Two domains of MyoD mediate transcriptional activation of genes in repressive chromatin: a mechanism for lineage determination in myogenesis

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Genetic studies have demonstrated that MyoD and Myf5 establish the skeletal muscle lineage, whereas myogenin mediates terminal differentiation, yet the molecular basis for this distinction is not understood. We show that MyoD can remodel chromatin at binding sites in muscle gene enhancers and activate transcription at previously silent loci. TGF- β , basic-FGF, and sodium butyrate blocked MyoD-mediated chromatin reorganization and the initiation of transcription. In contrast, TGF- β and sodium butyrate did not block transcription when added after chromatin remodeling had occurred. MyoD and Myf-5 were 10-fold more efficient than myogenin at activating genes in regions of transcriptionally silent chromatin. Deletion mutagenesis of the MyoD protein demonstrated that the ability to activate endogenous genes depended on two regions: a region rich in cysteine and histidine residues between the acidic activation domain and the bHLH domain, and a second region in the carboxyl terminus of the protein. Neither region has been shown previously to regulate gene transcription and both have domains that are conserved in the Myf5 protein. Our results establish a mechanism for chromatin modeling in the skeletal muscle lineage and define domains of MyoD, independent of the activation domain, that participate in chromatin reorganization.

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The myogenic family of basic helix-loop-helix (bHLH) proteins is composed of MyoD, Myf-5, myogenin, and MRF4 (Buckingham 1992; Edmondson and Olson 1993; Weintraub 1993). Each of these proteins recognizes a simple consensus sequence of CANNTG, termed an E box, present in the regulatory regions of many genes expressed in skeletal muscle (Blackwell and Weintraub 1990). In transfection assays, each of these proteins drives transcription of skeletal muscle reporters and can initiate expression of endogenous skeletal muscle genes, although some differences have been reported on specific enhancer elements (Asakura et al. 1993). Although these experiments suggested redundancy within the myogenic bHLH family, genetic studies demonstrated that MyoD and Myf-5 act to establish the myogenic lineage, whereas myogenin mediates the expression of the terminal phenotype (Weintraub 1993; Rudnicki and Jaenisch 1995). Specifically, mice lacking both MyoD and Myf-5 do not develop any identifiable myoblasts (Rudnicki et al. 1993). In contrast, mice lacking myogenin generate myoblasts, but show incomplete skeletal muscle differentia-

tion, with fewer and smaller myotubes (Hasty et al. 1993; Nabeshima et al. 1993; Venuti et al. 1995). The differences between the myogenic genes that confer these specific attributes are unknown, but could be encoded by their regulatory regions, their protein sequences, or both.

Initiating gene transcription at a previously silent locus is one of the hallmarks of establishing a lineage. Chromatin repression and derepression of gene transcription in a developmental context has been studied extensively, particularly with regard to genes expressed in a tissue-restricted fashion (Weintraub 1985; Wolffe 1992; Felsenfeld et al. 1996). It is generally believed that regions of repressive chromatin act to silence certain lineage-specific genes, suppressing basal levels of transcription in nonexpressing lineages, and that developmentally regulated chromatin remodeling can increase the access of transcription factors to *cis*-acting regulatory regions of genes expressed in a particular lineage. For example, chromatin contributes to the repression of β -globin gene transcription in nonerythroid cells, whereas during erythropoiesis the chromatin at specific regulatory regions of the *globin* loci is remodeled into an active, derepressed state that permits greater access of tran-

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scription factors, manifested by the appearance of regions that have increased accessibility and sensitivity of the DNA to nucleases (Weintraub and Groudine 1976; Stalder et al. 1980; Reitman and Felsenfeld 1990). Whereas the generation of DNase hypersensitive sites at regulatory regions during development is well described, the mechanism for remodeling the chromatin in any lineage remains poorly understood. In one of the best-characterized examples, DNA-binding sites for the lineage-restricted transcription factors GATA-1 and NF-E2 have been shown to be necessary for the formation of hypersensitive sites in the β -globin loci (Philipsen et al. 1993; Stamatoyannopoulos et al. 1995; Boyes and Felsenfeld 1996) and binding of GATA-1 and NF-E4 can act to protect a β -globin gene template from chromatin-mediated transcriptional repression in vitro (Barton et al. 1993), yet the transcription factor characteristics required to mediate chromatin remodeling remain unclear.

There are some notable examples, however, that demonstrate the mechanisms by which transcription factors can activate gene expression within a repressive chromatin context. Work in a number of model systems has supported the basic notion that some transcription factors can recognize binding sites within a positioned nucleosomal array and can direct modifications of chromatin structure that allow gene activation to take place (Archer et al. 1992; Morse 1993). For instance, when yeast cells are cultured in high phosphate, positioned nucleosomes are present at the *PHO5* gene. With phosphate starvation, the bHLH protein Pho4 binds to an intranucleosomal binding site. This causes a perturbation in nucleosome positioning that allows subsequent binding of Pho4 and other factors to sites that were occluded previously by the nucleosomal array, a process that is dependent on the Pho4 activation domain (Fascher et al. 1993; Svaren et al. 1994; Venter et al. 1994). In vitro chromatin studies with the GAL4 transcription factor suggest a two-phase process for nucleosome displacement (Cote et al. 1994; Owen-Hughes et al. 1996). An initial disruption of the nucleosome is caused by the binding of the transcription factor, which recruits a multiprotein complex, such as the SWI/SNF complex, that completes nucleosome eviction in a manner dependent on ATP utilization. Similarly, the *Drosophila* GAGA factor has been shown to regulate chromatin structure, and in vitro assays suggest that it may function with a multiprotein nucleosome remodeling factor (NURF) that contains a protein related to the SWI2/SNF2 protein (Granok et al. 1995; Tsukiyama and Wu 1995; Tsukiyama et al. 1995). Therefore, the general notion has emerged that sequence-specific transcription factors can recruit multiprotein nucleosome remodeling complexes to specific sites in the genome. Yet in a developmental context, very little is known regarding the relative abilities of transcription factors to initiate lineage-specific chromatin remodeling.

In the skeletal muscle lineage, the generation of muscle-specific chromatin alterations may be mediated by the MyoD family of transcription factors. Expression of MyoD in cultured cells activates both transfected re-

porter genes driven by skeletal muscle promoters and the cognate endogenous genes (Weintraub et al. 1989). Binding sites for numerous other transcription factors are found in combination with E boxes in the regulatory regions of muscle genes and may contribute to initiating or maintaining transcription (Biben et al. 1994; Catala et al. 1995); however, no factors other than MyoD family members have been demonstrated clearly to be capable of activating endogenous muscle gene expression. For example, myocyte enhancer factor 2 (MEF2) sites are found in tandem with E boxes in the regulatory regions of many muscle genes (Li and Capetanaki 1994; Malik et al. 1995) and are critical for high-level, muscle-specific activation of reporter constructs in transient transfections and in transgenic mouse models (Adolph et al. 1993; Cheng et al. 1993). Whereas members of the MEF2 family activate transfected muscle-specific reporters and cooperate with MyoD family members in the activation of endogenous muscle genes (Black et al. 1995; Molken- tin et al. 1995; Naidu et al. 1995), expression of MEF2 family members alone does not activate endogenous muscle gene expression (Molkentin et al. 1995), suggesting that MEF2 activity may be repressed by the native chromatin at transcriptionally silent skeletal muscle genes. Repressive chromatin may be the reason that many transcription factors enhance the expression of transfected reporter constructs but fail to activate expression of the endogenous copies of the target genes, because the chromatin packaging of transfected DNA does not recapitulate the native chromatin architecture (Reeves et al. 1985; Jeong and Stein 1994).

