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Spatial re-organization of myogenic regulatory sequences temporally controls gene expression

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ABSTRACT

During skeletal muscle differentiation, the activation of some tissue-specific genes occurs immediately while others are delayed. The molecular basis controlling temporal gene regulation is poorly understood. We show that the regulatory sequences, but not other regions of genes expressed at late times of myogenesis, are in close physical proximity in differentiating embryonic tissue and in differentiating culture cells, despite these genes being located on different chromosomes. Formation of these inter-chromosomal interactions requires the lineage-determinant MyoD and functional Brg1, the ATPase subunit of SWI/SNF chromatin remodeling enzymes. Ectopic expression of myogenin and a specific Mef2 isoform induced myogenic differentiation without activating endogenous MyoD expression. Under these conditions, the regulatory sequences of late gene loci were not in close proximity, and these genes were prematurely activated. The data indicate that the spatial organization of late genes contributes to temporal regulation of myogenic transcription by restricting late gene expression during the early stages of myogenesis.

INTRODUCTION

During tissue differentiation, lineage determining transcription factors initiate the onset of tissue-specific gene expression. These gene activation events generally involve cooperating transcription factors, RNA polymerase II and its associated factors, as well as a spectrum of co-activators, many of which post-translationally modify histones or alter chromatin structure in an ATP-dependent manner. While the binding interactions of these regulatory proteins are largely similar at different differentiation-specific genes, there nevertheless must be critical differences since most differentiation events involve temporally distinct waves of gene expression.

Skeletal muscle differentiation is marked by the synthesis of early genes, which include the regulatory protein myogenin that is required for terminal differentiation, and late genes that include the structural components of skeletal muscle. Previous ChIP analysis revealed that the myogenic determinant MyoD binds to regulatory sequences controlling late myogenic gene expression in differentiated cells (1–3), while a later study indicated that MyoD binds myogenic regulatory sequences prior to differentiation as well (4). These reports indicate that MyoD binding does not specify differences in differentiation-specific temporal gene expression. Many other factors and cofactors contribute to the induction of myogenic genes during differentiation, but few correlate well with the timing of gene expression. We hypothesized that coordinated temporal regulation of myogenic gene expression might instead involve higher order organization of myogenic genes.

Chromosome conformation capture (3C) utilizes cross-linking and proximity-mediated ligation to identify DNA sequences in close proximity despite being separated by large distances or being located on different chromosomes (5). Thus, interactions captured by 3C technology provide evidence for higher order chromatin organization. Of particular note, reports over the past several years have linked inter-chromosomal interactions to chromosome and genome organization, to regulation of gene expression, to the formation of chromosomal translocations and to other functions (6–16).

Fluorescence *in situ* hybridization (FISH) is a well-established approach to identifying the localization of specific DNA or RNA sequences within the nucleus of single cells and, as such, can provide spatial information about gene positioning relative to gene expression (17). Although

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the limits of detection preclude precise localization of short DNA stretches that typically comprise transcriptional regulatory elements, the identification of gene locus positioning in individual cells provides information about the frequency of a given event in a population, in contrast to molecular methods such as 3C, which identify data as the average of an event in a population of cells. Coupling these approaches, then, should provide complementary support for hypotheses involving biological functions associated with gene positioning.

Here, we utilized conventional 3C to document interchromosomal interactions between sequences upstream of myogenic late genes. These interactions were present in myoblasts or formed upon differentiation-mediated signaling in reprogrammed cells and required both the MyoD lineage-determining transcription factor and functional SWI/SNF chromatin remodeling enzymes. FISH studies provided independent corroboration of the interactions between loci at the onset of differentiation. Functional assessment of the formation of these interactions links them to the temporal control of myogenic gene expression during differentiation.

MATERIALS AND METHODS

Cell lines

B22 cells that inducibly express dominant negative Brg1 (18) were mock-infected or infected with a MyoD encoding retrovirus (19) as described (20). C2C12 cells were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) + 20% fetal calf serum. Differentiation was induce by replacing the media with DMEM + 2% horse serum. Treatment of C2C12 cells with MyoD siRNA was performed exactly as described (21).

Tissue preparation

Isolation of E10.5 (carcass with head and internal organs removed), E12.5 (limb buds) and E14.5 (limb skeletal muscle) was performed as described (2).

