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 Chrm81. 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 NH2- 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 (Sabbourin 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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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 NPI 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 by the primary myogenic factors (Braun et al., 1989; Thayer et al., 1989; Wyzykowski et al., 2002). To circumvent the potential problem of auto- and cross-activation, we reintroduced MyoD or Myf5 into a MyoD–/Myf5 double-null (double knockout [dbKO]) 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 was obtained by using chimeric MRFs that interchanged their NH2-terminal, bHLH, and COOH-terminal domains. The bHLH domains (DNA binding and dimerization) are highly conserved between MyoD and Myf5, whereas their NH2-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 NH2-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−/− (dbKO) 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 dbKO 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 >106 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 (Chrmβ1, Mcp18, Spp1, Six1, Runx1, Idb2, Ugcg, and Kdtd2), 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; Tgfb3, −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 Sntk, 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 tropomin-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 dbKO 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-
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 quantify 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.
| Gene                                      | GeneChip (U74Av2) | Real-time PCR |
|-------------------------------------------|-------------------|---------------|
|                                          | Fold1             | Call          | Fold1             |
| **Transcription factors**                 |                   |               |
| Myogenin                                  | 10.1              | 1.2           | 2.8               | A   | 27.0 | 8.4 |
| Sine oculis-related homeobox 1 (Six1)     | 3.8               | 3.5           | 2.2               | P/A | 2.6  | 2.2 |
| Inhibitor of DNA-binding 2 (Idb2)         | 3.6               | 2.4           | 2.7               | P   | –    | –   |
| Run-related transcription factor 1 (Runx1)| 3.4               | 2.9           | 3.3               | P/A | 4.3  | 2.7 |
| Hairy and enhancer of split 6 (Hes6)      | 2.7               | 0.9           | 1.1               | A   | 1.3  | 1.0 |
| Lung carcinoma myc-related oncogene 1 (Lmey)4 | 2.3               | 1.5           | 0.8               | A   | 4.7  | 2.6 |
| **Adhesion and receptors**                |                   |               |
| Cholinergic receptor, nicotinic, β-1      | 24.6              | 16.0          | 15.8              | A   | 5.0  | 6.1 |
| Cholinergic receptor, nicotinic, γ        | 9.3               | 2.0           | 4.8               | A   | 18.1 | 16.4|
| Cholinergic receptor, nicotinic, α-1      | 8.4               | 2.6           | 2.5               | A   | 3.2  | 1.9 |
| Cadherin 15 (macadherin)5                 | 4.2               | 0.5           | 1.9               | A   | 290.6| 85.4|
| Transmembrane 4 superfamily member 6      | 2.6               | 2.3           | 2.5               | P   | –    | –   |
| Discodin domain receptor family 1         | 2.3               | 1.6           | 2.6               | A   | –    | –   |
| **Secreted factors**                      |                   |               |
| Insulin-like growth factor–binding protein 5| 142.6             | 9.8           | 7.0               | A   | 123.1| 89.0|
| Mast cell protease β (Mcpβ)               | 11.7              | 11.1          | 14.4              | A   | 6.2  | 15.6|
| Secreted phosphoprotein 1 (Spp1)          | 8.3               | 5.7           | 4.6               | P   | 11.5 | 18.5|
| **ESTs**                                  |                   |               |
| RIKEN cDNA 1190002N15                      | 5.0               | 1.7           | 2.9               | P/A | –    | –   |
| cDNA clone                                | 3.2               | 1.3           | 1.9               | P   | –    | –   |
| RIKEN cDNA 2610201A13                     | 1.4               | 2.1           | 1.2               | P   | –    | –   |
| cDNA clone                                | 1.1               | 2.1           | 1.7               | P   | –    | –   |
| **Others**                                |                   |               |
| H19 fetal liver mRNA                      | 35.6              | 6.1           | 13.7              | P/M/A| –    | –   |
| CT1a/tumor necrosis factor-related protein 36 | 5.8               | 1.9           | 3.1               | P   | –    | –   |
| K+ channel tetramerization domain 126     | 4.9               | 6.0           | 3.2               | P/A | 1.1  | 1.3 |
| Protein kinase inhibitor, α ([PKia])      | 4.6               | 1.6           | 2.1               | A   | 1.8  | 1.0 |
| UDP-glucose ceramide glycosyltransferase  | 4.3               | 3.3           | 2.9               | P   | –    | –   |
| Paternally expressed 3 (Peg3)             | 3.6               | 1.0           | 1.9               | P   | –    | –   |
| Enolase 3, β muscle                       | 3.5               | 1.8           | 2.0               | P   | –    | –   |
| H2B and H2A histones                      | 3.3               | 0.6           | 1.7               | A   | –    | –   |
| α-methylacylCoA racemase                  | 3.0               | 2.0           | 1.7               | P/A | –    | –   |
| Ankyrin repeat domain 1 (cardiac muscle)  | 3.0               | 3.0           | 3.1               | P   | –    | –   |
| SH3 domain GRB2-like B1 (endophilin)      | 2.2               | 1.4           | 2.0               | P   | –    | –   |
| WNT1-inducible signaling pathway 1        | 2.2               | 2.3           | 2.6               | P   | –    | –   |
| ADP ribosylation factor-like 6 interacting protein 5 | 2.1               | 1.5           | 1.6               | P   | –    | –   |
| SKI-interacting protein (Skip)            | 1.2               | 4.8           | 3.6               | P/A | 1.0  | 0.5 |
| Enabled homologue ([Drosophila])           | 1.9               | 2.4           | 2.9               | P/A | –    | –   |
| **Differentiation markers**               |                   |               |
| Actin, α, cardiac                         | 240.7             | 3.1           | 9.6               | A   | 42.9 | 10.1|
| Troponin C, cardiac/slow skeletal         | 172.8             | 9.3           | 16.8              | A   | 1.8  | 1.0 |
| Actin, α-I, skeletal muscle               | 93.3              | 9.2           | 14.7              | M/A | 265.6| 130.9|
| Troponin T3, skeletal, fast               | 49.3              | 2.8           | 10.4              | A   | –    | –   |
| Myosin light chain, phosphorylatable, fast skeletal | 43.5             | 3.0           | 4.6               | A   | 3850 | 1540.0|
| Myosin, light polypeptide 4              | 28.5              | 2.1           | 6.8               | A   | –    | –   |
| Myosin, heavy polypeptide 3, skeletal     | 21.4              | 2.6           | 3.4               | P/A | –    | –   |
| Myosin, light polypeptide 1              | 17.0              | 0.8           | 2.0               | A   | –    | –   |
| Myosin, heavy polypeptide 3, skeletal     | 16.4              | 0.8           | 0.8               | A   | –    | –   |
| ATPase, Ca2+ transporting, cardiac fast 1 | 11.0              | 1.1           | 1.9               | A   | –    | –   |
| Troponin I, skeletal, slow                | 10.5              | 0.9           | 2.7               | A   | –    | –   |
| Myosin, light polypeptide 1              | 9.3               | 0.7           | 1.6               | A   | –    | –   |
| Retinoblastoma 1                         | 5.5               | 1.6           | 1.5               | A   | –    | –   |
| Myosin-binding protein H                  | 4.7               | 1.2           | 1.2               | A   | –    | –   |
| Sarcoglycan, β                           | 3.9               | 0.5           | 1.5               | A   | –    | –   |
| Myocyte enhancer factor 2A (MEF2A)       | 3.1               | 1.2           | 1.4               | P/A | 0.8  | 0.9 |
| Growth arrest and DNA damage-inducible 45 α| 2.5               | 0.9           | 1.1               | P/A | –    | –   |
| Cyclin-dependent kinase inhibitor 1A (P21)| 2.2               | 1.1           | 1.0               | P   | 1.4  | 1.4 |

