MyoD induces myogenic differentiation through cooperation of its NH_2- and COOH-terminal regions

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MyoD and Myf5 are basic helix-loop-helix transcription factors that play key but redundant roles in specifying myogenic progenitors during embryogenesis. However, there are functional differences between the two transcription factors that impact myoblast proliferation and differentiation. Target gene activation could be one such difference. We have used microarray and polymerase chain reaction approaches to measure the induction of muscle gene expression by MyoD and Myf5 in an in vitro model. In proliferating cells, MyoD and Myf5 function very similarly to activate the expression of likely growth phase target genes such as *L-myc*, *m-cadherin*, *Mcpt8*, *Runx1*, *Spp1*, *Six1*, *IGFBP5*, and *Chrnβ1*. MyoD, however, is strikingly more effective than Myf5 at inducing differentiation-phase target genes. This distinction between MyoD and Myf5 results from a novel and unanticipated cooperation between the MyoD NH_2- and COOH-terminal regions. Together, these results support the notion that Myf5 functions toward myoblast proliferation, whereas MyoD prepares myoblasts for efficient differentiation.

Introduction

The process of skeletal muscle differentiation is orchestrated by a family of four conserved basic helix-loop-helix (bHLH) transcription factors that are collectively known as myogenic regulatory factors (MRFs). Mice harboring single mutations of either *MyoD* or *Myf5* are viable and do not have overt muscle phenotypes, suggesting that MyoD and Myf5 have considerable overlap in their roles (Braun et al., 1992; Rudnicki et al., 1992). However, either MyoD or Myf5 is required for proper myogenesis during embryogenesis because compound *MyoD*/*Myf5*-null mice lack essentially all skeletal muscle tissue at birth (Rudnicki et al., 1993; Kassar-Duchossoy et al., 2004). In contrast, myogenin is important for the terminal differentiation and fusion of myoblasts into mature muscle fibers (Rawls et al., 1998; Vivian et al., 2000). 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 (Kassar-Duchossoy et al., 2004).

Postnatal growth and regeneration of skeletal muscle is mediated primarily by a pool of myogenic stem cells known as satellite cells, which reside adjacent to the fibers. In response to damage through injury or exercise, these satellite cells activate expression of MyoD and Myf5 and undergo numerous rounds of proliferation as myoblasts. A small number of myoblasts return to a quiescent state, thus replenishing the pool of satellite cells; the remainder continue their differentiation, fusing into existing or new myofibers and expressing myogenin and MRF4 while down-regulating Myf5. In contrast to wild-type myoblasts, *MyoD*-null myoblasts grow more quickly, show aberrant expression of muscle markers, and differentiate inefficiently (Sabourin et al., 1999), a phenotype that is the cause of the regeneration deficit exhibited by compound dystrophic (*mdx*) *MyoD*-null animals (Megeney et al., 1996). Conversely, *Myf5*-null myoblasts proliferate poorly and differentiate precociously (Montarras et al., 2000). The sequence of MRF expression in activated satellite cells, in conjunction with the phenotypes of single-null myoblasts and animals, argue that MyoD and Myf5 do not have identical roles in myoblast proliferation and induction of differentiation.

MyoD and Myf5 target genes have largely been examined after the onset of differentiation and, hence, are involved in producing the enormous phenotypic shift from a proliferating myoblast to a contractile, multinucleated muscle fiber. Although it has been suggested that MyoD and Myf5 transactivation is checked in growing myoblasts (whether by degradation [Thayer

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Abbreviations used in this paper: bHLH, basic helix-loop-helix; dblKO, double knockout; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MRF, myogenic regulatory factor.

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et al., 1989], modification [Lindon et al., 1998], signaling [Vaidya et al., 1989; Li et al., 1992], or interfering heterodimerization [Benezra et al., 1990]), the distinct phenotypic differences that are exhibited by growth phase *MyoD*- and *Myf5*-null myoblasts suggest that MyoD and Myf5 do have active roles in myoblasts. Indeed, Wyzykowski et al. (2002) identified *Id3* and *NP1* as target genes of MyoD under growth conditions.

Distinguishing the distinct functions of MyoD and Myf5 is complicated by their abilities to auto- and cross-activate expression from the endogenous loci (Tapscott et al., 1988; Braun et al., 1989; Thayer et al., 1989; Wyzykowski et al., 2002). Such a circular network could account for the stabilization and irreversibility of the commitment of a cell to a myogenic fate (Thayer et al., 1989; Weintraub et al., 1991a). However, gene expression changes resulting from the introduction of exogenous MyoD or Myf5 could be an indirect effect that is mediated through the other MRF. Expression of MyoD in the absence of Myf5 (and vice versa) permits the comparative evaluation of each factor's functions in myogenic commitment.

To this end, we reintroduced MyoD or Myf5 into a *MyoD−/Myf5* double-null (double knockout [dblKO]) fibroblast cell line that was maintained in high serum growth conditions. These cell lines are normally nonmyogenic but can be converted to skeletal muscle upon the exogenous expression of MyoD or Myf5. Microarray analysis identified numerous differentially regulated genes, which were further validated by examining specific candidates using real-time PCR. A number of growth phase targets were identified, demonstrating that MyoD and Myf5 are transcriptionally active in proliferating cells. Surprisingly, we did not find unique targets, and both MRFs were able to induce the expression of these genes.

MyoD, however, was strikingly more effective at activating differentiation markers than Myf5. Additional support for functional differences between MyoD and Myf5 were obtained by using chimeric MRFs that interchanged their NH_2-terminal, bHLH, and COOH-terminal domains. The bHLH domains (DNA binding and dimerization) are highly conserved between the two genes, whereas their NH_2- and COOH-terminal regions are more divergent. Whereas Myf5 was inefficient at inducing differentiation gene expression, the activation of a cohort of these markers by the chimeric MRFs provided strong evidence for cooperative gene activation by the NH_2-terminal and bHLH + COOH-terminal regions of MyoD. *MyoD*-null primary myoblasts have a greatly reduced expression of the same genes relative to wild-type myoblasts. Therefore, these data support the idea that Myf5 is biased toward myoblast proliferation, whereas MyoD promotes myoblast differentiation (Sabourin et al., 1999; Seale et al., 2001).

Results

MyoD and Myf5 activate skeletal muscle genes in growth conditions

To circumvent the potential problem of auto- and cross-activation by the primary myogenic factors (Braun et al., 1989; Thayer et al., 1989; Weintraub et al., 1991b; Hollenberg et al., 1993), we infected clonal double-null *MyoD−/−;Myf5−/−* (dblKO) embryonic fibroblast lines with retrovirus expressing MyoD or Myf5 or with empty control retrovirus. With this approach, we were able to assess which genes were common targets of MyoD and Myf5 versus those that could be uniquely regulated by one or the other primary MRF. These genes could be directly activated by MyoD or Myf5 or could be indirectly activated through an intermediate transcription factor; we considered both classes to be downstream targets of the primary MRFs.

Shortly after infection of the dblKO target cells with retrovirus, positive-expressing cells were purified by FACS based on GFP fluorescence expressed from the bicistronic retroviral transcript (Fig. 1, A and B). Pools of $>10^6$ cells were then harvested for total RNA after a further 24 h of culture in high serum growth conditions. Fluorescently labeled probes generated from biological–triplicate RNA samples were hybridized to MG-U74Av2 GeneChips, each containing probesets directed at ~6,000 genes and an additional ~6,000 ESTs. Comparison of MyoD or Myf5 arrays with control arrays produced candidate lists that were considered to contain genes potentially regulated by MyoD or Myf5 during growth phase (Table I and Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200502101/DC1).