In this study we show that MyoD can remodel chromatin in the regulatory regions of skeletal muscle genes, an activity that is regulated by transforming growth factor (TGF- β) and basic fibroblast growth factor (bFGF). The ability to initiate endogenous gene transcription and remodel chromatin depended on two regions of MyoD that previously had no defined activity. These regions are separate from the activation and DNA-binding domains of MyoD, but have elements that are highly conserved in the Myf5 protein. Consistent with their genetically determined role in establishing the skeletal muscle lineage, MyoD and Myf5 can initiate transcription from genes in repressive chromatin with a 10-fold higher efficiency than myogenin. We conclude that MyoD and Myf5 contain specific domains that mediate chromatin remodeling, and suggest that the chromatin structure established by these lineage-determining proteins constrains the activity of other transcription factors, including myogenin, that mediate the expression of genes during differentiation. The demonstration that MyoD can remodel chromatin under the regulation of growth factors establishes a mechanism for lineage determination during myogenesis.

Results

MyoD remodels chromatin at the myogenin locus

The proximal regulatory region of the *myogenin* gene has been characterized previously. A well-conserved

180-bp fragment immediately upstream from the *myogenin* start site is sufficient to recapitulate *myogenin* expression patterns in transgenic mice (Cheng et al. 1993; Yee and Rigby 1993). This fragment contains two E boxes and an AT-rich region that can bind MEF2. Faithful recapitulation of the *myogenin* expression pattern during development requires the proximal E-box and the MEF2 site, indicating that MyoD and MEF2 family members regulate the expression of *myogenin* (Cheng et al. 1993). We have used 10T1/2-MyoD-ER (estrogen receptor) cells, a fibroblast cell line that expresses a fusion protein combining MyoD with the hormone-binding domain of the estrogen receptor, to analyze chromatin structure at the *myogenin* locus. 10T1/2-MyoD-ER cells can be induced to differentiate synchronously into skeletal muscle by the addition of β -estradiol (Hollenberg et al. 1993). In 10T1/2-MyoD-ER cells, *myogenin* transcription can be activated by MyoD in the presence of cycloheximide (see Fig. 5B, below), indicating that MyoD, in concert with ubiquitously expressed factors, is capable of directly activating *myogenin* transcription.

As an initial step in characterizing chromatin structure at the *myogenin* locus, DNase I was used to determine whether hypersensitive sites were present either before or after induction of transcription. Before induction of 10T1/2-MyoD-ER cells with estradiol, DNase I-hypersensitive sites were not detected between about -2000 and +1000 bp (Fig. 1A), a region that includes sequences that are sufficient for *myogenin* expression in transgenic mice. However, after 48 hr of induction, two hypersensitive sites became evident (Fig. 1B, arrows). The proximal hypersensitive site mapped near the MyoD binding site at -15 bp, whereas the more distal hypersensitive site mapped near the MEF2 site located at -75 bp (Fig. 1C). This indicates that MyoD can induce *myogenin* transcription without a pre-existing hypersensitive site present in this region, and transcriptional activation is accompanied by regional chromatin reorganization.

We also analyzed nucleosome positioning around the *myogenin* promoter using micrococcal nuclease (MNase), which preferentially cleaves linker DNA. Identical banding patterns were observed with 10T1/2 cells treated with estradiol (Fig. 2A) and with uninduced 10T1/2-MyoD-ER cells (not shown). Regularly spaced areas of protection of ~200 bp, consistent with the presence of a core nucleosome at each protected region, extended >1 kb upstream from the start site (Fig. 2A). Clear regions of protection were centered at the start site and at -200 bp, indicative of specifically positioned nucleosomes. The nucleosome positioned at the start site covers the proximal E box and the AT-rich region. In contrast, after the induction of *myogenin* transcription by estradiol treatment of 10T1/2-MyoD-ER cells, MNase digestion resulted in a pattern that was less distinct than in 10T1/2 cells that did not express the MyoD-ER construct, suggesting that the nucleosomes were not positioned as specifically following induction (Fig. 2, cf. A and B). We conclude that MyoD induces a relaxation of nucleosome positioning at the *myogenin* promoter, po-

Figure 1. DNase hypersensitive sites develop at the *myogenin* locus after induction. Isolated nuclei from uninduced and induced 10T1/2 MyoD-ER cells were incubated with increasing concentrations of DNase I for 15 min at 37°C. Following DNase I digestion, reactions were stopped, and the DNA was purified, digested with *Hind*III and *Eco*RI, and analyzed by Southern blot as indicated in the schematic. (A) No estradiol treatment: (lane 1) No DNase; (lane 2) 0.2 units; (lane 3) 0.4 unit; (lane 4) 0.8 unit; (lane 5) 1.2 units; (lane 6) 1.6 units; (lane 7) 2.4 units. (B) Forty-eight hours of estradiol treatment. (lane 1) No DNase; (lane 2) 0.2 unit; (lane 3) 0.4 unit; (lane 4) 0.6 unit; (lane 5) 0.8 unit; (lane 6) 1.2 units; (lane 7) 1.6 units; (lane 8) 2.4 units. The arrows indicate hypersensitive site bands. (C) Approximate locations of probes, enzyme sites, and factor binding sites. (E) *Eco*RI; (H) *Hind*III; (*) E-box; (++) *Mef*II site. Arrows indicate approximate locations of HS sites, mapped using the blots in A and B, and additional Southern analysis not shown.

tentially increasing access of factors to the proximal E box and the MEF2-binding site that are incorporated in a nucleosome in uninduced fibroblasts.