Chromosome conformation capture

3C experiments were performed largely as described (5) except that samples were fixed with 1% formaldehyde for 5 min at room temperature and genomic DNA was extracted with a DNeasy kit (Qiagen). 1 ug of total crosslinked genomic DNA was used, and PvuII digestion was performed prior to the ligation at 20 ng/ul with Takara Ligation Kit Ver.2 (Takara). Data were obtained with runs of <35 cycles. Interaction frequencies were normalized to the Ct value of the corresponding ligated bacterial artificial chromosome (BAC) amplicon. The maximum interaction frequency for each experiment was then normalized to 1 and all other values are relative to that value. Primer sequences and BAC clone numbers used for 3C experiments are listed in Supplementary Table S1.

Chromatin immunoprecipitation and antibodies

ChIPs were performed as described (2,22) except that chromatin was sonicated to fragments of ∼200 bp and cross-linking was performed at room temperature. Recruitment is presented relative to input. The IgH enhancer, which contains an E box that does not bind to MyoD (2,22,23) was used as a negative sequence control for all experiments (data not shown). For the ChIP in Figure 6, the primers for *Acta1* sequences were 5′-CACA GGTTTTTATATAGTCCCGGGAGCTCT-3′ and 5′-GC AGACAGCTGGGGATACTCTCCATATACG-3′, which spanned −245 to −30. For the ChIP in Supplementary Figure S5, the *Acta1* primers were: −81 −110: 5′-TGTT GCTGCCCTTCCCAAGCCATATTTGGG-3′ and −236 −265: 5′-GCAGACAGCTGGGGATACTCTCCATATA CG-3′. All other ChIP primers were published (2). ChIP antibodies included HDAC2 (Invitrogen 51-5100), MyoD (22) and a rat monoclonal antibody against Brg1 (4E5) generated exactly as described (24).

Gene expression

Quantitative RT-PCR procedures were described (2). Primers used are listed in Supplementary Table S2.

Fluorescence *in situ* hybridization

B22 or C2C12 cells were grown on lamin coated glass coverslips. We used a standard paraformaldehyde fixation protocol (25–27). BAC clone DNAs (BACPAC Resource Center, Children's Hospital Oakland Research Institute, Oakland, CA, USA) were nick-translated with either biotin or digoxigenin-dUTP (Roche, Basel, Switzerland) and used as probes for each gene analyzed. BAC clone numbers are listed in Supplementary Table S1. DNA hybridization was performed as described previously (25,27). Hybridization was detected with either anti-digoxigenin antibody (Boehringer Mannheim) coupled with FITC rhodamine or, for biotin detection, avidin conjugated to Alexa Fluor–streptavidin 594 (red) (Boehringer Mannheim).

Digital images presented are single plane images acquired using AxioVision LE4.4 with either an Axiovert 200 or an Axiophot microscope (Carl Zeiss, Thornwood, NY, USA) equipped with a 100× PlanApo objective (NA 1.4; Carl Zeiss MicroImaging, Inc.) and multiband pass dichroic and emission filter sets (model 83000; Chroma Technology Corp.) set up in a wheel to prevent optical shift. The high 1.4 numerical aperture of this objective lens provides excellent X–Y resolution (∼0.2 μm) and results in a very shallow depth of field (∼0.6 μm). Two signals separated along the z-axis by more than this distance will not be in the same focal plane. Images were captured using an Orca-ER camera (Hamamatsu, Bridgewater, NJ, USA) or a cooled charge-coupled device (CCD) camera (200 series, Photometrics, Inc., Tucson, AZ, USA).

A range of images for associated loci was observed. We categorized gene loci that showed an obvious and predominant overlapping signal as 'co-localized', while gene loci that were abutting with no visible overlap or that showed minimal overlap that still had clear red and green signals were classified as 'adjacent'. For both the C2C12 and the MyoD-reprogrammed cells containing nuclei that were scored as positive, 30–40% had gene loci that were 'co-localized' and 60–70% had gene loci that were 'adjacent'.

Statistical methods

Standard deviation was calculated from three independent experiments for ChIP, gene expression and conventional 3C assays.

For B22 FISH experiments, counts were modeled using generalized linear mixed models (28–30) assuming underlying binomial distribution and a logit link function; this is a form of logistic regression modeling (31) without the necessity of an assumption of independent observations. Experiment was modeled as a random factor and virus (MyoD), time and tetracycline treatments as fixed factors. Models were built by first fitting saturated models (i.e. all main and interaction terms) then non-significant terms removed in descending order of complexity and then the reduced model refit. Significance was assessed using Type II *F*-tests. Modeling was performed using the GLIMMIX procedure (32) in the SAS statistical software package (SAS 9.2. Version 9.2. Cary, NC: SAS Inc.; 2008).