1Mean fold change for pairwise comparisons of MyoD/Myf5/myogenin versus GFP from log(fold) change in MAS 5.0 software (Affymetrix, Inc.).
2Present/marginal/absent call from MAS 5.0.
3Fold change based on ΔΔCt between MyoD/Myf5 versus puromycin alone real-time PCR, normalized to GAPDH expression.
4Added after manual inspection of dataset.
5Ct1a 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 1). 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 ChrmB1 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 1). These genes tended to be those that had modest fold changes in the GeneChip data (e.g., Hes6, Pki, 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 representative 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−/− primary myoblasts of the genes in Table 1 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 NH2 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
divergent NH2- and COOH-terminal domains rather than as a result of the bHLH recognition of discrete DNA sequences. To test this, chimeric MRFs were built, interchanging the corresponding NH2-terminal, bHLH-, and COOH-terminal regions of MyoD and Myf5. 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 ~105 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., Chrmβ1, 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 NH2- 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 (Chrmβ1 and Runx1, identified in the aforementioned GeneChip experiment) showed little relative difference between MyoD, Myf5, and the chimeras. In concordance with the Gene-
Chip results, however, the expression of MyoD produced a considerable activation of genes such as the cholinergic receptor \( \alpha \) and \( \gamma \) subunits, myogenin, \( \alpha \)-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 NH2 terminus alone (d55) also resulted in just moderate gene induction relative to full-length Myf5. Importantly, the concurrent presence of MyoD NH2- 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, 5d5, 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 NH2 terminus also had a noticeable effect on differentiation (Fig. 5 B, compare MyoD with 5d5, 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 5d5 with 55d) but had little effect otherwise. Overall, the combined NH2- and COOH-terminal portions of MyoD (MyoD and 5d5) 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 NH2- 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.
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 Chrm, Chrna1, 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-a7, Plgf, VCAM1, Igf4a, Terov13, laminin-a5, neurtin-1, and Kira18) 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 dbKO 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, Mcp8, 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