To test whether MyoD expression and the apparent relaxation of nucleosome phasing increased the access of factors to the *myogenin* promoter, we used restriction endonucleases to assess induction-associated changes in factor access at specific sites in *myogenin* chromatin, similar to restriction endonuclease access studies performed in other systems (McGhee et al. 1981; Loo and Rine 1994). Endonuclease access was assayed at eight sites ranging from a *Cl*aI site at about -6 kb to a *P*stI site at about +1100 bp. Figure 3A shows Southern blots of restriction endonuclease access for several representative sites. For each experiment, the amount of DNA cleavage was determined when the enzyme was added to isolated nuclei (lanes marked N) or to purified DNA (lanes marked P), and the ratio of the N/P band intensity is referred to as the percentage of enzyme access in nuclei (see Fig. 3; Materials and Methods). Following induction, enzyme access increases to ~50% in the proximal promoter region (Fig. 3B: -370 *P*vuII, -170 *P*stI, and -20 *B*anI) and in the coding region (Fig. 3B: +1040 *B*anI, +1070 *P*stI), indicating a significant relaxation of chromatin structure in this region. This is consistent with the MNase results that showed disrupted nucleosome

Figure 2. A phased nucleosomal array at the *myogenin* locus is modified with induction. Nuclei from 10T1/2 cells (A) and 10T1/2-MyoD-ER cells (B) were isolated after 48 hr induction with estradiol and incubated with 50 U/ml of MNase for increasing amounts of time as indicated. Following MNase treatment, DNA from each sample was purified, digested with *Bam*HI, Southern blotted, and hybridized to the probe indicated in the schematic (solid rectangle). Regions of clear protection from MNase digestion, which represent locations of nucleosome cores, are indicated by solid arrows and are evident in A, lanes 2–5. In B the open arrowheads indicate positions 0 and –200 bp, comparable to arrows in A. (*) Position of the proximal hypersensitive site seen in Fig. 1.

phasing in the proximal promoter region with induction, whereas increased enzyme access in the coding region is consistent with reports that the passage of the transcriptional machinery moves nucleosomes (Clark and Felsenfeld 1992). More modest increases in enzyme access were observed further upstream at the –1100-bp *Nde*I site and the –1565-bp *Xba*I site, and no change in access was observed at the –6000-bp *Cla*I site. Although a less extensive analysis was performed, similar results were obtained using NIH-3T3 cells, indicating that the chromatin remodeling is not specific to 10T1/2 cells. Considered together, the DNase I, MNase, and restriction endonuclease analyses indicate that MyoD expression disrupted a phased nucleosome array at the *myogenin* promoter and relaxed chromatin structure modestly >1 kb upstream of the promoter.

Although MyoD can activate *myogenin* transcription directly (Hollenberg et al. 1993), it was not clear whether the changes in chromatin structure described above were mediated by MyoD directly, or, alternatively, whether factors induced by MyoD, such as MEF2, effected the changes in chromatin. To address this issue, we assayed endonuclease access to the *myogenin* promoter using nuclei isolated from cells induced with estradiol in the presence of cycloheximide. Cells were induced with estradiol for 10.5 hr, at which time *myogenin* mRNA levels were similar in cells treated with estradiol alone or with estradiol plus cycloheximide (Fig. 4B, cf. lanes 2 and

4), and enzyme access was determined at the –170-bp *Pst*I site and the +1080-bp *Pst*I site (Fig. 4A). In the absence of induction, cycloheximide increased the restriction enzyme access approximately twofold (Fig. 4A, probe A, cf. lanes 1 and 5), and there was an additional twofold increase in access following induction in the presence of cycloheximide (cf. lanes 5 and 7). Although this twofold increase in access at the –170 bp *Pst*I site following induction in cycloheximide was notably smaller than the 6- or 10-fold increase at the –170-bp *Pst*I site seen without cycloheximide (Fig. 4A, probe A, cf. lanes 1 and 3), a two- to threefold increase in enzyme access following induction in cycloheximide was consistent among several experiments (see, e.g., Fig. 4C, cf. lanes 1 and 3). Access to the –370-bp *Pvu*II and the –20-bp *Ban*I sites showed a similar two- to threefold increase with induction in cycloheximide (data not shown), whereas in the coding region induction increased enzyme access at the +1070-bp *Pst*I site to similar levels with and without cycloheximide treatment (Fig. 4A, probe B, cf. lanes 3 and 7). Therefore, we conclude that MyoD initiates chromatin reorganization directly and that newly synthesized factors cooperate with MyoD to achieve complete remodeling of the chromatin structure at the *myogenin* promoter.

We next tested the effect of depleting ATP on the induction of chromatin reorganization by using the metabolic poisons sodium azide and deoxyglucose. These agents deplete cellular ATP (Lorico et al. 1995) and prevented the generation of detectable *myogenin* transcripts after estradiol induction (Fig. 4B, lane 6), presumably by inhibiting RNA polymerase II (Pol II), which has been shown to require ATP both for transcription initiation and elongation (Jiang et al. 1996). Treatment with sodium azide and deoxyglucose did not, however, prevent an increase of enzyme accessibility to the –170-bp *Pst*I site in the promoter (Fig. 4C, cf. lanes 5 and 7), similar to the results seen with cycloheximide (Fig. 4C, cf. lanes 3 and 7). Thus, MyoD can initiate chromatin reorganization at the *myogenin* promoter in the absence of detectable *myogenin* transcription and when cellular ATP is depleted.

MyoD alters chromatin structure at distal enhancer elements of other muscle genes

To determine whether MyoD alters chromatin structure at other skeletal muscle genes, we measured the accessibility of restriction endonucleases at sites adjacent to the MyoD-binding sites in the enhancer regions of two other genes regulated by MyoD, *muscle creatine kinase* (*MCK*) and *myoD* (Jaynes et al. 1988; Tapscott et al. 1992). Similar to *myogenin*, enzyme access increased in the regulatory regions of these genes following 48 hr of induction (Fig. 5A–C), indicating that chromatin alterations occur with induction at the *MCK* and *myoD* loci. The regulatory regions of *myoD* and *MCK* that were analyzed in these experiments are 5.2 and 1.2 kb upstream of the respective transcription start sites, demonstrating

Figure 3. Restriction enzyme access to the *myogenin* locus increases with induction. (A) Southern blot analysis assessing changes in enzyme activity at the *myogenin* locus with induction in 10T1/2–MyoD–ER cells. Nuclei were isolated from uninduced cells (estradiol–) and cells induced for 48 hr (estradiol +). Nuclei were incubated at 37°C for 30 min with 40 units of a given restriction endonuclease (assay enzyme). Reactions were stopped and the DNA was purified and digested to completion with flanking enzymes as depicted in the schematics below each blot. To express the accessibility of the site in isolated nuclei relative to purified DNA as a percentage, half the sample was digested further to completion with the assay enzyme. (P) Samples exposed to the assay enzyme as purified DNA; (N) samples exposed to the assay enzyme only as isolated nuclei. Bands generated by cleavage with the assay enzyme are indicated by arrows. The percent accessibility of each site was determined as the density ratio of the arrowed bands for each nuclei/purified sample pair using a Molecular Dynamics PhosphorImager. (B) Schematic showing percent accessibility to sites in the *myogenin* locus with and without induction, calculated using the procedure described above. Results shown are representative of the typical experiment.

that chromatin alterations can be induced at sites relatively distant from promoter elements.

Several control experiments were performed to demonstrate that chromatin changes with induction were attributable to MyoD activity and were specific to skeletal muscle genes. Access to a *BanI* site in the locus control region (LCR) of the β -globin gene, which is not transcribed in skeletal muscle, did not increase with induction in 10T1/2–MyoD–ER cells, and estradiol treatment of 10T1/2 cells that lack the MyoD–ER protein did not change the access of *BanI* to the *myogenin* promoter (Fig. 5D,E).

