MyoD Acetylation Influences Temporal Patterns of Skeletal Muscle Gene Expression

Monica Di Padova1,2, Giuseppina Caretti1,3, Po Zhao4, Eric P. Hoffman5, and Vittorio Sartorelli1,3

From the 1Laboratory of Muscle Stem Cells and Gene Regulation, NIAMS, National Institutes of Health, Bethesda, Maryland 20829 and the 2Research Center for Genetic Medicine and Children’s National Medical Center, Washington, D. C. 20010

MyoD is sufficient to initiate the skeletal muscle gene expression program. Transcription of certain MyoD target genes occurs in the early phases, whereas that of others is induced only at later stages, although MyoD is present throughout the differentiation process. MyoD acetylation regulates transcriptional competency, yet whether this post-translational modification is equally relevant for activation of all the MyoD targets is unknown. Moreover, the molecular mechanisms through which acetylation ensures that MyoD achieves its optimal activity remain unexplored. To address these two outstanding issues, we have coupled genome-wide expression profiling and chromatin immunoprecipitation in a model system in which MyoD or its nonacetylatable version was inducibly activated in mouse embryonic fibroblasts derived from MyoD+/−/Myf5+/− mice. Our results reveal that MyoD acetylation influences transcription of selected genes expressed at defined stages of the muscle program by regulating chromatin access of MyoD, histone acetylation, and RNA polymerase II recruitment.

MyoD, Myf5, myogenin, Mrf4, and the Mef2 family of MADS box transcription factors are the prominent regulators of skeletal myogenesis (1–3). MyoD and Mef2 recruit enzymes that introduce post-translational histone modifications at the chromatin of specific genomic loci to enable site-specific and temporally regulated muscle gene expression (4). MyoD engages at least two acetyltransferases, p300 and PCAF (5), which promote acetylation of both histones and of MyoD itself. p300 and PCAF acetylate evolutionarily conserved MyoD lysines, which, when mutated to arginines, prevent full and proper transcriptional activity (6–9). In vitro transcription experiments have indicated that although p300 acetylation is directed at histones H3 and H4, it is PCAF that acylates MyoD (10). The functional relevance of MyoD acetylation in the animal has been confirmed in a recent study. Knock-in embryos homozygous for a mutant MyoD allele in which lysines 99 and 102 were replaced by arginines had delayed muscle regeneration and increased number of myoblasts with reduced differentiation potential (11). Although these studies indicate an overall important role of MyoD acetylation in both cultured cells and in the animal, a detailed investigation of whether MyoD acetylation impacts the temporal activation of the individual components of the muscle program is lacking. Moreover, the molecular mechanisms leading to defective transcription of nonacetylable MyoD have not been clarified yet.

To evaluate the contribution of MyoD acetylation at distinct, temporal-specific, stages of muscle gene activation, we have performed reiterated genome-wide expression profiling and chromatin immunoprecipitation of mouse embryonic fibroblasts (MEFs)4 derived from MyoD+/−/Myf5+/− animals in which MyoD activity (wild type or nonacetylatable forms) was conditionally induced. Our results indicate that acetylation is relevant to regulate a discrete set of genes at distinct stages of muscle gene expression by allowing proper chromatin access to both MyoD and RNA polymerase II.

EXPERIMENTAL PROCEDURES

Cell Culture, Retroviral Constructs, Cells, and Cell Transduction—Mouse embryo fibroblasts (MEFs) null for both MyoD and Myf5 are described in Ref. 12. MEFs were transduced with either a retrovirus expressing MyoD wild type (WT) (13, 14) or MyoD with mutations at lysine residues at positions 99, 102, and 104 (6) fused to the estrogen receptor binding domain. Selection of transduced MEFs was obtained by culturing them in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal calf serum, 1% penicillin/streptomycin, and 1.5 μg/ml puromycin. Control MEFs were transduced with a virus carrying only the selectable marker. To induce ER-MyoD nuclear translocation and cell differentiation, transduced MEFs were switched to Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum, insulin, transferrin, selenium, and β-estradiol (10−7 M), as described in Ref. 14. C2C12 cells were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal calf serum.

4 The abbreviations used are: MEF, mouse embryo fibroblast; WT, wild type; ER, estrogen receptor; RT, reverse transcription; qPCR, quantitative PCR; PolI, polymerase II; HAT, histone acetyltransferase; CREB, cAMP-response element-binding protein; DM, differentiation medium; MHC, myosin heavy chain; ChIP, chromatin immunoprecipitation.
Plasmids, Transfection, and Luciferase Assay—The Ckm-luc, 4RE-luc, and cytomegalovirus-luc (pGL-2-luc; Promega) reporter constructs (0.5 μg each) (15) were transfected into MEFs using the Lipofectamine 2000 reagent (Invitrogen). After 16 h, MEFs were exposed to β-estradiol (10−7 M), and a luciferase assay was performed after an additional 24 h of culture in differentiation medium as reported in Ref. 16. In every transfection reaction, 50 ng of a cytomegalovirus-lacZ expression vector was added and assayed for β-galactosidase activity as an internal control. Reporter activity is expressed as relative luminescence units normalized for β-galactosidase activity. Transfections were performed in triplicate samples and repeated in at least three independent experiments.