MyoD expression in a *MyoD/Myf5* double-null background produced increases in 47 genes (including *L-myc* and *cadherin-15*), whereas only 17 genes were increased by Myf5. Of these genes, 11 were common targets of both MyoD and Myf5; eight were activated to similar degrees by both (*Chrnβ1*, *Mcpt8*, *Spp1*, *Six1*, *Runx1*, *Idb2*, *Ugcg*, and *Kctd12*), whereas the others were more strongly activated by MyoD (*IGFBP5*, *H19*, and *α-actin*). In contrast, only six genes were down-regulated by either MyoD or Myf5 (none by both), and only four had fold changes of <-2 (*Tcf20*, −4.9; *Dlk1*, −2.9; *Tgfbr3*, −2.7; and *S100a13*, −2.0). Relaxing the stringency of the selection criteria had only a moderate effect, producing 70 increases by MyoD and 32 increases by Myf5 versus 5 and 14 decreases, respectively (Tables S1–S4, available at http://www.jcb.org/cgi/content/full/jcb.200502101/DC1).

One goal of our study was to identify targets that are uniquely regulated by Myf5 but not by MyoD (and vice versa). However, very few genes were suggested by the microarray data to be increased by Myf5 and not by MyoD, and real-time PCR directed at several of those transcripts, in turn, did not support them as targets (*Skiip*, Table I; *Refbp1* and *Snk*, unpublished data). In contrast, 36 genes were up-regulated by MyoD but not by Myf5. The majority of these targets (e.g., *myogenin*, *myosin heavy chain*, and *troponin-T*), however, are associated with skeletal muscle differentiation.

At least six of the identified targets are transcription factors (Table I) and may themselves regulate the expression of other genes. Foremost amongst them is myogenin, which is an MRF that is activated by MyoD immediately upon the switch to differentiation conditions (Hollenberg et al., 1993; Bergstrom et al., 2002). Therefore, we examined (by GeneChip analysis) the possibility that our candidates were activated indirectly by myogenin using myogenin retrovirus–infected dblKO cells that were prepared as for MyoD and Myf5. 50% (13/26) of myogenin targets were also downstream from MyoD and Myf5. However, for most nondifferentiation class genes, our microarrays indicated that tar-

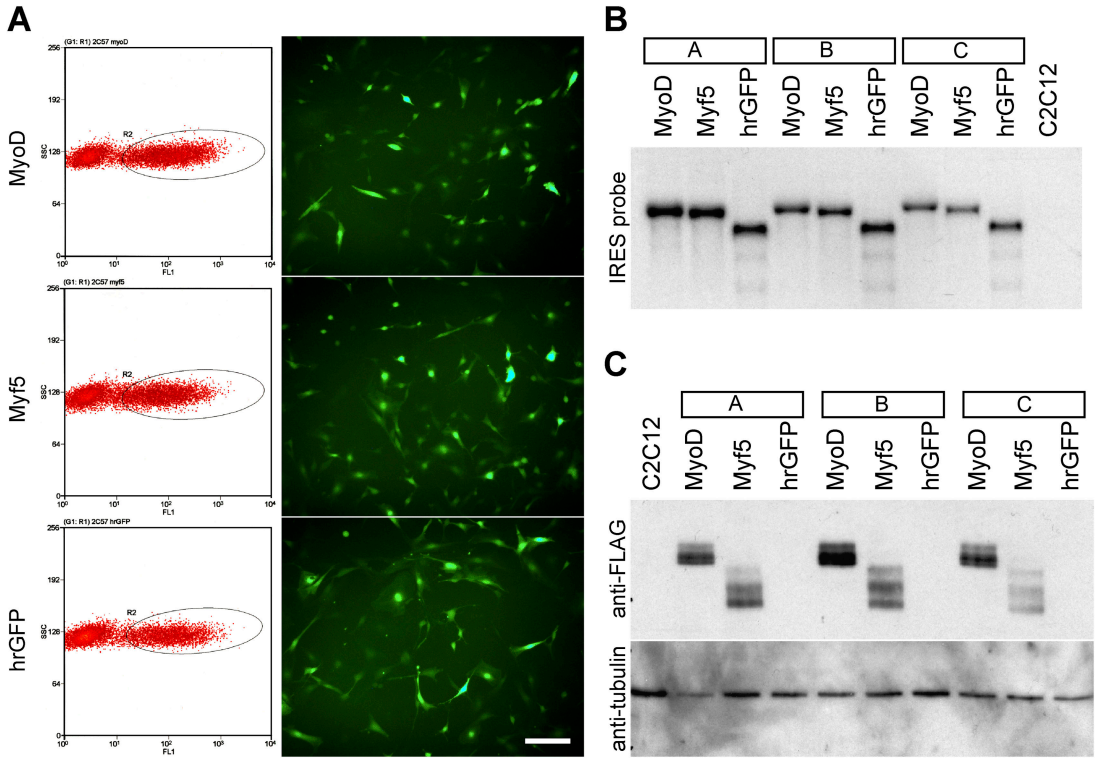

Figure 1. **Preparation of RNA for GeneChip analysis.** (A) Representative FACS plots of *MyoD−/−;Myf5−/−* fibroblasts infected with retrovirus expressing MyoD, Myf5, or no gene as well as GFP from an internal ribosomal entry site (IRES) within the same transcript. GFP expression amongst sorted cells after 24 h of culture was verified by fluorescence microscopy immediately before harvesting for total RNA. SSC, side scatter; FL1, fluorescence channel 1; G, gate; R, region. The circled regions denote the sorted populations. Bar, 100 μm. (B) Northern blot demonstrating equivalent levels of retroviral transcript expression amongst samples. (C) Western blots demonstrating robust MyoD or Myf5 expression in corresponding samples.

get activation was similar between MyoD, Myf5, and myogenin treatments, which contrasts with the considerable induction of myogenin by MyoD (10-fold) compared with Myf5 (1.2-fold; Table I). This suggests that these genes are common targets of MRFs rather than being strictly dependent on myogenin.

To identify potential growth phase targets of MyoD, probable differentiation markers were removed using previous work by Bergstrom et al. (2002). They used an inducible MyoD–ER fusion system to examine gene expression by microarray analysis during early differentiation in low serum conditions (Bergstrom et al., 2002). They identified nine subsets in their data by using a clustering algorithm to find coordinate patterns of temporal regulation, including those with transient increases, early or delayed increases, and decreases in expression. Of the 571 genes identified by Bergstrom et al. (2002), we mapped 298 to probesets on the MU74Av2 GeneChip on the basis of GenBank, Unigene, or LocusLink IDs. Of these 298, 22 genes were also found to be up-regulated by MyoD in our experiment, and an additional seven were also up-regulated by Myf5. This provided added support to our data. A portion of these genes fell within Bergstrom clusters 5 and 6 and primarily represented differentiation-specific targets such as structural genes (e.g., *myosin* and *troponin*). However, others within clusters 1–4 (early induction) and 7–9 (decreased expression through differentiation) were also seen to have increased expression in our GeneChip data and were considered as possible growth targets.

All of the genes identified in Table I are robustly expressed by proliferating wild-type primary myoblasts (Fig. 2). Most (47/53 = 89%) of these genes are decreased in *MyoD−/−* myoblasts, whereas the remainder (6/53) exhibit a mixture of increase/no change/decrease calls and moderate fold increases. Differentiation markers are vastly decreased (up to 140-fold for *myosin heavy chain-3* or *troponin T1*), whereas most putative growth-phase genes showed only moderate changes (e.g., approximately eightfold for *m-cadherin* or *L-myc* and ∼2.7–1.8-fold for *Six1* or *Runx1*).

Thus, in high serum conditions, MyoD and Myf5 are capable of regulating the transcription of numerous genes. The growth phase regulation of a selection of those candidates was then specifically examined.

Growth phase candidate validation by SYBR Green real-time PCR

The expression of these candidate genes was re-examined using a second set of independently derived RNA samples. This second set was produced by the infection of a distinct dblKO cell line with drug-selectable retrovirus, yielding proliferating puromycin-resistant pools of cells that were expanded for 2 wk under drug selection. SYBR Green real-time PCR was used to quantitate target gene transcript levels using PCR primers that were chosen to span at least one intron wherever possible. The specificity of the PCR was verified by denaturing curve analysis and direct sequencing of the products.