Previous work has shown that both MyoD and E proteins can bind to the E boxes in the enhancer of the *immunoglobulin heavy chain* (*IgH*) gene and regulate transcription of *IgH* reporter constructs in transfection assays (Weintraub et al. 1994). Because MyoD can interact functionally with these E boxes, yet the *IgH* gene is not transcribed in skeletal muscle, we wanted to determine whether MyoD expression would alter enzyme access at this enhancer. The enhancer contains three functional E boxes, one of which, E2, is identical to the E box in the *myogenin* promoter at –370 bp. In contrast to the

marked increase in *PstI* access to a site adjacent to the E box in the *myogenin* promoter (Fig. 3), access of *PstI* to a site directly adjacent to the E2 E box remained low following induction of 10T1/2–MyoD–ER cells with estradiol (Fig. 5F). Together, the data shown in Figure 5 indicate that MyoD relaxed chromatin in the region of MyoD-binding sites specifically at genes that are expressed in skeletal muscle.

Growth factors prevent MyoD-mediated chromatin relaxation

The activity of MyoD as a transcription factor can be inhibited by a number of agents in cell culture, potentially reflecting mechanisms that modulate the activity of MyoD in vivo. For example, sodium butyrate, which inhibits histone deacetylase, inhibits MyoD activity and myogenesis in cell culture (Johnston et al. 1992). Likewise, the growth factors TGF- β and bFGF, which are present in the developing limb bud and somite, can inhibit the activity of both MyoD and myogenin in cell culture (Hannon et al. 1992; Li et al. 1992; Martin et al. 1992). Furthermore, TGF- β has been shown recently to

Figure 4. MyoD induces changes in chromatin structure at the *myogenin* promoter when protein synthesis or ATP production are blocked. (A) Southern blot of *myogenin* locus showing enzyme access to the *Pst*I site in the promoter (probe 1) or the coding region (probe 2) of uninduced (estradiol-) and induced (estradiol +) cells treated with and without cycloheximide (CHX) (50 μ g/ml.) Nuclei were isolated and percent access to the *Pst*I site was determined as described in Fig. 3 and in Materials and Methods, using the flanking enzymes *Nco*I and *Eco*RI (schematic). The same blot was probed sequentially with probe 1 and 2. (*) Residual signal from the first probe; (N) nuclei; (P) purified. (B) Northern analysis using RNA from uninduced and induced cells treated with CHX or the metabolic inhibitors (MI), sodium azide (.1%), and deoxyglucose (5 mM) as indicated. (C) Southern blot showing enzyme access to the *Pst*I site in the *myogenin* promoter of cells treated as indicated. For A–C, cells were induced for 10.5 hr.

modulate the interaction between a domain of the CTF-I transcription factor and histone H3 (Alevizopoulos et al. 1995). To determine whether these agents could prevent MyoD-mediated relaxation of chromatin structure, 10T1/2–MyoD–ER cells were cultured in the presence of sodium butyrate, bFGF, or TGF- β , and enzyme access to the *Ban*I site in the *myogenin* promoter was assessed after 24 hr of estradiol induction (Fig. 6A). All three agents inhibited MyoD-induced increases in enzyme access to this site (cf. lanes 2, 4, 6, and 8). The accumulation of *myogenin* message also was inhibited by the addition of these agents at the time of induction (Fig. 6C, lanes 2,7; data not shown). Therefore, TGF- β , bFGF, and sodium butyrate inhibit the ability of MyoD to induce chromatin remodeling at the *myogenin* locus and block the initiation of *myogenin* transcription.

To test whether these agents were able to block transcription at the *myogenin* locus when an active chromatin conformation and transcription complex was already established, we assayed *myogenin* mRNA levels and enzyme access in 10T1/2–MyoD–ER cells that had been induced in differentiation medium supplemented with estradiol for 24 hr, followed by growth for an additional 24 hr in differentiation medium alone, or differentiation medium supplemented with either sodium butyrate or TGF- β (Fig. 6B,C). With estradiol treatment alone, high

levels of *myogenin* mRNA were present at 24 hr (Fig. 6C, lanes 1,6) and further increased at 48 hr (Fig. 6C, lanes 3,8). *Myogenin* expression between 24 and 48 hr in the absence of continued estradiol treatment reflects a stable activation state that is likely maintained by autoregulation. TGF- β and sodium butyrate added at the time of induction suppressed mRNA accumulation (lanes 2,7), whereas cells induced with estradiol for 24 hr and then exposed to TGF- β or sodium butyrate for 24 hr maintained the level of *myogenin* RNA present at 24 hr of induction (lanes 4,9). Because the half-life of *myogenin* mRNA is <8 hr (data not shown), the mRNA levels after 24 hr of treatment with TGF- β or sodium butyrate were not simply attributable to message stability, and we conclude that *myogenin* transcription persists in the presence of either inhibitor. Furthermore, the chromatin conformation at the *myogenin* locus remained accessible to restriction enzymes in cells treated with TGF- β or sodium butyrate following 24 hr of induction (Fig. 6B, lanes 3,5).

It is unlikely that induction makes the cells unresponsive to these agents, because the expression of muscle genes that had not been fully activated at 24 hr of induction were inhibited by subsequent addition of TGF- β or sodium butyrate. For example, Figure 6C shows that *myosin heavy chain* (*MyoHC*) mRNA increased dramati-

Figure 5. Chromatin structure is altered in the regulatory regions of several muscle genes with induction. (A–D) Isolated nuclei from uninduced (lane 1–estradiol) and induced (lane 2 +estradiol) 10T1/2 MyoD–ER cells were incubated with 40 units of *Ban*I enzyme for 30 min. Reactions were stopped and Southern blotted as indicated in the schematics, which show the approximate locations of probes, selected enzyme sites, and E boxes within each regulatory region. Lane 3 (naked) of each blot is purified DNA-digested to completion with *Ban*I and flanking enzymes. (E) Similar to Fig. 4A, except samples were isolated from 10T1/2 cells not expressing the *myoD*–ER construct. (F) Similar to above, except *Pst*I access to a site in the *IgH* enhancer was assessed. (Ap) *Apa*I; (B) *Bam*HI; (H) *Hind*III; (N) *Nco*I; (P) *Pvu*II; (R1) *Eco*RI; (X) *Xho*I; (*) E-box.

cally between 24 and 48 hr (cf. lanes 1 and 3), but addition of TGF- β at 24 hr blocked the increase in mRNA levels substantially (cf. lanes 3 and 4). Therefore, the ability of TGF- β and sodium butyrate to inhibit transcription depended on whether an active chromatin structure and transcription complex had been established at the target gene. This could represent a mechanism for differential regulation of gene transcription by these agents.