Expression Profiling and Data Analysis—Total RNA was extracted from transduced MEFs at 0, 6, 12, and 24 h after treatment with β-estradiol using the TRizol reagent (Invitrogen). Biotin-labeled cRNA was prepared and hybridized to GeneChip Mouse Genome 430_2 arrays (Affymetrix) as previously described (17). Every microarray time point is represented by three independent replicates. Primary data analysis was done using Affymetrix Microarray Suite 5.0. Data derived from Affymetrix analysis were input into GeneSpring (Agilent Technologies) for further analysis. The expression levels of each probe set across all time points for each group were normalized to time 0. Analysis was conducted with probe sets to compare ER-MyoD WT samples and ER-MyoD RRR samples with MyoD-none (control, MEFs transduced with empty vector) samples at each time point (6, 12, and 24 h), respectively. This analysis involves two steps. First, probe sets were required to be induced at least 2-fold more than MyoD-none samples. Second, the resulting probe sets were subjected to Student’s t tests to retain probe sets that survive a p value of <0.05, corrected with the Benjamini and Hochberg false discovery rate (0.05). Using these statistics, it is expected that no more than 5% of the genes identified as MyoD targets and as differentially affected by ER-MyoD WT versus ER-MyoD RRR represent false positives. In the microarray experiments, the ratios of the ER-MyoD WT versus ER-MyoD RRR transcripts were 1:1 at 6 h, 1:2:1 at 12 h, and 1:3:1 at 24 h. These differences were taken into consideration, and the values observed for the transcripts at different time points were corrected (divided) by the ER-MyoD WT/ER-MyoD RRR ratio observed at the corresponding time point.

FIGURE 1. ER-MyoD WT and ER-MyoD RRR (nonacetylatable) proteins have a comparable half-life and are expressed at similar levels in MyoD−/−/Myf5−/− MEFs. A, MyoD−/−/Myf5−/− MEFs were transduced with retroviruses expressing estrogen receptor hormone binding domain fused to MyoD wild type (ER-MyoD WT) or nonacetylatable ER-MyoD (ER-MyoD RRR). A retrovirus without the ER-MyoD insert was employed as negative control (empty vector). Nuclear translocation of the ER-MyoD proteins was induced by culturing the MEFs in DM supplemented with β-estradiol for 0, 6, 12, 24, and 36 h. ER-MyoD was detected by immunofluorescence with the anti-MyoD monoclonal antibody 5.8. B, ER-MyoD WT and ER-MyoD RRR MEFs were immunostained with the anti-MyoD 5.8 antibody after 12 h of β-estradiol treatment in DM. Cell nuclei were identified by 4′,6-diamidino-2-phenylindole staining. C, cell extracts derived from ER-MyoD WT and ER-MyoD RRR MEFs cultured with β-estradiol for 12 h in DM and then exposed to cycloheximide (CHX) for an additional 0, 1, and 2 h were immunoblotted with MyoD and tubulin antibodies. Densitometry of the bands corresponding to MyoD WT and MyoD RRR obtained in two independent experiments is shown in arbitrary units (right). D, immunoblotting of ER-MyoD WT and ER-MyoD RRR proteins at the indicated time after β-estradiol treatment. Tubulin is shown as loading control.
Antibodies—Antibodies used for immunoblotting and immunofluorescence were as follows: anti-MyoD (clone 5.8, Novocastra Laboratories Ltd.), anti-MyoD (M-318; Santa Cruz Biotechnology), anti-myogenin (M-225; Santa Cruz Biotechnology), tubulin E7, and anti-myosin heavy chain (MHC) (MF20) (Developmental Studies Hybridoma Bank).

Protein Half-life—ER-MyoD WT- and ER-MyoD RRR-transduced MEFs were treated with β-estradiol for 48 h. C, immunofluorescence detection of MHC in ER-MyoD WT- and ER-MyoD RRR-transduced MEFs cultured in DM supplemented with β-estradiol for 48 h. DAPI staining reveals cell nuclei. D, the percentage of cells co-expressing either MHC and ER-MyoD WT or MHC and ER-MyoD RRR was determined by co-staining with anti-MHC and anti-MyoD polyclonal after 48 h of β-estradiol treatment. Approximately 200 cells for either ER-MyoD WT or ER-MyoD RRR were evaluated (n = 3). Error bars, S.D.