Table I. **Candidate MyoD/Myf5 target genes**

Gene	GenBank	GeneChip (U74Av2) Fold[1] MyoD	Myf5	Myogenin	Call[2] GFP	Real-time PCR Fold[3] MyoD	Myf5
Transcription factors							
Myogenin	X15784	10.1	1.2	2.8	A	27.0	8.4
Sine oculis–related homeobox 1 (Six1)	X80339	3.8	3.5	2.2	P/A	2.6	2.2
Inhibitor of DNA-binding 2 (Idb2)	AF077861	3.6	2.4	2.7	P	–	–
Runt-related transcription factor 1 (Runx1)	D26532	3.4	2.9	3.3	P/A	4.3	2.7
Hairy and enhancer of split 6 (Hes6)	AW048812	2.7	0.9	1.1	A	1.3	1.0
Lung carcinoma myc-related oncogene 1 (L-myc)[4]	X13945	2.3	1.5	0.8	A	4.7	2.6
Adhesion and receptors							
Cholinergic receptor, nicotinic, β-1	M14537	24.6	16.0	15.8	A	5.0	6.1
Cholinergic receptor, nicotinic, γ	X03818	9.3	2.0	4.8	A	18.1	16.4
Cholinergic receptor, nicotinic, α-1	M17640	8.4	2.6	2.5	A	3.2	1.9
Cadherin 15 (m-cadherin)[4]	AJ245402	4.2	0.5	1.9	A	290.6	85.4
Transmembrane 4 superfamily member 6	AF053454	2.6	2.3	2.5	P/A	–	–
Discoidin domain receptor family, 1	L57509	2.3	1.6	2.6	A	–	–
Secreted factors							
Insulin-like growth factor–binding protein 5	L12447	142.6	9.8	7.0	A	123.1	89.0
Mast cell protease 8 (Mcpt8)	X78545	11.7	11.1	14.4	A	6.2	15.6
Secreted phosphoprotein 1 (Spp1)	X13986	8.3	5.7	4.6	P	11.5	18.5
ESTs							
RIKEN cDNA 1190002N15	AW125453	5.0	1.7	2.9	P/A	–	–
cDNA clone	AW120874	3.2	1.3	1.9	P	–	–
RIKEN cDNA 2610201A13	AA222883	1.4	2.1	1.2	P	–	–
cDNA clone	AA796831	1.1	2.1	1.7	P	–	–
Others							
H19 fetal liver mRNA	X58196	35.6	6.1	13.7	P/M/A	–	–
C1q/tumor necrosis factor-related protein 3[5]	AI315647	5.8	1.9	3.1	P	–	–
K+ channel tetramerization domain 12[5]	AI842065	4.9	6.0	3.2	P/A	1.1	1.3
Protein kinase inhibitor, α (Pkia)	AW125442	4.6	1.6	2.1	A	1.8	1.0
UDP-glucose ceramide glucosyltransferase	AI853172	4.3	3.3	2.9	P	–	–
Paternally expressed 3 (Peg3)	AF038939	3.6	1.0	1.9	P	–	–
Enolase 3, β muscle	X61600	3.5	1.8	2.0	P	–	–
H2B and H2A histones	X05862	3.3	0.6	1.7	A	–	–
α-methylacyl-CoA racemase	U89906	3.0	2.0	1.7	P/A	–	–
Ankyrin repeat domain 1 (cardiac muscle)	AF041847	3.0	3.0	3.1	P	–	–
SH3 domain GRB2-like B1 (endophilin)	AI842874	2.2	1.4	2.0	P	–	–
WNT1-inducible signaling pathway 1	AF100777	2.2	2.3	2.6	P	–	–
ADP ribosylation factor-like 6 interacting protein 5	AW049647	2.1	1.5	1.6	P	–	–
SKI-interacting protein (Skiip)	AW046671	1.2	4.8	3.6	P/A	1.0	0.5
Enabled homologue (Drosophila)	D10727	1.9	2.4	2.9	P/A	–	–
Differentiation markers							
Actin, α, cardiac	M15501	240.7	3.1	9.6	A	42.9	10.1
Troponin C, cardiac/slow skeletal	M29793	172.8	9.3	16.8	A	1.8	1.0
Actin, α-1, skeletal muscle	M12347	93.3	9.2	14.7	M/A	265.6	130.9
Troponin T3, skeletal, fast	L48989	49.3	2.8	10.4	A	–	–
Myosin light chain, phosphorylatable, fast skeletal	AV290649	43.5	3.0	4.6	A	3850	1540.0
Myosin, light polypeptide 4	M19436	28.5	2.1	6.8	A	–	–
Troponin T2, cardiac	L47600	21.4	2.6	3.4	P/A	–	–
Myosin, heavy polypeptide 3, skeletal	M74753	17.0	0.8	2.0	A	–	–
Troponin T1, skeletal, slow	AV213431	16.4	0.8	0.8	A	–	–
ATPase, Ca^{2+} transporting, cardiac fast 1	X67140	11.0	1.1	1.9	A	–	–
Troponin I, skeletal, slow 1	AJ242874	10.5	0.9	2.7	A	–	–
Myosin, light polypeptide 1	X12973	9.3	0.7	1.6	A	–	–
Retinoblastoma 1	M26391	5.5	1.6	1.5	A	–	–
Ryanodine receptor 1, skeletal muscle	D38216	4.7	1.2	1.2	A	–	–
Myosin-binding protein H	U68267	3.9	0.5	1.5	A	–	–
Sarcoglycan, β	AB024921	3.5	1.0	1.8	A	–	–
Myocyte enhancer factor 2A (MEF2A)	AW045443	3.1	1.2	1.4	P/A	0.8	0.9
Growth arrest and DNA damage-inducible 45 α	U00937	2.5	0.9	1.1	P/A	–	–
Cyclin-dependent kinase inhibitor 1A (P21)	AW048937	2.2	1.1	1.0	P	1.4	1.4

[1]Mean fold change for pairwise comparisons of MyoD/Myf5/myogenin versus GFP from log(fold) change in MAS 5.0 software (Affymetrix, Inc.).
[2]Present/marginal/absent call from MAS 5.0.
[3]Fold change based on ΔCt between MyoD/Myf5 versus puromycin alone real-time PCR, normalized to *GAPDH* expression.
[4]Added after manual inspection of dataset.
[5]*C1q* and *K+ channel* identified in current annotations; previously listed as ESTs.

The majority of the genes that were selected as possible growth phase target genes in the GeneChip data were also found by real-time PCR to be up-regulated (Table I). The estimates of the fold up-regulation of *Six1*, *Runx1*, *L-myc*, *IGFBP5*, and *Mcpt8* were similar by either technique. *Spp1* was increased more, and *Chrnβ1* was increased slightly less as estimated by real-time PCR; nonetheless, each was significantly increased by MyoD and Myf5. The increases in *m-cadherin* and *myogenin* levels by real-time PCR were much greater than by microarray, probably as a result of the lower background of the PCR assay. Again, however, the consistency of the changes in target expression that were produced by MyoD and Myf5 contrast with the variable induction of *myogenin* by MyoD and Myf5, arguing that this is not a strictly indirect effect.

Approximately one third of the candidates that were selected for real-time PCR validation did not exhibit significant changes in the stable pool samples (Table I). These genes tended to be those that had modest fold changes in the GeneChip data (e.g., *Hes6*, *Pkia*, *Skiip*, and *p21*). Probesets for differentiation markers that exhibited larger changes by microarray (such as *cardiac troponin C* and *MEF2A*) were likely seen as a result of spontaneous differentiation in the original GeneChip samples and were minimized in these proliferating cultures. These observations support the conclusion that the stable pools lacked the spontaneously differentiating cells that were observed in our original samples.

Wyzykowski et al. (2002) used the same inducible MyoD system as Bergstrom et al. (2002) to generate inputs for a representational difference analysis protocol. They identified *Id3* and *NP1* as growth phase targets of MyoD (Wyzykowski et al., 2002). Their cells were maintained in a high serum growth medium during MyoD induction, suggesting that *Id3* and *NP1* are not differentiation targets that are expressed simply as a consequence of serum deprivation. Rather, they are induced in the presence of cyclohexamide, indicating that they are likely to be direct targets of MyoD that do not require intervening protein synthesis for activation.