MyoD is less sensitive to chromatin repression than myogenin

Although there is functional redundancy within the MyoD family (Wang et al. 1996), MyoD and Myf-5 act preferentially in myoblast specification, whereas myogenin and MRF4 drive terminal differentiation (Nabeshima et al. 1993; Venter et al. 1994; Venuti et al. 1995). To determine whether MyoD and myogenin had equal abilities to activate transcription of genes in inactive chromatin, we analyzed the relative abilities of MyoD and myogenin to activate endogenous muscle genes. Initially, we characterized activation of endogenous *desmin* and *MyoHC* genes in 3T3 cells transfected

with expression plasmids containing the enhancer of the murine sarcoma virus driving MyoD or myogenin, pEMSV–MyoD, or pEMSV–myogenin. We used 3T3 cells because the endogenous *myoD* gene is not autoactivated following transfection of *myoD* expression vectors (Thayer et al. 1989); however, similar results were obtained using 10T1/2 cells. Cells were also transfected with pCMV–*lacZ* to control for transfection efficiency and p4RTK–CAT, which consists of four multimerized E boxes driving *chloramphenicol acetyltransferase* (CAT) (Weintraub et al. 1991). The simultaneous assay of transcription factor activity on a transfected reporter and on an endogenous gene was used to determine the relative efficiency of the factor to activate a gene that is in a nontranscribed region of genomic chromatin. Following overnight calcium phosphate transfection, cells were placed in differentiation medium for 24 hr and then double-stained with antibodies to MyoHC and CAT or MyoHC and desmin. MyoD and myogenin activated transcription from the p4RTK–CAT plasmid with similar efficiencies, based on counts of CAT-positive cells in equal areas of each dish and quantitative CAT assays from sister plates (Table 1). MyoD activated transcription of the endogenous *MyoHC* and *desmin* genes at a frequency equal to its activation of p4RTK–CAT, with a

Figure 6. Effects of growth factors and sodium butyrate on chromatin structure and muscle gene expression. (A) Southern analysis of access to the *BanI* site in the *myogenin* promoter with and without induction, and with and without basic FGF (25 ng/ml), TGF- β (0.2 nM), or sodium butyrate (5 mM) treatment as indicated. (B) Southern analysis of access to the *BanI* site at the *myogenin* promoter with TGF and butyrate treatment following 24 hr of induction. (C) Northern analysis of myogenin and myosin heavy chain expression with TGF and butyrate treatment. Agents were added at either the time of induction or following 24 hr of induction as indicated.

ratio of MyoHC/CAT-positive cells of 0.9 and a desmin/CAT ratio of 1.03. In contrast, myogenin showed many fewer MyoHC- and desmin-positive cells, with a MyoHC/CAT ratio of 0.08. The overall transfection efficiencies were similar based on staining of duplicate

plates for MyoD, myogenin, or LacZ (data not shown). Northern analysis using a probe to *MyoHC* confirmed that the number of cells expressing each protein reflected levels of transcript present in the cell population (data not shown), supporting our interpretation that the

Table 1. Activation of endogenous muscle genes compared to transfected reporters by MyoD and myogenin

Experiment 1 ^a	MyoHC	4RTK-CAT	Ratio (MyoHC/4RTK-CAT)		Desmin	Ratio (desmin/4RTK-CAT)			
MyoD	86	96 (9.9×)	0.90		99	1.03			
Myogenin	12	151 (7.5×)	0.08		3	0.02			
Experiment 2 ^b	MyoHC	4RTK-CAT	Ratio	Desmin	Des-CAT	Ratio	MCK	MCK-CAT	Ratio
MyoD	61	52 (7.4×)	1.2	70	33 (8.8×)	2.12	6	29	0.21
Myogenin	10	114 (6.8×)	0.09	6	28 (8.9×)	0.21	0	21	0

^aCells were cotransfected overnight with either pEMSV-MyoD or pEMSV-myogenin and p4RTK-CAT, and prepared for immunohistochemistry after 24 hr in differentiation medium. The integers are counts of cells staining positive with antibody to the indicated muscle marker or to CAT within a standard area of a dish. Totals for MyoHC and CAT reflect cells within the same area on the same dish, whereas for desmin, equivalent areas of sister dishes were counted. The ratio was calculated using the cell count totals. Numbers in parentheses reflect fold activation attained in a quantitative CAT assay using sister dishes, relative to the parent EMSV vector.

^bCells were cotransfected with either pEMSV-MyoD or pEMSV-myogenin, and either p4RTK-CAT or pDesmin-CAT or p3300MCK-CAT. As in experiment 1, integers are counts of cells staining positive as indicated within a standard area of each dish, and parenthetical numbers reflect fold activation in quantitative CAT assays. For MyoHC and CAT, the same areas on the same dish were counted, whereas for desmin vs. CAT and MCK vs. CAT, equivalent areas on sister dishes were counted. The muscle marker to CAT ratios were calculated using the cell count totals.

indirect immunofluorescent assay of protein expression represented relative gene activity.

Therefore, relative to their activity on p4RTK-CAT, MyoD was more efficient than myogenin in activating the endogenous *desmin* and *myoHC* genes, which may reflect differences in the abilities of MyoD and myogenin to activate genes in an inactive region of chromatin. Alternatively, the *MyoHC* and *desmin* genes may be activated preferentially by MyoD as a result of binding-site specificity for MyoD or interaction with other transcription factors that bind the enhancer, as has been described previously for E boxes in the *myosin light chain* regulatory region (Asakura et al. 1993). To address the latter possibility, we determined the relative abilities of MyoD and myogenin to activate transcription of the endogenous *desmin* gene compared with a transfected p1.7des-CAT reporter gene, and the endogenous *MCK* gene compared with a transfected p3300MCK-CAT reporter gene. The *desmin* CAT reporter contains 1.7 kb of sequence 5'-' of the transcription start site (Pieper et al. 1987), and the MCK-CAT has 3.3 kb of upstream sequence (Jaynes et al. 1988). Cultured 3T3 cells were transfected in triplicate with the CAT reporter plasmid and either pEMSV-MyoD or pEMSV-myogenin. Plates were immunostained with a mouse monoclonal antibody to MyoHC and a rabbit polyclonal antibody to either CAT, desmin, or MCK proteins and positive cells counted in a standard area (Table 1). Activation of the p1.7des-CAT and the p3300MCK-CAT reporters was similar with either pEMSV-myD or pEMSV-myogenin transfection; yet many more desmin- and MCK-staining cells were detected in the plates transfected with pEMSV-myD than in the plates transfected with pEMSV-myogenin, similar to results obtained comparing activation of p4RTK-CAT with endogenous *MyoHC*. Therefore, MyoD and myogenin have comparable abilities to enhance transcription of transfected templates, but MyoD activates endogenous muscle genes more efficiently than myogenin.

We also compared the relative ability of Myf5 to activate transcription of endogenous genes. We transfected 3T3 cells with the p4RTK-CAT reporter and the pEMSV vector driving *myoD*, *myogenin*, or *Myf5*. The ratio of MyoHC-positive/CAT-positive cells, or myogenin-positive/CAT-positive cells, was used to calculate the relative activity of each transcription factor on endogenous genes, expressed as the percentage of activity relative to MyoD (Table 2). Myf5 was comparable to MyoD in its ability to induce transcription of an endogenous gene relative to its activity on the transfected reporter construct, and both MyoD and Myf5 were ~10-fold more efficient on endogenous genes than myogenin.