Quantitative Real Time PCR—Quantitative real time PCR was performed using the Mx3000P system (Stratagene) with a SyberGreen MasterMix (Applied Biosystems). Each data point was obtained from at least three independent experiments. Transcripts for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a reference. To ensure specific PCR amplification, every real time PCR run was followed by a dissociation phase analysis (denaturation curve). Specific primer sequences are reported in the supplemental materials. The amplicons corresponding to myogenin, me2A, Ckm, and Tnnt2 were analyzed by agarose gel electrophoresis, isolated, and sequenced to confirm their identity.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP was performed as described in Ref. 16. Immunoprecipitations were performed with 2 μg of normal rabbit IgG (sc-2027; Santa Cruz Biotechnology), MyoD (sc-760X), or RNA polymerase II (sc-9001X) antibodies or 1 μg of acetylated histone H3 antibody (06-599; Upstate Biotechnology, Inc.). The immunoprecipitated DNA was amplified by quantitative real time PCR using specific oligonucleotides reported in the supplemental materials. The reported data represent real time PCR values normalized to input DNAs and to the values obtained with normal rabbit IgG, which were set as one unit in each calculation. Data are presented as -fold differences relative to input and values obtained by normal rabbit IgG with the formula, $2^{{(Ct_{IgG} - Ct_{input}) - (Ct_{Ab} - Ct_{input})}}$, where Ct represents threshold cycles, IgG is normal rabbit IgG, Ab is...
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FIGURE 3. Venn diagrams of genes regulated by ER-MyoD WT and ER-MyoD RRR. A, Venn diagram representation of absolute number and percentage of genes activated by ER-MyoD WT at 6, 12, and 24 h of β-estradiol treatment. The percentage refers to how many genes are activated by MyoD over the total number of transcripts interrogated. B, Venn diagrams representing absolute number of genes regulated by ER-MyoD WT (outer circle) and nonacetylatable MyoD (ER-MyoD RRR; inner circle) at 6, 12, and 24 h of β-estradiol treatment.

specific antibody, and input is input genomic DNA. To ensure specific PCR amplification, every real time PCR run was followed by a dissociation phase analysis (denaturation curve), which indicated the presence of a single dissociation peak. Real time PCR was performed at least three times with DNA samples obtained from three independent ChIP experiments. In selected experiments, the PCR-amplified immunoprecipitated DNAs were sequenced and confirmed to correspond to the expected target loci.

RESULTS

The quantitative study of temporal gene expression occurring during skeletal myogenesis requires synchronization of the process. Cultures of either established (i.e. C2C12 cells) or primary skeletal muscle cells undergo differentiation in an asynchronous manner. Moreover, a significant proportion of the cells, “reserve cells,” escape terminal differentiation (18). To overcome this limitation, we have employed a cellular system in which uniform and synchronized nuclear translocation and activation of a protein composed by the hormone-binding domain of the estrogen receptor fused to MyoD (ER-MyoD) is obtained by adding β-estradiol to the culture medium. Although we cannot formally exclude the possibility that the addition of estradiol may influence some aspects of gene expression, this system has been extensively employed and convincingly shown to recapitulate, with a good approximation, the transcriptional and chromatin-binding behavior of MyoD in skeletal muscle cells, including regulated temporal activation of muscle gene transcription (12–14, 19, 21–23).

Nonacetylatable MyoD Is Defective in Conferring the Skeletal Muscle Phenotype to MEFs Derived from MyoD−/−/Myf5−/− Animals—MEFs obtained from MyoD−/−/Myf5−/− animals (12) were transduced with retroviruses expressing either ER-MyoD WT or ER-MyoD bearing three substitutions (lysine to arginine) at amino acid residues 99, 102, and 104 that render it no longer acetylatable (ER-MyoD RRR) (6). A retrovirus without the ER-MyoD insert was also employed as negative control. Disruption of both the MyoD and Myf5 loci in the transduced MEFs eliminates the potentially confounding effects due to the autoregulatory and redundant activities of the endogenous genes (14). MEFs transduced with either ER-MyoD WT or ER-MyoD RRR retroviruses were cultured in a medium that favors differentiation (DM) supplemented with β-estradiol for 0, 6, 12, 24, and 36 h. The addition of β-estradiol to the culture medium caused nuclear translocation of ER-MyoD WT and ER-MyoD RRR with analogous efficiencies and kinetics (Fig. 2, A–D). Moreover, transcription driven by either a naturally occurring muscle regulatory region, the muscle creatine kinase enhancer (Ckm-luc), or an artificial construct bearing four multimerized MyoD binding sites (4RE-luc) was impaired in ER-MyoD RRR cells, whereas expression of the control cytomegalovirus-luc reporter was not influenced by either ER-MyoD WT or ER-MyoD RRR (Fig. 2E). Expression of exogenous ER-MyoD WT and ER-MyoD RRR was within physiological range, since the levels of ER-MyoD WT and ER-MyoD RRR in MEFs approximated that of endogenous MyoD found in C2C12 skeletal muscle cells (Fig. 2, F and G). Previous experiments conducted with a vector expressing MyoD RRR without the ER moiety in C3H10T1/2 mouse embryonic fibroblasts were consistent with the results reported here (impaired muscle-specific transcription and muscle conversion) (6), indicating that the effects observed with the ER-MyoD RRR retroviral construct are not due to a peculiar activity of the ER-MyoD RRR fusion protein or the specific genetic make-up of the MyoD−/−/Myf5−/− MEFs.