In contrast, our GeneChip experiments did not reveal an induction of *Id3* or *NP1* in dblKO cells by either MyoD or Myf5. To assess whether this was a consequence of low sensitivity to those genes, we used SYBR Green real-time PCR to specifically examine the expression of *Id3* and *NP1* in both the GeneChip RNA samples and in RNA from drug-selected pools; however, no significant changes were detected (unpublished data). It is likely that these inconsistencies are attributable to the numerous differences in our experimental systems, including the method of MyoD expression, the type of host cell used, and possibly the presence/absence of endogenous *MyoD* and *Myf5* genes.

From this combination of GeneChip and real-time PCR analyses, we defined a validated set of growth phase targets. Notably, MyoD and Myf5 were both capable of regulating each of these genes, and none were strictly associated with one MRF. These targets were then applied to examine the differences in function between corresponding regions of MyoD and Myf5.

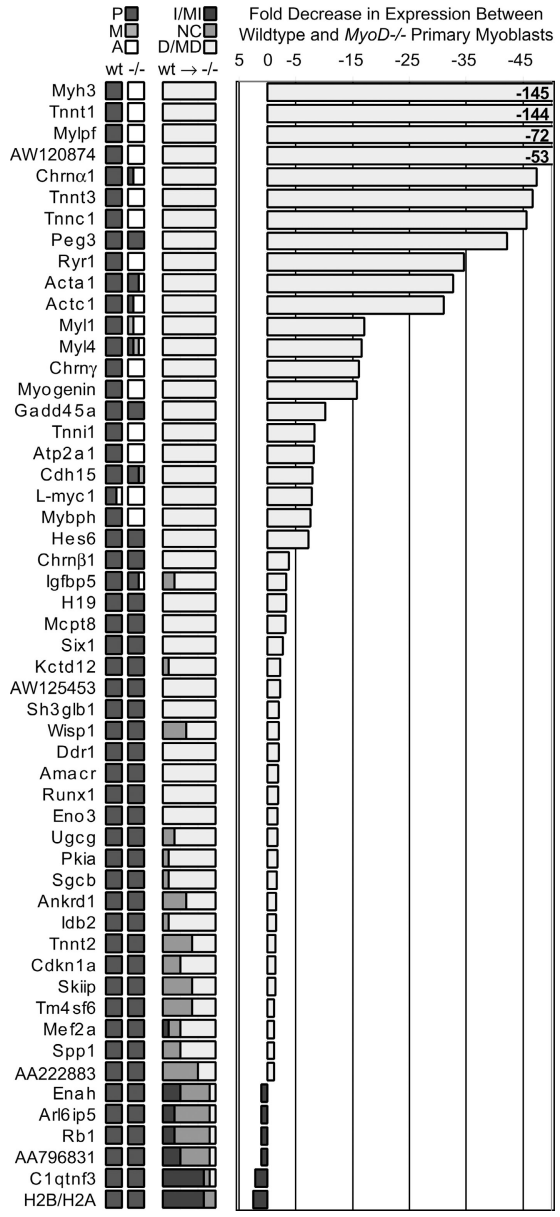

Figure 2. **Decreased expression of differentiation markers by *MyoD−/−* primary myoblasts**. Expression levels in *MyoD*-null primary myoblasts of the genes in Table I show that the majority of differentiation markers are greatly reduced relative to wild-type myoblasts (e.g., *Myh3* to *myogenin*). In contrast, genes that are regulated in growth phase by MyoD and Myf5 are reduced to a lesser degree, if at all (e.g., *Mcpt8*, *Six1*, and *Runx1*). Calls are shown for wild-type ($n = 3$) or *MyoD−/−* ($n = 3$) myoblasts. P, present; M, marginal; A, absent. Change calls are shown for nine pairwise comparisons between wild-type and *MyoD−/−* myoblasts. I, increase; MI, marginal increase; NC, no change; MD, marginal decrease; D, decrease.

Association of growth phase gene activation with MyoD and Myf5 domains

Within their bHLH regions, Myf5 and MyoD exhibit 88% identity and >95% homology at the amino acid level. In contrast, the regions that are NH_2 and COOH terminal to this highly conserved DNA-binding region exhibit considerably more differences in sequence and function (Gerber et al., 1997). Therefore, we hypothesized that functional differences between MyoD and Myf5 would be a consequence of their

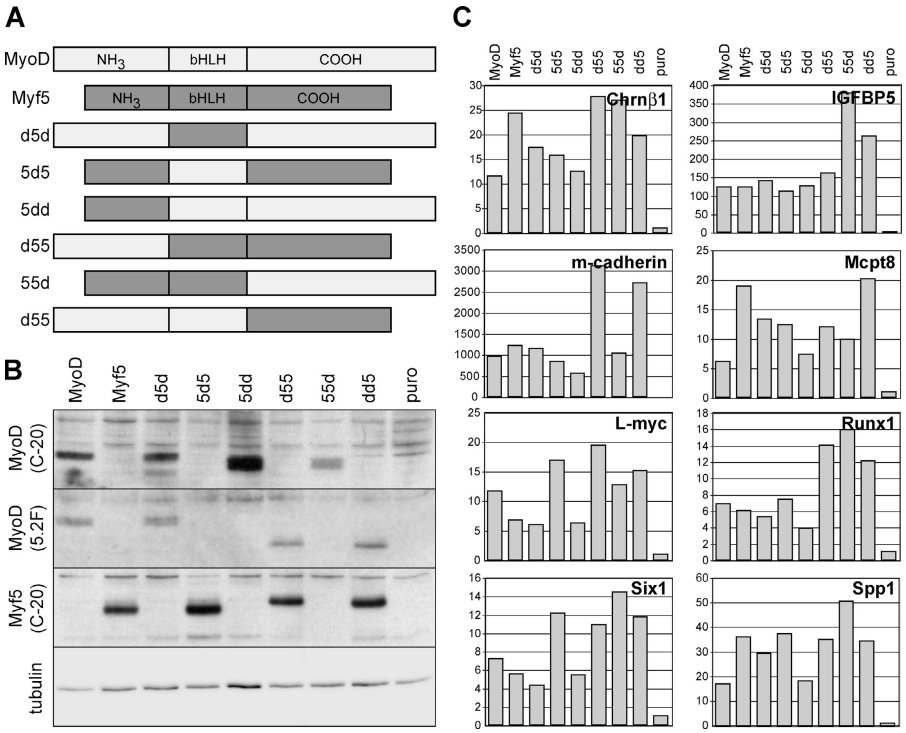

Figure 3. **Gene expression induced by chimeras in growing dblKO cells.** (A) Schematic of MyoD/Myf5 chimeras. Chimeric MRFs were constructed by interchanging the corresponding NH_2-terminal, bHLH-, and COOH-terminal regions of MyoD and Myf5. (B) Levels of MRF and tubulin protein expression in each pool. The combination of three different epitopes that were recognized by MyoD and Myf5 antibodies was used to normalize the expression results in C for relative MRF expression. Puro, puromycin-resistant empty vector negative control. (C) Induction of transcripts for potential growth phase targets by each of MyoD, Myf5, and the chimeric MRFs (normalized to MRF protein and *GAPDH* transcript levels). Numbers on y axis indicates fold changes.

divergent NH_2- and COOH-terminal domains rather than a result of the bHLH recognition of discrete DNA sequences. To test this, chimeric MRFs were built, interchanging the MyoD and Myf5 NH_2- and COOH-terminal regions around their bHLH domains. This yielded six chimeric MRF genes (termed d5d, d55, 55d, 5d5, 5dd, and dd5, where d denotes the portion derived from MyoD and 5 denotes the portion derived from Myf5) in addition to the full-length wild-type MyoD and Myf5 (Fig. 3 A).