Efficient activation of endogenous genes depends on two regions of MyoD that are separate from the acidic activation and bHLH domains

A series of deletion mutations of the MyoD protein (Tapscott et al. 1988) were used to determine whether a domain of MyoD conferred its ability to activate endog-

Table 2. Two domains of MyoD mediate activation of endogenous genes

Plasmid	MyoHC/CAT (% MyoD activity) ^a	Myogenin/CAT (% MyoD activity) ^a
MyoD	100	100
Myogenin	7	N.D.
Myf-5	75	86
MyoD(Δ3-51)	64	215
MyoD(Δ63-99)	30	0.5
MyoD(Δ167-318)	44	19
MyoD(Δ270-318)	129	218
MyoD(Δ170-209)	N.D.	733
MyoD(Δ218-269)	N.D.	22

3T3 Cells were cotransfected overnight with the indicated expression plasmid and p4RTK-CAT, and prepared for immunohistochemistry after 24 hr in differentiation medium. Cells were stained with antibodies to MyoHC (monoclonal) and CAT (polyclonal), or myogenin (monoclonal) and CAT. The number of cells staining positive for the indicated muscle marker or CAT in standard areas of each dish was used to determine MyoHC/CAT and myogenin/CAT ratios for each plasmid. The ratio obtained with MyoD transfection for each experiment was set at 100%, and the other percentages were normalized accordingly. Results from independent experiments were averaged.

^aNot determined.

enous genes. Following transfection of 3T3 cells with p4RTK-CAT and various MyoD mutants in the pEMSV expression vector, we assessed the relative efficiency of activating *MyoHC* or the endogenous *myogenin* gene for each deletion mutant of MyoD (Table 2). Deletion of the amino-terminal acidic activation domain [MyoD(Δ3-51)] and the carboxyl terminus [MyoD(Δ270-318)] did not impair the ability of the mutant MyoD to activate expression of the endogenous *myogenin* gene. In contrast, deletion of residues 63-99 [MyoD(Δ63-99)] decreased the activation of the endogenous *myogenin* gene nearly 100-fold relative to the number of CAT-positive cells. A second region between residues 218 and 269 also contributed to activating the endogenous *myogenin* gene, because MyoD(Δ218-269) has a lower activity than either wild-type MyoD or MyoD(Δ270-318). A similar trend in the activity of the MyoD deletions was observed for the ratio of MyoHC-positive/CAT-positive cells (Table 2), but the differences between constructs were not as large.

It is interesting that deletion of amino acids 170-209 apparently increases the activity of MyoD on the endogenous *myogenin* gene. This increased activity may depend on the presence of amino acids 218-269, because the termination mutant MyoD(Δ167-318) has an activity similar to MyoD(Δ218-269), suggesting that the region between 170 and 209 may negatively regulate the activity of the region between 218 and 269.

Quantitative assays of reporter activity were also performed for each construct by use of a p4RTK-luciferase reporter (Fig. 7). Whereas MyoD(Δ63-99) and MyoD(Δ218-269) were slightly less active than the wild-type MyoD (60% and 70% of the activity of MyoD, respectively), they were both more than twice as active as

Figure 7. Activity of MyoD deletion mutants on transiently transfected p4RTK-luciferase. 3T3 cells were transfected with the indicated expression vector following the protocol described in Table 2, using a luciferase reporter instead of a CAT reporter. Transfections were performed in triplicate and numbers represent the luciferase activity relative to vector alone. A cotransfected *pCMV-lacZ* vector was used to normalize values for transfection efficiency.

the MyoD(Δ 3-51), which efficiently induced expression of the endogenous *myogenin* gene. Because saturation of the activity of wild-type MyoD could obscure a greater difference between the mutant proteins, we also performed a titration of wild-type MyoD and MyoD(Δ 63-99) comparing activity on a transfected p4RTK-luciferase reporter and the endogenous *myogenin* gene with similar results (data not shown). Therefore, the ability of the deletion mutants to activate endogenous genes does not correlate with their transcriptional activity on a transfected reporter, and we conclude that specific regions of MyoD increase the efficiency of initiating transcription of endogenous genes.

The region of MyoD between amino acids 63 and 99 is necessary for chromatin remodeling

The region between amino acids 63 and 99 is rich in cysteine and histidine residues, and has been designated the C/H region (Tapscott et al. 1988; Weintraub et al. 1991). Because deletion of this region had the largest effect on the activation of endogenous genes, we next tested whether deletion of this region influenced chromatin remodeling. The role of the amino-terminal activation domain was also evaluated, because transcription factor activation domains have been implicated previously in chromatin remodeling (Svaren et al. 1994). We constructed MyoD(Δ 3-51)-ER and MyoD(Δ 63-99)-ER, analogous to MyoD-ER, and analyzed *Pst*I access at the *myogenin* promoter in 10T1/2 cells. Following 10.5 hr of induction, enzyme access to the -170-bp *Pst*I site in the *myogenin* promoter was increased only weakly in 10T1/2-MyoD(Δ 63-99)-ER cells (Fig. 8, lanes 5-8), whereas cells expressing MyoD(Δ 3-51)-ER (Fig. 8A, lanes 1-4) or

MyoD-ER (see Fig. 4) exhibited marked increases in access to this site. Thus, deletion of the C/H region impaired the ability of MyoD to remodel chromatin. In contrast to its weak activity on chromatin remodeling, MyoD(Δ 63-99)-ER showed activity similar to that of MyoD-ER and MyoD(Δ 3-51)-ER on transfected p4RTK-CAT reporters (Fig. 8B; data not shown). The relatively higher activity of the MyoD(Δ 3-51)-ER in this assay, compared with that shown in Figure 7, most likely reflects the efficient activation of endogenous *myogenin* that contributes to reporter activation, although the ER domain may also affect its activity (see Discussion). We conclude that the C/H region allows MyoD to access binding sites efficiently in repressed chromatin and initiate chromatin remodeling.

Discussion

This study has shown that MyoD remodels chromatin at target genes. Chromatin relaxation and initiation of gene transcription were induced by MyoD at the *myogenin* locus in the absence of protein synthesis, indicating that

Figure 8. Chromatin remodeling is impaired with deletion of the C/H region, but not the activation domain. 10T1/2-MyoD(Δ 3-51)-ER and 10T1/2-MyoD(Δ 63-99)-ER cells were transiently transfected with either pTK-CAT or p4RTK-CAT and subsequently induced with estradiol for 10.5 hr. (A) Enzyme access was analyzed at the -170 *Pst*I site in the *myogenin* promoter as described in Fig. 3. (B) CAT assays from sister plates represented as the fold activation of CAT activity comparing p4RTK-CAT (containing multimerized MyoD-binding sites) with pTKCAT. (B) *Bam*HI; (R) *Eco*RI; (N) nuclei; (P) purified DNA.