Identification of MyoD Target Genes Whose Temporal Expression Is Influenced by MyoD Acetylation—To identify genes whose expression is influenced by MyoD acetylation at different stages of cell differentiation, we wished to compare
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**TABLE 1**

Representative list of genes activated by MyoD

MyoD WT-FC indicates average -fold change at 6, 12, and 24 h of MyoD activation with β-estradiol relative to noninduced MyoD (MyoD WT at 0 h of β-estradiol treatment set to 1). —, either the transcript was not detected, or the values did not meet statistical criteria.

| UniGene     | Name and function                      | 6 h MyoD WT-FC | 12 h MyoD WT-FC | 24 h MyoD WT-FC |
|-------------|----------------------------------------|----------------|----------------|----------------|
| **Structural-cytoskeleton** | | | | |
| Mm.219450   | Actin, α1, skeletal muscle (Acta1)      | —              | —              | 8.5            |
| Mm.686     | Actin, α, cardiac (Actc1)              | 3.3            | 12             | 79             |
| Mm.258670   | Troponin T1, skeletal, slow 1 (Tnnt1)  | —              | 56             | 165            |
| Mm.712     | Troponin C, cardiac/slow skeletal (Tnc) | 5.7            | 12.8           | 30.4           |
| Mm.247470   | Troponin T2, cardiac (Tnnt2)           | 4.6            | 7.5            | 13.4           |
| Mm.1716     | Troponin C2, fast (Tnn2c)              | —              | —              | 188            |
| Mm.154546   | Troponin T3, skeletal, fast (Tnnt3)    | —              | —              | 86             |
| Mm.260998   | Myosin Vb (Myo5b)                      | —              | —              | 5.5            |
| Mm.14526    | Myosin light chain, phosphorylatable (Mylpf) | —              | —              | 93             |
| Mm.46242    | Titin (Ttn)                            | —              | —              | 23             |
| Mm.29733    | LIM domain binding 3 (Ldb3)            | —              | —              | 4              |
| **Nuclear regulatory factors** | | | | |
| Mm.16528    | Myogenin (Myog)                        | 3.1            | 80             | 797            |
| Mm.132788   | Myocyte enhancer factor 2A (MEF2A)     | 6.9            | 9.7            | 25             |
| Mm.280029   | Hairy and enhancer of split 6 (Hes6)   | 2.1            | 3.6            | 9.9            |
| Mm.27962    | Zinc finger protein 238 (Zfp238)       | 14.6           | 22.6           | 36.6           |
| Mm.234274   | SET and MYND domain containing 1 (Smyd1) | 1.5           | 6.4            | 75             |
| Mm.37159    | Ankyrin repeat and SOCS box-containing protein 2 (Asb2) | —              | —              | 18.7           |
| Mm.347398   | B-cell leukemia/lymphoma 6 (Bcl6)      | 1.5            | 2.4            | 2.6            |
| Mm.291928   | Catenin β (Catenb)                     | 2.7            | 3.7            | 2.4            |
| Mm.206218   | Histone deacetylase 11 (Hdac11)        | —              | 19             | 88             |
| **Receptors/Signaling** | | | | |
| Mm.1272    | Receptor-associated protein of the synapse (Rapsn) | 5.7          | 21.8           | 49.8           |
| Mm.29660    | Srho homology 3-binding kinase (Sbk)   | 1              | 2.8            | 7.3            |
| Mm.330594   | ProSAP-interacting protein 1 (ProSAPIP1) | 1.9          | 3.5            | 21.5           |
| Mm.247956   | Sushi domain-containing 2 (Sussd2)     | —              | 1.9            | 10.3           |
| **Cell cycle/DNA replication** | | | | |
| Mm.2214    | Septin 4 (Sept4)                       | —              | 1.6            | 14.6           |
| Mm.273862   | Retinoblastoma 1 (Rb1)                | 10.2           | 16.8           | 26.4           |
| Mm.1236    | Growth arrest and DNA-damage-inducible 45 α (Gadd45a) | 3.4          | 2.9            | 9              |
| Mm.281298   | Growth arrest and DNA-damage-inducible 45 γ (Gadd45g) | 4.4          | 2.9            | 1.5            |
| **Adhesion/Matrix** | | | | |
| Mm.1976    | Cadherin 15 (Cdh15)                    | 9.3            | 19.1           | 11.5           |
| Mm.234266   | Syndecan 2 (Sdc2)                     | —              | 0.5            | 0.7            |
| Mm.44075    | Fibronectin type III domain containing 5 (Fn1c5) | —              | —              | 15.4           |
| **Others** | | | | |
| Mm.2375    | Creatine kinase, muscle (Ckm)          | —              | —              | 39             |
| Mm.1824344 | Follistatin-like 1 (Fst1l)             | 0.7            | 0.3            | 0.3            |
| Mm.5142    | Neuronal pentraxin 1 (Nptx1)          | 15.3           | 18.4           | 10.1           |
| Mm.281763   | Cardiac lineage protein 1 (Clp1)      | 7              | 6.5            | 4.8            |
| Mm.2187994  | Wingless-type MMTV integration site 9A (Wnt9A) | 13.3         | 10.5           | 4.8            |