Retrovirus expressing one of the chimeric MRFs were introduced into dblKO fibroblasts, yielding drug-selected pools of $>10^5$ clones from which RNA and protein were extracted for analysis. The growth phase targets identified previously were then assayed within these samples by SYBR Green real-time PCR.

Both *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) transcript (input RNA quantity; reverse transcription) and MRF protein levels (MRF expression) were used to enable equitable comparisons by providing normalization between samples. *GAPDH* transcripts were quantified by real-time PCR. Relative protein expression levels of full-length and chimeric MRFs were derived by direct digital densitometry of Western blots probed with three primary antibodies that each recognized unique but overlapping sets of four MRFs (Fig. 3 B). All of the chimeras were demonstrated to be capable of regulating the genes identified in the previous analysis, producing substantial increases in expression compared with the puromycin-alone negative control pool.

After normalization, 5dd tended to be the poorest relative activator, whereas 55d was (in most cases) the best (Fig. 3 C). However, in contrast to the other samples, these two chimeras were subject to significant normalization corrections (Fig. 3 B), which may have tended to overstate their effects. Nonetheless, no single region of MyoD or Myf5 strictly correlated with enhanced or decreased relative activation. In certain cases (e.g., *Chrnβ1*, *m-cadherin*, and *Spp1*), there is a tantalizing suggestion that the Myf5 bHLH domain could have greater activity than that of MyoD, although this pattern is not borne out amongst the others. Chimeras dd5 and d55 have a greater effect than MyoD or d5d. This might suggest that an interaction between the flanking portions of MyoD acts to suppress transactivating activity, which is an effect that is disrupted when a portion of Myf5 is substituted. A similar effect was observed in deletion studies of MyoD (Weintraub et al., 1991b). Thus, for growth-phase genes, the corresponding domains of MyoD and Myf5 are otherwise interchangeable, and differences emerge only with respect to the degree of target induction.

The MyoD NH_2- and COOH-terminal domains cooperate to induce differentiation

A significant number of potential MyoD target genes that were identified by GeneChip analysis were differentiation markers. Wild-type MyoD and Myf5 and chimeras were expressed in dblKO cells using retrovirus and were maintained for 3 d in growth conditions before harvest. The proportion of infected cells was very similar between pools (see Fig. 5 A). Differentiation marker expression was examined by real-time RT-PCR (Fig. 4 A). This set of vectors included a COOH-terminal FLAG epitope tag that allowed for the normalization of gene expression against MRF protein levels (Fig. 4 B). Two growth phase markers (*Chrnβ1* and *Runx1*, identified in the aforementioned GeneChip experiment) showed little relative difference between MyoD, Myf5, and the chimeras. In concordance with the Gene-

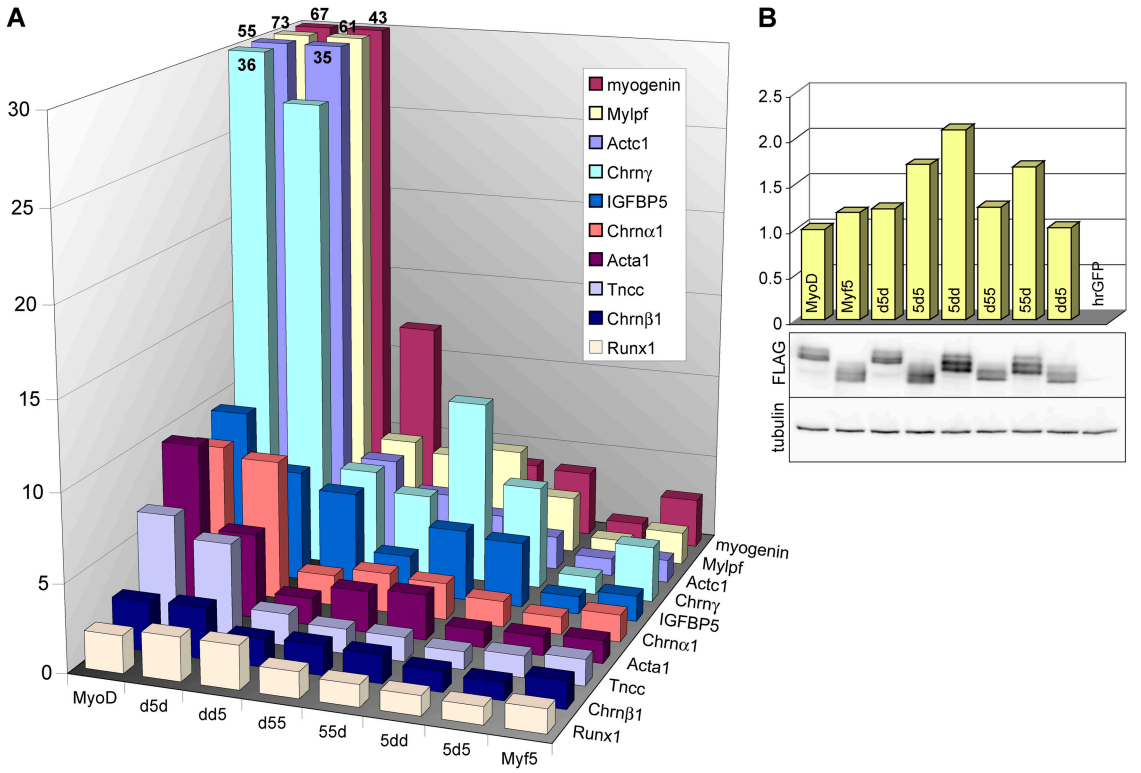

Figure 4. **MyoD NH_2 and COOH termini cooperate to activate differentiation marker expression.** Amongst a variety of differentiation genes, the d5d chimera had near wild-type activity, whereas the separated NH_2 terminus and COOH terminus of MyoD had much less. In contrast, growth-phase genes *Chrnβ1* and *Runx1* were induced similarly by MyoD, Myf5, and the chimeras. (A) Gene expression measured by real-time PCR and normalized to MRF protein expression (B) and *GAPDH* transcript levels. Plotted as relative activity between MRFs. (B) Relative expression levels of FLAG-tagged chimeric MRFs in growth phase (normalized to tubulin). Numbers on y axis indicate fold changes.

Chip results, however, the expression of MyoD produced a considerable activation of genes such as the *cholinergic receptor* α and γ subunits, *myogenin*, *α-actin*, *myosin*, and *troponin* (Fig. 4; Table II shows unnormalized changes vs. empty vector controls). In comparison, the level of Myf5 induction of these genes was moderate relative to the empty vector control.

Substitution of the MyoD COOH terminus into Myf5 (55d) was not sufficient in growth phase to cause differentiation and could produce only modest increases in the expression of numerous differentiation markers. Replacement with the MyoD NH_2 terminus alone (d55) also resulted in just moderate gene induction relative to full-length Myf5. Importantly, the concurrent presence of MyoD NH_2- and COOH-terminal regions (d5d) resulted in activity approaching that of full-length MyoD.

The same pools of dblKO cells expressing one of the wild-type or chimeric MRFs were challenged to differentiate under reduced serum conditions. When the MyoD COOH terminus was present (MyoD; d5d, 5dd, and 55d), there was enhanced differentiation compared with the corresponding Myf5 region (Myf5; dd5, d55, and 5d5; Fig. 5 B). Deletion of the putative cdk4-interacting domain (Zhang et al., 1999) from the COOH-terminal region of full-length MyoD or insertion of this region into the corresponding location of Myf5 does not significantly affect these results based on protein (myosin heavy chain immunostaining) or RNA (real-time PCR) markers (Punch, V., personal communication). The MyoD NH_2 terminus also had a noticeable effect on differentiation (Fig. 5 B, compare MyoD with 5dd, d5d with 55d, dd5 with 5d5, and d55 with Myf5). The MyoD bHLH domain, which has previously been connected to cell cycle arrest (Crescenzi et al., 1990; Sorrentino et al., 1990), enhanced differentiation when combined with the MyoD COOH-terminal region (Fig. 5 B, compare MyoD with d5d and 5dd with 55d) but had little effect otherwise. Overall, the combined NH_2- and COOH-terminal portions of MyoD (MyoD and d5d) were most effective in producing robust differentiation, whereas the two MRFs lacking both (Myf5 and 5d5) were the poorest. Thus, in both growth and differentiation conditions, the MyoD NH_2- and COOH-terminal regions cooperate to strongly activate the myogenic differentiation program.

