A protein was below the limit of detection by Western and CHIP analysis using several commercial preparations of antibody. Furthermore, ITF-2 protein was undetectable at any point during our myoblast differentiation time course (data not shown). However, a recent proteomic study of C2C12 myoblast differentiation found that ITF-2 protein was detectable beginning 6 days after initiation of differentiation, at which point HEB protein is no longer detectable (26). We also observed a decline in HEB protein levels 4 days after initiation of differentiation, even though the mRNAs for both HEB $\alpha$  and HEB $\beta$  were present (Fig. 1). Taken together, this suggests that E-proteins are targeted for degradation or that translation of the mRNA is specifically inhibited.

Notch signaling induces E47 ubiquitination and degradation by inducing formation of an E47-CHIP complex (22, 38). CHIP, a cochaperone protein with ubiquitin ligase activity, promotes the association of E47 with Skp2 and Cul1, resulting in ubiquitination of E47 and eventual degradation. CHIP is highly expressed in skeletal muscle (2) and interacts with specific amino acids in EHD2 (E-protein homology domain) and EHD3 (22), which are highly conserved between E-protein family members. Specifically, 81% of amino acids in these regions of E2A and HEB are identical, including two serines within EHD2 and a tryptophan in EHD3, which are required for the E2A-CHIP interaction. This suggests that HEB and



E2A may share similar mechanisms of targeted protein degradation. However, the 24-amino acid insert specific to HEB $\beta$ , not found in HEB $\alpha$  or other E-protein family members, lies 18 amino acids C-terminal of EHD3, introducing the possibility that this domain may interfere with the CHIP interaction and targeted degradation of HEB $\beta$  specifically.

Moreover, E2A degradation is induced by interaction of amino acids 476 to 494 and 505 to 513 of E2A with mUbc9 (23, 25, 30), a ubiquitin-conjugating enzyme also expressed in skeletal muscle (17, 19). Deletion of amino acids 476 to 494 or 505 to 413 dramatically stabilizes E2A protein. Importantly, this region of E2A is not conserved within HEB—only 5 of the 28 amino acids in the corresponding region of HEB are identical to those of E2A, even though amino acids N-terminal and C-terminal to this region are highly conserved between the two E-proteins. Therefore, it is possible that HEB and E2A protein stability is regulated by distinct mechanisms, causing E2A degradation and allowing HEB protein stability within the same cell.

In C2C12 myoblasts and 10T1/2 fibroblasts expressing MyoD or Myf5, neither E2A (E12 or E47) nor ITF-2 (ITF-2A or ITF-2B) augmented the activity of *MLC-Luc* or *4R-Luc* (data not shown). By contrast, HEB is abundantly expressed in primary and C2C12 myoblasts and synergistically enhances MyoD and myogenin activity on *MLC-Luc* and *Mgn-Luc* (Fig. 3) (9, 43). Taken together, these data provide a compelling argument that, in myoblasts and early differentiation, HEB serves as the E-protein partner of the MRFs.

Determining the precise role of HEB in myogenic gene transcription is complicated by the presence of two isoforms, HEB $\alpha$  and HEB $\beta$ . Our experiments indicate that differentiation-dependent alternative splicing of *HEB* plays a hitherto unappreciated regulatory role during myogenic differentiation. While *HEB $\alpha$*  is expressed in both proliferating and differentiating myoblasts, *HEB $\beta$*  expression is upregulated exclusively during myogenic differentiation (Fig. 1B). Knockdown experiments demonstrated that HEB $\beta$  is essential for myogenin expression and differentiation into multinucleate myotubes. Loss of HEB did not appear to completely inhibit expression of myosin heavy chain but did alter differentiation, as the average number of nuclei per myotube is considerably reduced in cells positive for HEB siRNA. Moreover, cells transfected with HEB $\beta$ -specific siRNA displayed a higher level of Myf5 expression (Fig. 7, lane 4). This is reminiscent of primary myoblasts lacking *MyoD*, which express more *Myf5* and remain primarily mononuclear after mitogen withdrawal (46). Taken together, this suggests that, in the absence of HEB $\beta$ , the transcriptional activity of MyoD is markedly reduced. Therefore, restricted expression of HEB $\beta$ , which governs formation of a MyoD-HEB $\beta$  complex, serves as an important means of regulating myogenin expression.

Mice lacking HEB $\alpha$  and HEB $\beta$  die perinatally, surviving no longer than 2 weeks after birth (61). These mice display defects in B-cell development but have yet to be analyzed specifically for skeletal muscle development and satellite cell function. An initial analysis of muscle structure and satellite cell function *ex vivo* suggest that loss of HEB $\alpha$  and HEB $\beta$  has profound effects on skeletal muscle development as well as satellite cell survival, proliferation, and differentiation (M. H. Parker and M. A. Rudnicki, unpublished data). However, targeting of both iso-

forms by siRNA results in a reduction in the number of Cy3-positive cells after induction of differentiation, suggesting that loss of both HEB $\alpha$  and HEB $\beta$  affects survival of differentiating myocytes (data not shown). Targeting of HEB $\beta$  alone does not alter the number of Cy3-positive cells after initiation of differentiation relative to the GAPDH control, specifically implicating HEB $\alpha$  in maintaining survival after serum withdrawal. Therefore, to accurately assess the role of HEB $\beta$  *in vivo* and thus validate our results in a murine system, a mouse lacking only HEB $\beta$  would need to be generated.