genome-wide expression profiling of MEFs transduced with either ER-Myod WT or ER-Myod RRR cultured in the presence of a medium that promotes skeletal muscle differentiation supplemented with β-estradiol for 0, 6, 12, and 24 h, respectively. A comprehensive annotation of the transcripts identified by genome-wide expression profiling has been deposited in the GEO data base (available on the World Wide Web). The accession numbers for the individual experiments are provided under “Microarray Data Information Access” in the supplemental materials. Expression profiling of ER-Myod WT cells at 0 h of β-estradiol induction did not differ from that of cells transduced with the control retrovirus. After 6 h of β-estradiol treatment, ER-Myod WT induced 768 of the 44,224 interrogated transcripts (1.7%). At 12 and 24 h, the transcripts induced by ER-Myod WT were 785 of 44,189 (1.7%) and 1,092 of 43,754 interrogated transcripts (2.5%), respectively (Fig. 3A). Table 1 shows a list of representative genes induced by ER-Myod WT. Gene activation induced by ER-Myod WT at different time points was calculated as -fold change over the values observed for ER-Myod WT at 0 h of β-estradiol treatment. Among others, transcription of genes, such as those for retinoblastoma 1 (Rb1), cadherin 15 (Cdh15), transcription factor zinc finger 238 (Zfp238), neural pentraxin1 (Nptx1), and wingless-type MMTV integration site 9A (Wnt9A), was robustly induced as early as 6 h after β-estradiol treatment. Transcription of myogenin, already detectable at 6 h, continued to increase at 12 and 24 h and was accompanied by the appearance of the transcripts corresponding to actins (α-cardiac and α-skeletal actins), troponins (Tnnt1, Tncc, Tnnt2, Tnnc2, and Tnnt3), myosins (Myo5b and Mylpf), titin, and muscle creatine kinase (Ckm). A similar gene activation kinetics was observed in both the C2C12 skeletal muscle cell line (26, 27) and developing embryos (28, 29), indicating that the MEF ER-Myod model retains temporal characteristics of physiological muscle gene expression. Interestingly, transcription of the early induced genes Nptx1 and cardiac lineage protein 1 (Clp1) diminished over time, and, as reported (14), MyoD repressed transcription of the follistatin-like 1 (Fst1l) and syndecan 2 (Sdc2) genes (Table 1). We then compared the percentage of genes differentially activated by ER-Myod WT and ER-Myod RRR. Only transcripts that passed two criteria for statistical significance (p
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Representative list of genes affected by MyoD acetylation

MyoD WT/MyoD RRR-FC indicates the fold change induced by MyoD wild type relative to the MyoD RRR mutant observed at 6, 12, and 24 h after that MyoD nuclear translocation, and transcriptional activation was induced with β-estradiol. —, either the transcript was not detected or values did not meet statistical criteria.

| UniGene | Name and Function | 6 h MyoD wt/MyoD RRR-FC | 12 h MyoD wt/MyoD RRR-FC | 24 h MyoD wt/MyoD RRR-FC |
|---------|------------------|-------------------------|-------------------------|-------------------------|
| Acta1   | Actin, α1, skeletal muscle | — | — | 2 |
| Actc1   | Actin, α, cardiac (Acta1) | 2 | 1.7 | 1.7 |
| Tnnt1   | Troponin T1, skeletal, slow 1 (Tnnt1) | — | 2.6 | 1.3 |
| Tncc    | Troponin C, cardiac/slow skeletal (Tncc) | 2.6 | 3 | 1.2 |
| Mef2A   | Myoglobin (Myog) | 2.6 | 2.6 | 1.7 |
| Mef2B   | Myocyte enhancer factor 2A (MEF2A) | 2.6 | 1 | 1 |
| Gadd45a | Hairy and enhancer of split 6 (Hes6) | 1.7 | 1.5 | 1.2 |
| Gadd45g | Zinc finger protein 238 (Zfp238) | 3 | 1 | 1 |
| Mef2C   | Ankyrin repeat and SOCS box-containing protein 2 (Ash2) | — | — | 2 |
| Bcl-6   | B-cell leukemia/lymphoma 6 (Bcl6) | 2 | 1 | 1 |
| Hes6    | Histone deacetylase 11 (Hdac11) | — | 1.8 | 1.3 |
| Rapsn   | Receptor-associated protein of the synapse | 1.6 | 2.2 | 1.5 |
| Sbk     | Srb homology 3-binding kinase (Sbk) | 1 | 1.5 | 1.5 |
| Susd2   | ProSAP-interacting protein 1 (ProSAP1) | 1.5 | 1.6 | 1.8 |
| Ssd2    | Sushi domain-containing 2 (Ssd2) | — | 1 | 1 |
| Gadd45a | Growth arrest and DNA-damage-inducible 45 α (Gadd45a) | 2.3 | 1 | 1 |
| Gadd45g | Growth arrest and DNA-damage-inducible 45 γ (Gadd45g) | 1 | 1 | 1 |
| Cdh15   | Cadherin 15 (Cdh15) | 2.5 | 1.4 | 1.2 |
| Sdc2    | Syndecan 2 (Sdc2) | — | 0.6 | 0.5 |
| Fndc5   | Fibronectin type III domain-containing 5 (Fndc5) | — | — | 2.1 |
| Ckm     | Creatine kinase, muscle (Ckm) | — | — | 2 |
| Fstl1   | Follistatin-like 1 (Fstl1) | 1.2 | 0.6 | 0.5 |
| Nptx1   | Neuronal pentraxin 1 (Nptx1) | 1 | 1 | 1 |
| Cln1    | Cardiac lineage protein 1 (Cln1) | 1 | 1 | 1 |
| Wnt9A   | Wingless-type MMTV integration site 9A (Wnt9A) | 1 | 1 | 1 |