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Table II. Fold activation of myogenic genes by real-time PCR1

| Gene   | MyoD | d5d | Sdd | Ssd | dds | d5S | S5d | Myf5 | GFP |
|--------|------|-----|-----|-----|-----|-----|-----|------|-----|
| Acta1  | 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 |
| Myf5   | 2645.3| 2727.9| 249.2 | 356.0 | 211.1 | 234.3 | 62.4 | 81.8 | 1.0 |
| Chrm1  | 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 |
| ChrmA1 | 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 | 1.6 | 1.8 | 1.4 | 1.3 | 1.0  | 1.0 |
| ChrmB1 | 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 | 2.3 | 2.8 | 2.0 | 1.9  | 1.0 |

1Relative to GAPDH and normalized to GAPDH but not to FLAG protein.
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 NH2-, 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 chimeric MyoD bHLH domain levels of differentiation gene expression, whereas the other chimeras, Myf5, and myogenin were many times less effective. The MyoD NH2- 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 NH2- 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 NH2-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.)

Figure 5. MyoD NH2 and COOH termini cooperatively promote differentiation. The expression of MyoD/Myf5 chimeric MRFs that included the MyoD NH2- 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.
al., 2004). Therefore, MyoD may use its COOH-terminal chromatin-remodeling domain to provide access to silenced muscle genes for the NH2-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 G1 of the cell cycle, whereas Myf5 levels are high in S/G2, and G0 in association with proliferation and a failure to differentiate for differentiation.

Levels of MyoD occur only at a cell cycle point that is appropriate (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 dbKO 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 (Megemen et al., 1996; Sabourin et al., 1999).

Myf5, MyoD, chimeric MRFs, or myogenin were introduced into dbKO 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 (IRE5hrGFP) sequence (Stratagene). MRFs that were expressed using the retroviral plasmid were COOH-terminally tagged with a 3X FLAG epitope. Empty control virus expressing only puromycin resistance or hrGFP. 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 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**

2 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 RNasy Mini Kit (QiAGEN) according to the manufacturer’s instructions and quantitated by OD260 or by Bioanalze (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 Otawa 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. Probescets 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. Probescet annotations were obtained from http://www.affymetrix.com/analysis/index.affx. Microarray data is available from StemBase (http://www.sccp.ca/SB080/StemBase/; Ontario Genomics Innovation Centre; Perez-fratke 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 2X TaqSYBR Green SuperMix (BioRad 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. G 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 NH2- and COOH-terminal regions to the central bHLH domain (Fig. 3 A). The 318 aa of Myf5 (available from GenBank/EMBL/DDJB under accession no. NM_010866) were divided into aa 1–96 (NH2 terminus), 97–161 (bHLH), and 162–318 (COOH terminus). The 255 aa of Myf5 (available from GenBank/EMBL/DDJB under accession no. NM_008656) were divided into aa 1–70 (NH2 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).
Immunochemistry and Western blot analysis

Primary antibodies that were used are listed as follows: mouse anti-FLAG (M2 and M5; Sigma-Aldrich), rabbit anti-Myc5 (C-20; Santa Cruz Biotechnology, Inc.), rabbit anti-MyoD (C-20; Santa Cruz Biotechnology, Inc.), mouse anti-MyoD (S2F; Sigma-Aldrich), mouse antmyogenin (F5D) and mouse anti-myosin heavy chain (MF20; hybridoma supernatant), mouse antdesmin (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 dMS/PBS. Secondary antibodies were applied in 5% goat serum–PBS. Secondary antibodies were applied in 5% goat serum–PBS. Secondary antibodies were applied in 5% goat serum–PBS. Secondary antibodies were applied in 5% goat serum–PBS. Secondary antibodies were applied in 5% goat serum–PBS. Secondary antibodies were applied in 5% goat serum–PBS. Secondary antibodies were applied in 5% goat serum–PBS. Secondary antibodies were applied in 5% goat serum–PBS. Secondary antibodies were applied in 5% goat serum–PBS. Secondary antibodies were applied in 5% goat serum–PBS. Secondary antibodies were applied in 5% goat serum–PBS. Secondary antibodies were applied in 5% goat serum–PBS. 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