MyoD is capable of accessing binding sites within transcriptionally inactive chromatin, and initiating both chromatin remodeling and transcription. MyoD was more effective than myogenin at activating genes within repressed chromatin, in part as a result of a region rich in cysteine and histidine residues and a region in the carboxyl terminus of MyoD. Both of these regions have areas that are highly conserved in the Myf5 protein, and Myf5 has an ability to activate endogenous genes that is comparable to MyoD. These findings suggest a model in which the myogenic bHLH proteins that determine the skeletal muscle lineage, MyoD and Myf5, can remodel chromatin at target genes into an open, or active, conformation, whereas the transcription factors that mediate the expression of muscle genes in the differentiated cell, such as myogenin, are relatively inefficient at activating genes in repressive chromatin and are therefore constrained to act on genes that have been preset in an open, or active, chromatin conformation.

Our data are consistent with a process in which the induction of chromatin reorganization by MyoD is not attributable solely to local nucleosome displacement associated with movement of the RNA Pol II holoenzyme. Chromatin alterations were observed at the *MCK* and *myoD* loci at sites 1.2 and 5.2 kb upstream of transcription initiation, making it unlikely that passage of the transcriptional machinery had displaced nucleosomes in these areas. In addition, chromatin modifications were detected when transcription was inhibited by ATP depletion, indicating that transcriptional elongation is not required for MyoD-initiated chromatin relaxation. We cannot conclude that low levels of ATP are not required for chromatin rearrangement because residual amounts of ATP may be present following treatment with the metabolic inhibitors.

The presence of MyoD-binding sites within target genes is not sufficient for MyoD to initiate chromatin reorganization. MyoD expression did not modify chromatin structure at the *IgH* locus despite the presence of multiple E boxes in the region that are capable of binding MyoD. We cannot at this time determine whether MyoD binds to the E boxes in the *IgH* enhancer without modifying chromatin, or whether the sites cannot be recognized by MyoD because of the special features of the chromatin composition. Sequences flanking the pE5 E box in the *IgH* enhancer have been shown to repress transactivation by MyoD (Weintraub et al. 1994) and may function to recruit a factor to this site that prevents MyoD activity. Although the mechanism is not known, our results raise the possibility that this element blocks the capacity for MyoD to alter chromatin structure in the region.

Chromatin modifications occurred at the *myogenin* promoter in the presence of either metabolic inhibitors or cycloheximide, although more extensive chromatin remodeling was attained when de novo protein synthesis was allowed. It is not likely that this is simply the result of limiting amounts of MyoD, because the amount of *myogenin* RNA was similar in cycloheximide-treated and -untreated cultures. Furthermore, chromatin modi-

fications were similar in the coding region with and without protein synthesis. Thus, our results suggest that the activity of MyoD and ubiquitous factors can cause a limited degree of chromatin relaxation at the *myogenin* locus, and that newly synthesized proteins are required for full chromatin remodeling to occur. We have not determined whether the partial increase in enzyme access represents incomplete chromatin remodeling at a majority of sites or complete remodeling at only a limited number of sites, as has been suggested for partial enzyme access to β -globin associated hypersensitive sites with GATA-1- or NFE2-binding site mutations (Boyce and Felsenfeld 1996). The detection of a hypersensitive site at the MEF2 binding site after 48 hr of induction suggests that MEF2, which is induced by MyoD and acts as a coactivator with MyoD, may participate in the completion of chromatin remodeling at the *myogenin* locus following the initial MyoD-mediated chromatin relaxation. Multistep nucleosomal rearrangement has been described in a number of other systems, such as the induction of *PHO5* by Pho4 (Venter et al. 1994) and the cooperation of the SWI/SNF complex with transcription factors to displace nucleosomes (Owen-Hughes et al. 1996). Further work will be necessary to determine whether similar nucleosome remodeling complexes interact with MyoD.

It should be noted that the studies on chromatin remodeling relied on a chimeric protein that incorporated the hormone-binding domain of the estrogen receptor into MyoD and it is possible that this domain may alter the characteristics of the native MyoD protein. For example, p300 has been shown to interact with the ligand-binding domain of the estrogen receptor and related nuclear receptors (Chakravarti et al. 1996; Hanstein et al. 1996; Kamei et al. 1996) and may be artificially incorporated into a DNA-binding complex; however, p300 also interacts with the bHLH region of MyoD (Eckner et al. 1996; Yuan et al. 1996) and may be in the complex in the absence of the estrogen receptor domain. Whereas the potential of the artificial incorporation of a coactivator, such as p300, into the complex with the chimeric MyoD-ER tempers our interpretation of the ability of the MyoD(Δ 3-51)-ER to remodel chromatin, for each of the MyoD-ER chimeras tested [MyoD-ER, MyoD(Δ 3-51)-ER, and MyoD(Δ 63-99)-ER] the ability to remodel chromatin was the same as the ability of the non-fusion proteins [MyoD, MyoD(Δ 3-51), and MyoD(Δ 63-99)] to induce transcription of endogenous genes, suggesting that the incorporation of the ER domain is not fundamentally altering the activity of the MyoD proteins.

Our analysis of activation of endogenous versus transfected templates revealed that myf-5 and MyoD were more potent than myogenin in activating endogenous muscle gene expression. Because genetic studies in mice have shown that MyoD and Myf-5 determine the muscle lineage (Braun et al. 1994), whereas myogenin is critical for driving terminal muscle differentiation (Venuti et al. 1995), our data suggests that the ability to access nucleosomal binding sites efficiently and relax chromatin structure is an attribute common to factors involved in

lineage determination. In contrast to our results, however, Wang et al. (1996) demonstrated that myogenin expressed from the *myf-5* locus can rescue the rib-defect phenotype and activate the expression of endogenous muscle genes in *myf-5* null mice, but the levels of gene expression relative to wild-type mice were not determined quantitatively. In our experiments, the different abilities among the myogenic factors to activate muscle gene expression within repressive chromatin were quantitative, rather than qualitative, and the relative differences among the factors diminished when transfected cells were left in differentiation medium for several days (data not shown). Based on our studies, we would predict that myogenin will not completely rescue myogenesis in a *myoD* and *myf-5* double knock-out mouse because of its relative inability to activate genes in repressed chromatin.

Local chromatin structure established during development may subsequently influence gene expression within a lineage by determining the access of other transcription factors to binding sites. In this regard, MyoD and *myf-5* are believed to establish separate lineages during mouse development, which together comprise the muscle compartment (Braun and Arnold 1996). Therefore, if MyoD and *myf-5* have slightly different target specificities, then the lineage identity that each establishes can be maintained despite the subsequent expression of shared transcription factors, such as MEF2 and myogenin, which are relatively constrained to act at genes with relaxed chromatin structure. We would predict that other tissue-specific bHLH families, such as the NeuroD family of neurogenic bHLH proteins, will be shown to contain early expressed members that alter chromatin structure at target genes and later expressed members that regulate gene expression within the constraints of a pre-established chromatin structure. In this manner, the combinatorial action of a relatively small number of transcription factors acting both temporally and spatially could generate a high degree of diversity.