value <0.05 and false discovery rate of 0.05) were considered. A gene was considered not properly induced by ER-MyoD RRR if the average expression of multiple replicates was one-half or less the average induced by ER-MyoD WT in at least one of the time points analyzed. Of the 768 genes induced by ER-MyoD WT at 6 h, 232 (30%) genes were also fully activated by ER-MyoD RRR. At 12 and 24 h, ER-MyoD RRR fully activated 48% (380 of 785) and 51% (563 of 1,092) of the genes activated by ER-MyoD WT, respectively (Fig. 3B). Therefore, ER-MyoD RRR failed to fully activate 70% (at 6 h), 52% (at 12 h), and 49% (at 24 h) of the genes induced by ER-MyoD WT. The data illustrated in the Venn diagram in Fig. 3B indicate that the genes fully activated by ER-MyoD RRR are a subset of those activated by ER-MyoD WT. Moreover, our microarray analysis has not detected genes whose expression was reliably induced more by ER-MyoD RRR than by ER-MyoD WT (data not shown). The lists of the genes activated by either ER-MyoD WT or ER-MyoD RRR, respectively, at 6, 12, and 24 h are reported in the supplemental materials (Tables S1–S6). The abilities of ER-MyoD WT and ER-MyoD RRR to activate gene transcription of a representative list of genes were compared and expressed as the ratio of the values observed for ER-MyoD over those observed for ER-MyoD RRR (ER-MyoD WT/ER-MyoD RRR) (Table 2). With the false discovery rate set to 0.05, it is expected that no more than 5% of the transcripts identified as differentially affected by ER-MyoD WT versus ER-MyoD RRR may be false positive. Therefore, we further evaluated the expression of some of the transcripts identified as differentially regulated (Table 2) by RT-qPCR using independent triplicate sets of RNA, different from those employed for the profiling analysis (Figs. 4–7). We found that, for the transcripts analyzed, there was good agreement between the RT-qPCR and the microarray results. After 6 h of β-estradiol induction, ER-MyoD RRR was defective in activating transcription of several genes, including Rb1, Cdh15, myogenin, Mef2A, Zfp238, Actc1, Tnnt2, growth arrest, and DNA damage-inducible 45 α (Gadd45a), Bcl-6, and Hes 6, although differences were noted for individual genes (Table 2 and Fig. 4). After 12 h of β-estradiol induction, myogenin remained comparatively lower in ER-MyoD RRR cells (Table 2 and Fig. 5). Consistent with a role of myogenin in activating genes expressed in differentiated cells, transcripts for Tncc, Tnnt1, Tnnt2, and Actc1 were also lower in ER-MyoD RRR cells (Table 2 and Fig. 5). By this time, expression of Mef2A,
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FIGURE 4. Analysis of gene expression and recruitment of MyoD, PolII, and histone H3 acetylation at different genomic loci in ER-MyoD WT and ER-MyoD RRR MEFs after 6 h of β-estradiol treatment. RT-qPCR was employed to determine the expression levels of selected transcripts at 6 h after β-estradiol induction in ER-MyoD WT (black bars) or ER-MyoD RRR (gray bars) MEFs. Each experiment was performed with three independent RNA samples (n = 3), and the values were normalized to those obtained for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. The error bars represent S.D. A ChIP assay was performed with chromatin derived from either ER-MyoD WT (black bars) or ER-MyoD RRR (gray bars) MEFs. The antibodies employed are indicated at the bottom of each graph. The immunoprecipitated DNA was amplified by real time qPCR with specific primers for regulatory regions of the genes indicated at the top of each graph. The -fold enrichment was obtained after correcting for the values observed with the DNA derived from immunoprecipitations performed with nonspecific IgG, which were set as one unit in each calculation, as described by Caretti et al. (16). qPCR runs were performed with triplicate samples and were repeated with chromatin obtained from at least three independent experiments (n = 3 or >3). The error bars indicate S.D. For both the mRNA expression and ChIP experiments, p < 0.05.

Zfp238, and Bcl6 in ER-MyoD RRR became comparable with that in ER-MyoD WT cells. Myogenin expression never recovered in ER-MyoD RRR cells (Table 2 and Figs. 2 and 6), and the transcripts for Ckm, α-skeletal actin, Actc1, Tnnt1, Tncc, Tnn2, Tnn3, Myo5b and Mylpf, titin, Asb2, and sep-tin 4 also remained lower in ER-MyoD RRR cells (Table 2 and Fig. 6). Beside activating transcription, MyoD also represses expression of selected genes (14). In the case of Fstl1 and utrophin, MyoD represses gene expression by inducing transcription of miR-206, which targets sequences in the 3′-untranslated region of Fstl1 and utrophin mRNAs (30). The transcripts for Fstl1 and Sdc2 were reduced in β-estradiol-induced ER-MyoD WT cells when compared with noninduced conditions (Table 1). Such reduction was less pronounced in ER-MyoD RRR (Table 2), indicating that ER-MyoD RRR is less effective in either activating transcription of a repressor, such as miR206 in the case of Fstl1, or promoting repression of Sdc2. Finally, transcription of some genes was equally induced by either ER-MyoD WT or ER-MyoD RRR (Table 2; neural pentraxin1; cardiac lineage protein1; Wnt19a; Gadd45γ). Overall, the results described in this paragraph indicate that MyoD acetylation affects the kinetics and strength of transcription of discrete subsets of genes.