Discussion

Previous work has suggested that there are unique roles for MyoD and Myf5 in adult myogenesis (Megeney et al., 1996; Sabourin et al., 1999; Montarras et al., 2000). To investigate the possibility that these phenotypes are a result of the activation of unique target genes by each of these transcription factors, we conducted genome-wide surveys of gene expression changes in response to MRF expression. The embryonic fibroblast cells that were used are normally nonmyogenic but can be converted to myoblasts by ectopic MRF expression. Importantly, they were derived in a *MyoD−/−;Myf5−/−* background, precluding cross-activation between MyoD and Myf5.

Table II. **Fold activation of myogenic genes by real-time PCR**[1]

Gene	MyoD	d5d	5dd	55d	dd5	d55	5d5	Myf5	GFP
Actc1	89.2	68.5	6.5	7.8	8.9	7.6	2.8	2.5	1.0
Myogenin	145.0	114.8	17.4	14.5	26.2	18.4	3.7	7.4	1.0
Mylpf	2645.3	2727.9	249.2	356.0	211.1	234.3	62.4	81.8	1.0
Chrng	48.5	45.3	16.9	24.4	8.0	8.0	2.3	5.2	1.0
Acta1	35.4	21.8	7.7	16.1	5.7	10.8	6.9	5.6	1.0
ChrnA1	8.7	10.0	3.1	3.6	1.8	2.9	1.7	1.9	1.0
IGFBP5	127.4	104.0	104.0	91.4	74.2	35.9	22.8	23.0	1.0
Tncc	5.1	5.1	1.6	1.8	1.4	1.3	1.6	1.3	1.0
Chrnb1	11.7	14.9	10.2	11.2	6.7	8.8	7.1	7.7	1.0
Runx1	2.4	3.4	2.6	2.3	2.8	2.0	1.9	1.8	1.0

[1]Relative to GFP and normalized to *GAPDH* but not to FLAG protein.

Our microarray data indicates that MyoD expression induces more myogenic genes than Myf5, often to a greater degree. Comparison of our data with a previous study (Bergstrom et al., 2002), however, demonstrated that the majority of these MyoD-regulated targets are markers of differentiated skeletal muscle. Thus, a unique function of MyoD (vs. Myf5) is a strong ability to induce differentiation. *MyoD*-null primary myoblasts have vastly reduced levels of differentiation marker gene expression (Fig. 2), which is consistent with the phenotypic data on single-null primary myoblast cultures; in the absence of MyoD, the myoblasts proliferate well and differentiate poorly (Sabourin et al., 1999), whereas in the absence of Myf5, the opposite is true (Montarras et al., 2000). The moderate reduction of levels of growth-phase genes in *MyoD*-null myoblasts likely indicates a preference for activation by MyoD that Myf5 compensates for only partially.

A previous study by Seale et al. (2004) used representation difference analysis to identify potential satellite cell markers and MyoD target genes using primary myoblasts from *MyoD−/−* mice as a model for early satellite cell activation. The decreased expression of many genes (including *Chrnγ*, *Chrnα1*, *myogenin*, *troponin T1*, *H19*, and *Peg3*) was seen (Seale et al., 2004); these genes were up-regulated by MyoD expression in our experiments, supporting the contention that MyoD regulates these genes and promotes differentiation. However, Seale et al. (2004) also showed that levels of numerous genes (e.g., *k-cadherin*, *integrin-α7*, *Plgf*, *VCAM1*, *Igsf4a*, *Tcrαv13*, *laminin α5*, *neuritin-1*, and *Klra18*) are increased in *MyoD−/−* myoblasts, which express considerable Myf5. None of these genes was identified in the current study as a Myf5 target. Thus, although MyoD and Myf5 have critical roles in the myogenic program, other transcription factors (e.g., Pax3 or Pax7) are likely needed to produce the full spectrum of normal myoblast gene expression.

We initially focused on the genes that were activated in the proliferating population by pruning our microarray results of differentiation genes. To assist in this, we leveraged the work of Bergstrom et al. (2002), who used an inducible MyoD in differentiating dblKO cells. Their identified target genes were those induced by serum deprivation, growth arrest, and MyoD activity in the context of those conditions. However, their data also included genes that are induced immediately before growth arrest but remain up-regulated either as a result of a failure of their RNA levels to decay to baseline during the early times of the experiment or because expression continues in differentiation. Thus, we identified a set of genes that were growth phase targets of MyoD and Myf5. Furthermore, real-time PCR validation confirmed that they were not unique targets but, rather, that they all could be induced by either of these MRFs.

These similarities in function are perhaps not surprising. Both *MyoD*- and *Myf5*-null mice possess grossly normal skeletal muscle, indicating that there is redundancy and compensation for the loss of either factor during development. Indeed, only with the concurrent loss of MRF4 activity does the defect in skeletal myogenesis become fully penetrant (Kassar-Duchossoy et al., 2004). Therefore, rather than MyoD and Myf5 each having a unique set of target genes that they are responsible for activating, it is reasonable that these two factors are capable of regulating the same downstream targets. Differences in MyoD and Myf5 function in proliferating cells may instead vary in the strength of their effect on similar sets of genes. Whether MyoD and Myf5 function similarly during differentiation is a distinct question.

Previous work by Wyzykowski et al. (2002) suggested that *Id3* and *NP1* were both activated by MyoD in myoblast growth phase. Although our data does not show this, the differences between our methods and theirs are considerable and are more than adequate to explain the discrepancy. The cell types in which the experiments were conducted are a primary example: Wyzykowski et al. (2002) used 10t1/2 fibroblasts (genetically wild type), whereas our fibroblasts were derived from *MyoD*/*Myf5* compound-mutant animals (it is also likely that our cells have disrupted MRF4 function; Kassar-Duchossoy et al., 2004). It is quite conceivable that the presence of other myogenic factors could be capable of modulating MyoD activity.

An important conclusion to be drawn from these data is that both MyoD and Myf5 are active transcription factors in proliferating myoblasts (previously shown for MyoD by Wyzykowski et al., 2002). Rather than passively awaiting a differentiation signal, these two factors induce the expression of myoblast growth-phase genes. MyoD and Myf5, therefore, act not only to commit the cell to a myoblast identity but also to prepare the myoblast for skeletal muscle differentiation.

The expression of MyoD or Myf5 led to the down-regulation of only a very small number of genes. This could indicate that they act primarily as transcriptional activators in high serum conditions. However, our experimental system involved converting a fibroblast cell type to the myogenic lineage and, thus, was incapable of detecting MRF-mediated suppression of any genes that were not initially expressed in the control cells.