The chromatin structure within a lineage may also influence the transcriptional response to agents that antagonize differentiation. When added at the time of MyoD induction, sodium butyrate, TGF- β , and basic-FGF blocked chromatin reorganization and the initiation of *myogenin* gene transcription. In contrast, TGF- β and sodium butyrate did not block *myogenin* transcription when added after chromatin remodeling had occurred. We conclude that once transcription is initiated and chromatin relaxation is accomplished, then either MyoD or other transcription factors, such as MEF2, can maintain an active state in the presence of myogenic inhibitors. In particular, whereas the *myoD* family is subject to both auto- and cross-activation (Thayer et al. 1989), our data suggest that active chromatin conformations can allow stable maintenance of *myoD* family member expression in the presence of inhibitory factors. This would explain the maintenance of myogenic identity in myoblasts following exposure to myogenic inhibitors in cell culture and during development. Together with previous results demonstrating that active chromatin conforma-

tions can be propagated to daughter cells (Groudine and Weintraub 1982), the timing of exposure to growth factors relative to gene activation could lock in a particular developmental state, providing a mechanism for the expansion of specific cell populations that represent an intermediate phenotype relative to terminal differentiation.

The ability of MyoD to activate genes within inactive chromatin mapped to amino acids 63–99, a region rich in cysteines and histidines (the C/H region), and amino acids 218–269. With the exception of the C/H region interacting with the negative regulator I-mf (Chen et al. 1996), neither region had a function identified previously. Both the C/H region and the carboxy-terminal region encompass motifs that are highly conserved between MyoD and Myf-5, supporting the notion that the ability to modify chromatin may be a feature common to factors involved in lineage determination. Deletion of either region significantly weakened the ability of MyoD to activate endogenous muscle genes, and deletion of the C/H region impaired the induction of chromatin remodeling by MyoD. The effects of the MyoD(Δ 63–99) deletion and the MyoD(Δ 218–269) deletion are not simply attributable to a reduction in the strength of MyoD as a transcriptional activator, because their activity was comparable to wild-type MyoD and greater than MyoD(Δ 3–51) when assayed on a transfected reporter. Furthermore, our previous work using GAL4/MyoD fusion proteins to map regions of the MyoD protein that enhance transcription from transfected reporters identified an activation domain in the region between amino acids 3 and 51, but showed no activation potential for the C/H or carboxy-terminal regions (Weintraub et al. 1991). In fact, deleting either of these two regions increased the activation potential of the GAL4/MyoD fusion protein. We conclude that the C/H domain and the carboxy-terminal domain do not function as typical activation domains, but rather mediate an interaction between MyoD and factors that remodel chromatin, increasing the probability that MyoD will initiate chromatin relaxation and activate gene expression. One intriguing possibility is that these domains participate in the recruitment of chromatin remodeling complexes such as the SWI/SNF complex, a member of which has been shown recently to have a muscle-specific isoform (Wong et al. 1996). It will be of interest to identify factors that interact with these regions and to determine whether members of other bHLH families involved in lineage determination contain functionally similar domains that initiate lineage-specific chromatin remodeling.

Materials and methods

Plasmids

pEMSV-myogenin was a gift of A. Chen (Harvard Medical School, Boston, MA). pMyo1565-CAT was a gift from E. Olsen (Edmondson et al. 1992). pEMSV-MyoD(Δ 3–51)-ER and pEMSV-MyoD(Δ 63–99)-ER were constructed by inserting the hormone-binding domain of the estrogen receptor into the *Nar*I site of pEMSV-MyoD, as described for the construction of

pEMSV-MyoD-ER (Hollenberg et al. 1993). Other plasmids are as referenced in the text.

Cell culture

10T1/2 and 3T3 cells were obtained from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% or 15% calf serum (Hyclone), penicillin, and streptomycin. Differentiation medium was DMEM supplemented with 10 μ g/ml of insulin and transferrin. Inductive medium was differentiation medium supplemented with 10^{-7} M β -estradiol.

Transfections

Transfections were performed by calcium phosphate precipitation as described (Weintraub et al. 1991). In the experiments to determine relative activation efficiency of endogenous versus transfected genes, cells in 6-cm TC dishes (Corning) were transfected for 12 hr with 0.5 mg pCMV-LacZ, 2 mg of CAT reporter, and 5 mg of myogenic factor expression plasmid per 6-cm dish, after which the medium was replaced with fresh growth medium for 2 hr, followed by differentiation medium for 24 hr. Immunohistochemistry, X-gal staining, CAT assays, and luciferase assays were performed as described previously (Weintraub et al. 1991; Alevizopoulos et al. 1995; Gerber and Tapscott 1996). Stable transformants were generated with pmyoD(Δ 3-51)-ER by coelectroporation with SV2-Neo and G418 selection, and with pmyoD(Δ 63-99)-ER by cotransfection with pY3, a vector containing the hygromycin resistance gene, and hygromycin selection. Clones were picked and analyzed for MyoD immunoreactivity.

Chromatin analysis

Cells were trypsinized, suspended in DMEM plus 15% bovine calf serum (BCS), centrifuged at 2000 rpm for 5 min, and washed once in ice-cold reticulocyte suspension buffer (RSB: 10 mM Tris at pH 7.4, 10 mM NaCl, 5 mM MgCl₂). Cells were resuspended in ice-cold lysis buffer (RSB plus 0.1% NP-40) at a concentration of 1.5 million cells per ml and incubated on ice for 10 min. Nuclei were isolated by centrifugation at 2000 for 5 min at 4°C. The pelleted nuclei were washed once in lysis buffer. For DNase analysis, the lysis buffer was removed and the nuclei were resuspended in RSB containing various concentrations of RNase-free DNase I (GIBCO), and incubated at 37°C for 10 min. The reaction was stopped with addition of an equal volume of stop buffer (0.6 M NaCl, 20 mM Tris base at pH 7.4, 10 mM EDTA, 1% SDS, and 2 mg/ml proteinase K). For MNase analysis, the lysis buffer was removed and nuclei were resuspended in RSB plus 0.1 mM CaCl₂ with 50 U/ml MNase (Pharmacia) for 0, 5, 10, 20, or 40 min, and the reaction was stopped with an equal volume of stop buffer. For restriction endonuclease analysis, isolated nuclei were suspended in 200 μ l of appropriate restriction endonuclease buffer plus 40 units of restriction enzyme (assay enzyme) per 10^6 nuclei. The buffer used for digestions with *Ban*I, *Cla*I, and *Nde*I was 20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT at pH 7.9. The buffer used for digestions with *Pst*I, *Pvu*II, and *Xba*I was 10 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT at pH 7.9. Following 30 min of incubation at 37°C, an equal volume of stop buffer was added to each reaction. Samples treated with DNase I, MNase, or restriction endonucleases were incubated overnight at 37°C following the addition of stop buffer, and the DNA was purified by phenol-chloroform extraction and ethanol precipitation. The purified DNA was digested with flanking

enzymes as indicated in the figure legends, and Southern blotted as described (Otten and Tapscott 1995). For quantitative analysis of endonuclease access, following digestion of the purified DNA with flanking enzymes, 50% of each sample was digested to completion with the assay enzyme. The percent access of the assay enzyme to sites in the isolated nuclei was obtained by dividing the density of bands from assay enzyme digestion of nuclei (N) with the density of the band generated with digestion of purified DNA (P). Densities for the N/P percentage were obtained using a Molecular Dynamics PhosphorImager.

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