Reduced Chromatin Binding of Nonacetylatable MyoD Correlates with Histone Hypoacetylation, Reduced Recruitment of RNA Polymerase II, and Impaired Transcriptional Activation of Selected Genes—Productive gene transcription can be monitored by recruitment of RNA polymerase II (PolII), histone acetylation, and the presence of mRNA transcripts. We started investigating the molecular mechanisms underlying the different transcriptional behavior of ER-MyoD and ER-MyoD RRR by performing a ChIP assay. Chromatin was immunoprecipitated with nonspecific IgG (control), MyoD, RNA PolIII, and acetylhistone H3 antibodies, respectively, and recruitment of the corresponding proteins interrogated at several genomic loci by quantitative real time PCR. After 6 h of β-estradiol treatment, recruitment of MyoD, PolIII, and histone H3 acetylation at the myogenin, Mef2A, Tnnt2, Zfp238, and Actc1 promoters was reduced in ER-MyoD RRR MEFs compared with ER-MyoD WT MEFs (Fig. 4). These findings correlated with impaired transcription of their corresponding genes in ER-MyoD RRR MEFs at 6 h (Table 2 and Fig. 4). The difference in MyoD recruitment was not imputable to different expression, since the levels of MyoD protein in MEFs ER-MyoD WT and MEFs ER-MyoD RRR were comparable (Fig. 7B). The amylase 2 promoter was employed as negative control, since it is not regulated by MyoD (1). After 12 h of β-estradiol treatment, recruitment of MyoD and PolII at the myogenin, Tnnt1, and Acta1 promoters remained defective and was associated with histone H3 hypoacetylation in ER-MyoD RRR MEFs (Fig. 5). At this time, transcription of myogenin, Tnnt1, and Actc1 was reduced in ER-MyoD RRR MEFs (Table 2 and Fig. 5). By contrast, transcription of Mef2A and Zfp238 at 12 h was comparable in ER-MyoD WT and RRR MEFs (Table 2 and Fig. 5), and, consistently, no difference was noted in the recruitment of either MyoD or PolII and histone H3 acetylation at the Mef2A and Zfp238 promoters (Fig. 5). At 24 h, MyoD and PolII recruitment was defective at the myogenin, Ckm, and Tnn2 regulatory regions and correlated with suboptimal H3 acetylation in ER-MyoD RRR MEFs (Fig. 6). Myogenin, Ckm, Tnn2, and Tnnt1 expression at 24 h was
reduced in ER-MyoD RRR MEFs (Table 2 and Fig. 6). As observed at 12 h, expression of Zfp238 continued to be comparable in ER-MyoD WT and ER-MyoD RRR MEFs at 24 h (Fig. 6), and this finding was mirrored by comparable recruitment of MyoD, PolII, and H3 acetylation at the Zfp238 promoter (Fig. 6). Recruitment of MyoD, PolII, and H3 acetylation at the investigated loci was at background levels in control MEFs (data not shown). As indicated by the microarray experiments (Table 2), RT-qPCR confirmed that transcription of both Fstl1 and Sdc2 was less efficiently repressed in ER-MyoD RRR MEFs (Fig. 7A). Overall, the results reported in this paragraph indicate that nonacetylatable MyoD can penetrate or is retained less efficiently at specific gene regulatory regions and that this phenomenon is associated with impaired histone H3 acetylation, defective recruitment of PolII, and reduced gene transcription.

DISCUSSION

The histone acetyltransferases p300, CREB-binding protein, and PCAF and several members of class I, II, and III histone deacetylases interact with MyoD and Mef2 to be deployed at specific genomic loci and induce chromatin modifications that are regulatory to gene expression (4). In addition to introducing and removing acetyl groups at amino acid residues of histone tails, histone acetyltransferases and histone deacetylases also modify discrete lysine residues of MyoD and Mef2 (6, 7, 31) (15, 32, 33, 34, 35). Acetylation of MyoD and Mef2 exerts regulatory functions on gene expression. MyoD acetylation increases its affinity for the bromo-domain of CREB-binding protein and stabilizes its interaction with both CREB-binding protein and p300 (36). Moreover, recombinant MyoD, in vitro acetylated by PCAF, displays, in association with its partner E12 (37, 38), an increased affinity for a DNA oligonucleotide spanning a MyoD-binding site within the Ckm enhancer, as assayed by gel
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![Figure 7](Image)

FIGURE 7. A, the follistatin-like 1 (Fst1) and syndecan 2 (Sdc2) transcripts were evaluated at 0 (uninduced), 6, 12, and 24 h after β-estradiol induction in ER-MyoD WT and ER-MyoD RRR MEFs, respectively. B, immunoblotting of proteins derived from empty vector (○), ER-MyoD WT (□), and ER-MyoD RRR (●) MEFs with MyoD and tubulin antibodies at the indicated time after β-estradiol treatment in differentiation medium.