Myogenin is not usually found in proliferating myoblasts and is induced at the onset of differentiation (Hollenberg et al., 1993; Weintraub, 1993; Bergstrom et al., 2002). The non-differentiation targets that were assayed (e.g., *Six1*, *Runx1*, *Mcpt8*, and *Spp1*) were induced to similar levels by either MyoD or Myf5, whereas *myogenin* was induced strongly by MyoD (10-fold) but only weakly by Myf5 (1.2-fold). Thus, although myogenin can activate similar targets to MyoD (Wyzykowski et al., 2002) and Myf5 (Table I), indirect regulation through myogenin is not dominant. The overexpression

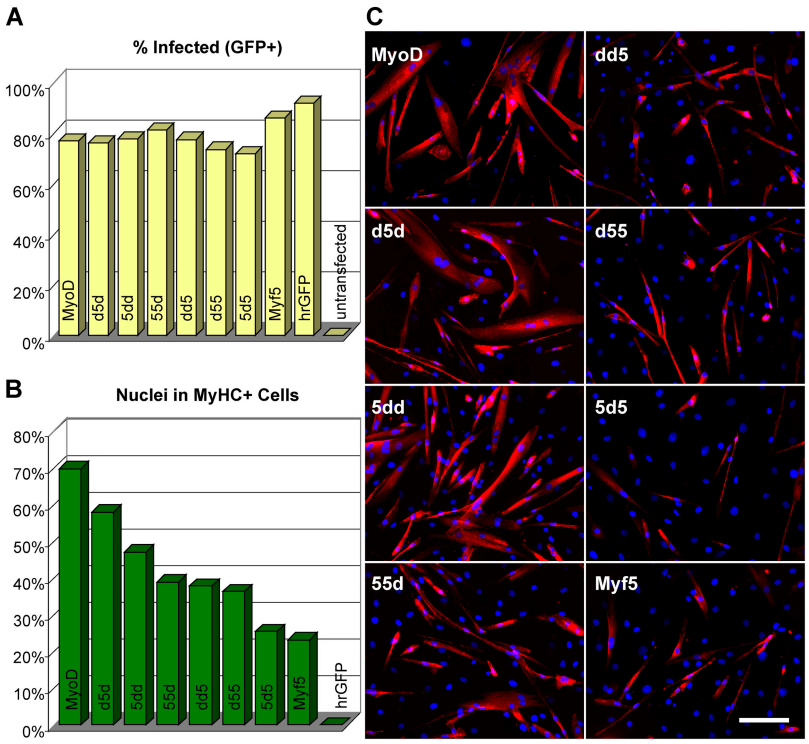

Figure 5. **MyoD NH_2 and COOH termini cooperatively promote differentiation.** The expression of MyoD/Myf5 chimeric MRFs that included the MyoD NH_2- or COOH-terminal regions in *MyoD−/−;Myf5−/−* fibroblasts produced more efficient differentiation in low serum conditions than those with the corresponding Myf5 region. (A) Percentage of infected cells in each pool based on GFP expression immediately before differentiation. (B) Percentage of total nuclei ($n > 1{,}000$) found within a differentiated myosin heavy chain+ cell, normalized to A. (C) Myosin heavy chain immunostaining of differentiated pools (MF20, red; DAPI, blue). Bar, 100 μm.

of myogenin alone was much less effective than MyoD at inducing differentiation marker transcripts, also demonstrating that MyoD has distinct functions that are not simply consequences of myogenin induction.

The high level of structural conservation between MyoD and Myf5, particularly in the bHLH domain, provides a strong rationale for our observation that there are few, if any, unique growth phase target genes of MyoD or Myf5 (this might be extended to myogenin—the conserved bHLH domain may allow the induction of growth targets, although myogenin would not usually be found in growing cells). It has been shown previously that just three differing amino acids between the MyoD and E12 bHLH regions encode specificity for the myogenic program (Davis and Weintraub, 1992). However, our results suggest that the three nonconservative and five conservative amino acid substitutions between MyoD and Myf5 bHLH domains are not likely to be involved in overt target gene selection, although they could potentially provide sufficient variation for more subtle types of regulation.

Chimeric MRFs, in which the NH_2-, bHLH-, and COOH-terminal domains of MyoD and Myf5 were interchanged, were used to explore which portions of MyoD might provide for enhanced myogenic differentiation. Strikingly, only the d5d chimera approached MyoD levels of differentiation gene expression, whereas the other chimeras, Myf5, and myogenin were many times less effective. The MyoD NH_2- and COOH-terminal regions had much less activity when separated, thus indicating a functional interaction between them.

In contrast to growth conditions (Fig. 4), differentiation conditions allowed the MyoD NH_2- and COOH-terminal regions to function independently (Fig. 5, B and C), with the concurrent presence of both producing the greatest differentiation. Therefore, each region is effective when growth signals are naturally reduced under low serum conditions. However, cooperation between these two MyoD regions was required to overcome the strong growth signals that were provided by high serum medium. Full-length MyoD, unlike Myf5, could, therefore, bias a cell toward differentiation by sensitizing it to a moderate reduction in growth signals.

Interestingly, the MyoD bHLH domain enhanced differentiation at the molecular and phenotypic levels (Figs. 4 and 5), but only when combined with the MyoD COOH terminus. Despite its exceptional similarity with the Myf5 bHLH, the MyoD bHLH domain functionally differs in its interaction with the MyoD COOH terminus. However, this MyoD bHLH effect modulates gene expression levels rather than target gene selection (Fig. 4).

The NH_2-terminal activity is likely to be the MyoD transcriptional activation domain, which was previously mapped between amino acids 3–56 (Weintraub et al., 1991b). It is interesting to speculate that the COOH-terminal function is provided by the chromatin remodeling amphipathic α-helix (helix III) domain that was previously described by Bergstrom and Tapscott (2001). Whereas MyoD can induce muscle marker expression at repressed loci, their studies demonstrated that a similar motif in myogenin was ineffective at this task. This α-helix motif is conserved in Myf5 (Gerber et al., 1997), but Myf5 is similar to myogenin in being less effective than MyoD at initiating muscle gene expression. Thus, MyoD might be more efficient than Myf5 for inducing differentiation because of a greater ability to remodel chromatin at lineage-restricted loci. A similar domain appears in MRF4 (Rhodes and Konieczny, 1989; Bergstrom and Tapscott, 2001) and could mediate the residual skeletal myogenesis that is found in the absence of MyoD and Myf5 (Kassar-Duchossoy et

al., 2004). Therefore, MyoD may use its COOH-terminal chromatin-remodeling domain to provide access to silenced muscle genes for the NH_2-terminal activation domain. In support of this, the substitution of either portion alone into Myf5 produced only moderate increases in gene expression, whereas together they strongly activated a variety of differentiation markers (Fig. 4).

If MyoD is inclined to promote differentiation, whereas Myf5 activates growth (but not differentiation) targets, then it is consistent with their cell cycle regulation in growing myoblasts. MyoD levels peak at the differentiation checkpoint in G_1 of the cell cycle, whereas Myf5 levels are high in S/G_2 and G_0 in association with proliferation and a failure to differentiate (Kitzmann et al., 1998). This complementary pattern of expression allows for the maintenance of expression of growth-phase genes and myogenic identity while ensuring that high levels of MyoD occur only at a cell cycle point that is appropriate for differentiation.

That MyoD and Myf5 activate the same downstream target genes, but to differing degrees, does much to explain the phenotypes observed in single knockout mice and cells. Our data reinforce the partitioning of myogenic factors into primary and secondary MRFs and, furthermore, add support to the concept of a role for Myf5 in myoblast proliferation versus MyoD instigating myogenic differentiation. MyoD's ability to activate differentiation marker expression despite the presence of high levels of serum is a distinguishing biochemical function that is not found in Myf5 and involves the cooperation of separate domains of MyoD. These regions may allow MyoD to interact with coactivators for which Myf5 has much less affinity. In the future, studies to further understand the distinct roles played by MyoD and Myf5 in growing and differentiating myoblasts, as well as the structural constraints and intermolecular interactions upon which those roles are built, will be essential to our understanding of the mechanism by which damaged skeletal muscle is efficiently regenerated.

Materials and methods

Cell culture

MyoD-null/*Myf5*-null dblKO mouse embryo fibroblasts were isolated, and clonal lines were selected and expanded (designated 2C5/7 and 4C5/2). Fibroblasts were cultured in subconfluent conditions in growth medium of DME supplemented with 10% FCS and 1% penicillin/streptomycin. Proliferating primary myoblasts from wild-type and *MyoD*−/− mice were isolated and cultured as described previously (Megeney et al., 1996; Sabourin et al., 1999).