The ability of HEB to synergize with MyoD and myogenin required the context of a natural promoter, as neither MyoD nor myogenin could be synergistically activated by HEB on the *4R-Luc* reporter plasmid (data not shown). Although this may be the result of differences in core E-box sequence, it is more likely to be indicative of the importance of the context, or the position, of the E-box within a promoter. As such, MyoD-HEB $\beta$  complexes require the E1 E-box of *Mgn-Luc* to transcriptionally activate the *myogenin* promoter (Fig. 5). The E1 E-box is proximal to the TATA box and the initiator (Inr) element of the promoter. Therefore, the ankyrin-like motif of HEB $\beta$  may play a role in recruiting the preinitiation complex to the E1 E-box.

Although transfection of MyoD alone was able to activate a *myogenin* promoter containing only the E2 E-box (*Mgn-LucE1<sup>mut</sup>*), cotransfection of either HEB $\alpha$  or HEB $\beta$  repressed MyoD activity on this reporter construct (Fig. 6C). Moreover, the activity of the E1-complex was regulated through the E2-complex, given that MyoD-HEB $\beta$  synergy was enhanced approximately twofold in the absence of the E2 E-box (Fig. 6C, *Mgn-LucDE2*). One interesting possibility is that HEB $\alpha$  and HEB $\beta$  each participate in the formation of transcriptional complexes with distinct activities. For example, the complex bound to the E2 E-box may be an inhibitory MyoD-HEB $\alpha$  complex, since HEB $\alpha$  is the predominant HEB isoform expressed during myoblast proliferation, and both MyoD and HEB are able to bind the *myogenin* promoter in proliferating cells, yet *myogenin* is not expressed (Fig. 2; also data not shown). Upon initiation of myogenic differentiation, HEB $\beta$  is expressed and an active MyoD-HEB $\beta$  complex binds to the E1 E-box to stimulate transcription of *myogenin*. Reporter assays with an activated MEK1 mutant suggest that active MEK1, present in proliferating cells, further regulates myogenin expression by forming a complex with MyoD-HEB $\alpha$  and inhibiting its activity (Fig. 6) (43).

A number of studies have demonstrated that HEB is able to function both as an activator and a repressor on a given promoter, depending on the presence of other factors (31, 45, 54). In particular, HEB represses activation of the GAP-43 gene in PC12 cells, pending expression of Nex1/MATH2, a member of the neuroD family of bHLH factors (50). Nex1/MATH2 binds to the GAP-43 promoter, alleviates HEB-mediated repression, and activates expression of the gene, resulting in neurite outgrowth and neuronal differentiation. Although the authors do not discriminate between HEB $\alpha$  and HEB $\beta$ , it is interesting to hypothesize that alleviating the repression may involve a switch from a HEB $\alpha$ -specific complex to a HEB $\beta$ -specific complex or vice versa.

The E2 E-box may also bind myogenin to maintain expression of the gene. MyoD and myogenin bind to distinct portions

of the *MLC* enhancer and function cooperatively to fully activate transcription of *MLC* (1). As such, HEB $\alpha$  or HEB $\beta$  may utilize this E-box in association with myogenin in later differentiation to sustain expression of myogenin. During later stages of myogenic differentiation, both HEB $\alpha$  and HEB $\beta$  activated the *myogenin* promoter synergistically and specifically with myogenin (Fig. 3). This observation is consistent with the notion that MyoD plays a central role in regulating the switch from myoblast proliferation to initiation of differentiation. By contrast, myogenin functions to enforce terminal differentiation by enhancing transcriptional activity of differentiation-specific target genes (4, 8). Therefore, MyoD initiates expression of myogenic genes, but once myogenin is expressed, the two MRFs function cooperatively and synergistically with HEB to fully activate transcription.

The ability of HEB to activate *Mgn-LucE1<sup>mut</sup> $\Delta$ E2*, which contains no canonical E-box, is intriguing. It strongly suggests that, in the absence of MyoD, HEB binds a noncanonical E-box to activate the *myogenin* promoter. This is consistent with data demonstrating that in vitro-translated MyoD and E12 bind to the noncanonical E-box adjacent to the canonical E2 E-box (5). Berkes and colleagues further assert that MyoD, in complex with Pbx1/Meis1, is able to activate the myogenin promoter lacking E1 and E2 through this noncanonical E-box. These data raise the possibility that HEB and MyoD do not form heterodimers on E-boxes but rather participate in the formation of a larger complex, which depends on the presence of sequences surrounding the E-box. This hypothesis is further supported by data that demonstrated that HEB was unable to synergistically activate the MRFs on multimerized canonical E-boxes (*4R-Luc*) (data not shown).

Therefore, HEB plays an important role in the formation of promoter-specific complexes on muscle-specific genes. HEB $\beta$  is expressed exclusively after initiation of differentiation in myoblasts and specifically forms a distinct activation complex in cooperation with MyoD to induce myogenin transcription. This study provides the first evidence that E-proteins, specifically HEB, directly regulate the activity of the MyoD family of bHLH transcription factors in an isoform- and promoter-specific manner. Determining the factors that regulate differentiation-specific expression of HEB $\beta$  will be important for understanding the early aspects of myogenic differentiation. Indeed, splicing is regulated in other tissues, such as neurons, and the ratio of expression of HEB $\alpha$  to HEB $\beta$  is characteristic of each cell type. Altering the ratio in these tissues potentially plays an essential role in the switch from proliferation to differentiation, as it does in skeletal muscle.

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