Mobility shift (6) and promotes transcription from a chromatin-reconstituted muscle-specific template (10). Indicating that MyoD acetylation is a functionally relevant modification, conversion of the acetylated lysines to arginines impaired (without completely abolishing) the ability of MyoD to promote transcription of either transiently transfected muscle reporter constructs or endogenous muscle-specific genes in skeletal muscle cells (6, 7). Finally, mice with knocked-in alleles for non-acetylatable MyoD have muscle regeneration defects and myoblasts derived from them exhibit reduced differentiation (11). The goal of the present study was 2-fold: to determine whether MyoD acetylation is equally relevant for transcription of all its target genes and to clarify the molecular mechanisms employed by acetylation to confer optimal transcriptional competency to MyoD in the cell. The results of our investigation indicate that MyoD discriminates among different gene targets by revealing a variegated temporal and graded susceptibility of the individual components of the muscle program to MyoD acetylation. Differences were noted in the ability of MyoD acetylation to regulate expression of defined genes at distinct time points. After 6 h from being activated, nonacetylatable MyoD was defective in inducing full expression of ~70% of the targets activated by MyoD WT. At later times of differentiation, the percentage of the genes refractory to full activation diminished to 52 and 49% at 12 and 24 h, respectively. It is likely that this phenomenon reflects, at least in part, the compensatory role exerted by activation of endogenous myogenin. Nonetheless, the lack of full induction of myogenin and of other transcripts, even at 24 h of differentiation, suggests that the cooperation between MyoD and myogenin in gene expression may be defective in cells expressing nonacetylatable MyoD. At the myogenin promoter, myogenin binds poorly and is ineffective in promoting histone acetylation and activating gene expression. On the contrary, MyoD is efficiently recruited and robustly promotes transcription (23). The combination of MyoD and myogenin has an additive effect on myogenin expression, since binding of MyoD is necessary to increase the amount of bound myogenin (23). It is therefore conceivable that the decreased binding of nonacetylatable MyoD would result in defective myogenin recruitment and consequent defective myogenin expression. The identification of myogenin and Ckm as genes whose transcription is susceptible to MyoD acetylation reported here is corroborated by other studies in which endogenous acetylated MyoD was detected at both the myogenin promoter and Ckm enhancer of differentiated C2C12 cells (39). Moreover, myogenin and Ckm expression was found to be impaired in muscles derived from nonacetylatable MyoD knock-in animals (11). In this respect, the relatively large numbers of genes whose transcription was here shown to be affected by MyoD acetylation (rather than the magnitude of the effect on the individual genes) may explain the drastic effects on regeneration observed in the nonacetylatable MyoD knock-in animals (11).

A mechanistic insight of how acetylation may influence MyoD activity was obtained by correlating the ChIP results with gene expression profiling. The analysis of these experiments revealed that a less efficient chromatin recruitment of nonacetylatable MyoD at specific muscle regulatory regions, at distinct time points, coincided with defective PolII engagement, histone H3 hypoacetylation, and defective gene expression. The mutations introduced in MyoD RRR do not, per se, decrease its intrinsic ability to recognize the DNA target sites. Bacterially expressed MyoD WT and MyoD RRR recognize their DNA targets with comparable efficiencies. However, acetylation increases the DNA binding ability of MyoD WT but not that of MyoD RRR (6). These findings are consistent with the crystal structure of the MyoD-DNA complex, which indicates that the MyoD amino acids 99, 102, and 104 do not make direct contact with the DNA (40). Therefore, we interpret the defective chromatin recruitment of MyoD RRR as a consequence of its inability to be acetylated, not as the result of the introduced mutations that would prevent its DNA interaction.

Interestingly, when expression of selected genes (i.e. Mef2A and Zfp238 at the 12 h time point) was comparable in ER-MyoD WT and RRR, also MyoD, PolII, recruitment and histone H3 acetylation became similar. Overall, the ChIP results support the hypothesis that nonacetylatable MyoD has a decreased affinity (or reduced stability) for its DNA binding site (6). They also suggest that such differences in DNA affinity/retention may be influenced by either the specific organization of the DNA modules within different gene regulatory regions or by the presence and structure of nucleosomes that render more or less accessible MyoD binding. MyoD acetylation seems to affect expression of selected genes activated at either earlier (myogenin, Cdhl5, and Mef2A) or later (Ckm and Tnnc2) stages of the myogenic program. Nonetheless, arguing against a generalized temporal delay of the myogenic program in cells expressing nonacetylatable MyoD is the observation that some of the earliest genes (Rb, Mef2A, and myogenin) maintain distinct behaviors. Although Rb and Mef2A transcription was simply...
delayed, myogenin expression did not recover. It is becoming increasingly evident that MyoD (and perhaps other myogenic regulatory factors), rather than indiscriminately activating transcription of all of its possible targets at once, differentially regulates muscle gene expression. Discrete signaling cascades and specific domains of MyoD impart transcription of distinct subsets of genes (14, 22, 41). Moreover, MyoD-associated cofactors influence its ability to activate some targets but not others (16, 42). MyoD has evolved different strategies to establish temporal specificity of gene activation (43), and acetylation may be one of them. Finally, several other transcription factors involved in the regulation of cell lineage-specific gene expression programs and circadian rhythms are acetylated (20, 44). It is therefore tempting to speculate that acetylation of other transcription factors may be employed to regulate temporal expression of distinct subsets of genes in relevant biological processes.

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