Myf5, *MyoD*, chimeric MRFs, or myogenin were introduced into dblKO cells using retrovirus based on the three-plasmid HIT system (provided by V. Sartorelli, National Institutes of Health, Bethesda, MD; Soneoka et al., 1995), including expression plasmids based on the pHAN backbone (with puromycin resistance driven from a distinct SV40 promoter) or a modified pHAN backbone in which the puromycin cassette was replaced by an internal ribosomal entry site and humanized Renilla GFP (IRES-hrGFP) sequence (Stratagene). MRFs that were expressed using the latter retroviral plasmid were COOH-terminally tagged with a 3× FLAG epitope. Empty control virus expressed only puromycin resistance or hrGRP. Retrovirus was produced by calcium phosphate transient cotransfection of *gag-pol* (pHIT60), *env* (pHIT456), and expression (pHAN) plasmids into 293T cells; virus-containing medium was harvested 48 h from the beginning of the transfection and was filtered through a 0.45-μm syringe filter (Millipore). Cells were infected overnight with diluted, filtered viral supernatant plus 8 μg/ml polybrene (hexadimethrine bromide; Sigma-Aldrich). Drug selection, where appropriate, was conducted with 1 μg/ml puromycin (Sigma-Aldrich) in growth medium for at least 1 wk; uninfected controls were obliterated after 4–5 d of selection. Differentiation was induced by replacing growth medium with DME + 2% horse serum (GIBCO BRL) and 1% penicillin/streptomycin.

FACS

3 d after infection with hrGFP-expressing retrovirus, pools of cells were trypsinized, centrifuged, resuspended in PBS + 5% FCS, filtered through a MACS filter (Miltenyi Biotec) to remove aggregates, and placed on ice. Cell sorting was performed using a MoFlo sorter (DakoCytomation), with gating on hrGFP+ populations set by a comparison with an uninfected negative control pool. A small portion of the sorted cells was reanalyzed to assess purity, and the remainder was replated in growth medium for an additional 24 h before harvesting for total RNA.

RNA and protein isolation

Total RNA was purified using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions and quantitated by OD_{260} or by RiboGreen (Invitrogen). RNA samples used for microarray analysis were ethanol precipitated in order to reach a minimum concentration of 2 μg/ml. Protein extracts were made by lysis of pelleted cells in radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with MiniComplete protease inhibitors (Roche).

Microarray analysis

Triplicate RNA samples for microarray analysis were submitted to the Ottawa Genome Centre for hybridization to MG-U74Av2 GeneChips (Affymetrix, Inc.). Manufacturer's quality controls were verified by the Centre. Raw data files were processed with MicroArray Suite (MAS 5.0; Affymetrix, Inc.) using the statistical algorithm (Affymetrix, Inc.) to derive signals and present/marginal/absent calls for each sample; all possible pairwise comparisons were also performed between experimental and control replicates, producing log(fold) ratio estimates of change and increase/no change/decrease calls. Processed results were then exported from MAS 5.0 and imported into Excel and Access (Microsoft) for further manipulation. Probesets showing consistent statistically significant changes between MRF and control samples were screened for detectable signal values (present/absent calls) and absolute log(fold) changes of at least one. Probeset annotations were obtained from http://www.affymetrix.com/analysis/index.affx. Microarray data is available from StemBase (http://www.scgp.ca:8080/StemBase/; Ontario Genomics Innovation Centre; Perez-Iratxeta et al., 2005) under experiments E223 (samples S361-4) and E59 (S78-9) and from the National Center for Biotechnology Information's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; Edgar et al., 2002; Barrett et al., 2005) under series accession no. GSE3245 (GSM73053...64) and GSE3244 (GSM73026, -29, -32, -35, -38, and -41).

Real-time PCR

RNA samples were reverse transcribed using random hexamer primers that were included in the RNA PCR Core Kit (PerkinElmer) according to the manufacturer's instructions. Reverse transcription reactions were diluted (1:10 in Fig. 3 C and 2:5 in Fig. 4 A) with 10 mM Tris, pH 8.0, yielding master samples of reverse-transcribed products from which related PCR reactions were drawn. Real-time PCR reactions included the following: 2 μl of diluted reverse transcription product, 10 μl of 2× iQ SYBR Green SuperMix (Bio-Rad Laboratories), 30 nM ROX passive reference dye (Stratagene), and 50 nM of each forward and reverse PCR primer. Real-time data was gathered using a system (MX4000; Stratagene) over 40 cycles (30 s at 94°C, 60 s at 58°C, and 30 s at 72°C) followed by a denaturation curve from 54 to 94°C in 30-s increments of 0.5°C to ensure amplification specificity. C_t values were calculated with the MX4000 software by using moving window averaging and an adaptive baseline. Fold changes, other calculations, and chart plotting were performed in Excel. A PCR efficiency of 85% was assumed. Primer sequences can be found in Table S2.

Chimeric MRF construction

Chimeric *MyoD/Myf5* MRFs were created by using an overlapping PCR approach to seamlessly fuse the NH_2- and COOH-terminal regions to the central bHLH domain (Fig. 3 A). The 318 aa of MyoD (available from GenBank/EMBL/DDBJ under accession no. NM_010866) were divided into aa 1–96 (NH_2 terminus), 97–161 (bHLH), and 162–318 (COOH terminus). The 255 aa of Myf5 (available from GenBank/EMBL/DDBJ under accession no. NM_008656) were divided into aa 1–70 (NH_2 terminus), 71–135 (bHLH), and 136–255 (COOH terminus). Each PCR product was cloned into an appropriate expression plasmid and verified by sequencing (Applied Biosystems).

Immunocytochemistry and Western blot analysis

Primary antibodies that were used are listed as follows: mouse anti-FLAG (M2 and M5; Sigma-Aldrich), rabbit anti-Myf5 (C-20; Santa Cruz Biotechnology, Inc.), rabbit anti-MyoD (C-20; Santa Cruz Biotechnology, Inc.), mouse anti-MyoD (5.2F; Sigma-Aldrich), mouse antimyogenin (F5D) and mouse anti-myosin heavy chain (MF20; hybridoma supernatants), mouse antidesmin (D33; DakoCytomation), and mouse anti–α-tubulin (Sigma-Aldrich).

For immunostaining, cells were fixed with 4% PFA, permeabilized with Triton X-100, and blocked with 5% normal goat serum in PBS. Primary and secondary antibodies were applied in 5% goat serum–PBS. Secondary detection used appropriate fluorescein- or rhodamine-conjugated antibodies (Chemicon). 0.25 μg/ml DAPI was included in a final wash step to highlight nuclei. Samples were mounted in fluorescence mounting medium (DakoCytomation), coverslipped, and imaged using a microscope (Axiophot 2; Carl Zeiss MicroImaging, Inc.), a 10× NA 0.30 plan-Neofluar (Ph1; ∝ /0.17) or 20× NA 0.75 plan Apochromat (∝ /0.17) objective, and a digital camera (Axiocam; Carl Zeiss MicroImaging, Inc.). Digital images were captured by using Axiovision (Carl Zeiss MicroImaging, Inc.) and were processed with Photoshop (Adobe). Enumeration was assisted by ImageJ software (http://rsb.info.nih.gov/ij/).

Western blots were made by electroblotting standard SDS-PAGE gels onto Immobilon-P membranes. Membranes were blocked in 5% skimmed milk in PBS; secondary detection was performed with an appropriate HRP-conjugated antibody (Bio-Rad Laboratories) visualized by ECL (GE Healthcare). For densitometry, blots were digitally imaged with a 16-bit GeneGnome chemiluminescence gel-doc system (Syngene), and bands were quantified with the bundled GeneTools software (Syngene) except parts of Fig. 3 B, for which the MyoD (C-20), Myf5 (C-20), and corresponding tubulin (not depicted) blots were exposed to film, scanned, and analyzed by using ImageJ software.

Online supplemental material

Tables S1–S4 show the expansion of Table I, where candidate gene lists were derived by testing for consistent pairwise increase/decrease changes (at least six of nine) as well as minimum mean threshold log(fold) changes of ±1 (twofold). Increases for MyoD, increases for Myf5, decreases for MyoD, and decreases for Myf5 correspond to Tables S1, S2, S3, and S4, respectively. Table S5 shows the sequences and targets for primers that were used for SYBR Green real-time PCR. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200502101/DC1.

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