For C2C12 experiments, trends across time points were evaluated using the generalized Cochran Mantel-Haenszel (CMH) test for a singly ordered rxc contingency table (33). In the presence of significant time effects, pairwise comparisons between times were made using the CMT test with two time points. A Bonferroni adjustment (34) was made for multiple comparisons. Analyses were performed with the StatXact statistical software program (Version 7. Cambridge: Cytel Software Inc.; 2005).

RESULTS

Promoter sequences from some myogenic genes are in close physical proximity to each other

To assess whether there might be a higher-order organization to loci encoding genes expressed during skeletal muscle differentiation, we asked whether myogenic genes that are induced at late times of differentiation, such as *skeletal alpha-actin (Acta1)*, and *muscle creatine kinase* (*Ckm*), were in close physical proximity. 3C analysis of embryonic mouse tissue at E10.5, E12.5 or E14.5 using a primer against the *Acta1* promoter (chromosome 8) pairwise with primers spanning the *Ckm* locus (chromosome 7) showed that sequences containing the *Acta1* promoter were in close physical proximity with the *Ckm* regulatory region (Figure 1A). Association between the *Acta1* promoter and other regions of the *Ckm* locus were minimal. No associations between these loci were observed in liver tissue, indicating tissue specificity and linking regulatory sequence association and gene expression.

These results were recapitulated in culture using fibroblasts differentiated via MyoD introduction (35). The *Acta1* and *Ckm* regulatory regions were in close enough proximity to be cross-linked in MyoD-differentiated fibroblasts at the onset of differentiation but the interaction frequency was greatly reduced 8–24 h post-differentiation (Figure 1B). Mock-differentiated cells showed no associations (Figure 1B), indicating that regulatory sequence clustering depended on MyoD-induced differentiation. In this tissue culture model for differentiation, time 0 is 24 h after MyoD introduction via retroviral infection and the time at which low mitogen media is added to promote differentiation. Therefore, the associations occurred prior to induction of gene expression, which was 8 h post-differentiation in these cells (2). This is consistent with the data in Figure 1A showing that late gene associations could be observed prior to late gene expression in embryonic tissue. A similar pattern of associations was observed when the promoter of the *Desmin* (*Des*) gene, located on chromosome 1, was analyzed across the *Ckm* locus (Figure 1C). The *Des* gene is expressed early during embryogenesis but is expressed with late gene kinetics in MyoD-differentiated fibroblasts (2,23,36,37) and is substantially induced in immortalized myoblasts after the induction of myogenin (38,39). Control experiments with digested and ligated BAC DNAs determined that the lack of signal obtained outside the regulatory regions was not due to inefficient PCR amplification from those specific primer pairs (Supplementary Figure S1A–C; relative differences in the amplification efficiencies of different primer sets were incorporated into the calculated interaction frequencies).

We noted no association between the *myogenin (Myog)* promoter (chromosome 1), which is expressed at early times, and the *Ckm* locus (Figure 1D). Interactions between other pair-wise combinations of these candidate genes were tested; the *Des* and *Acta1* promoters interacted while *Myog* did not interact with either *Des* or *Acta1* (Supplementary Figure S2). We also determined that no interactions occurred between the *eukaryotic elongation factor 1-alpha (Eef1A1)* promoter (chromosome 9) and any regions of the *Ckm or Des* loci at any timepoint (not shown). *Eef1A1* is constitutively expressed and unaffected by myogenic differentiation (2), which provides further evidence for the specificity of the associations between the myogenic late gene regulatory sequences.

We also examined inter-genic interactions in differentiating C2C12 myoblasts. As in tissue and in MyoD-differentiated fibroblasts, the *Acta1* promoter interacted with the *Ckm* regulatory sequences at the onset of differentiation, with the interaction frequency decreasing drastically by 12 h and dropping to near background after 24 h (Figure 1E). Unlike the reprogrammed fibroblast system, use of C2C12 cells permitted us to evaluate the existence of inter-genic interactions in proliferating myoblasts prior to the onset of differentiation. Analysis of *Acta1* or *Des* promoter interactions with the *Ckm* promoter showed that similar interaction frequencies were obtained for cells in growth media as for cells at the onset of differentiation (Figure 1F–G). qPCR analysis of the expression of these genes confirmed that expression of these genes was not significantly induced at the times that the inter-genic interactions were observed (Supplementary Figure S3). These results suggest that the organization of gene localization is a consequence of the initial commitment to the skeletal muscle lineage and not the result of signaling for terminal differentiation. Finally, we repeated the experiment with primers to the *Des* enhancer region (40,41) and to the *Ckm* promoter. The results were similar (Figure 1H), indicating that the *Des* enhancer sequences were also in proximity to the *Ckm* promoter.

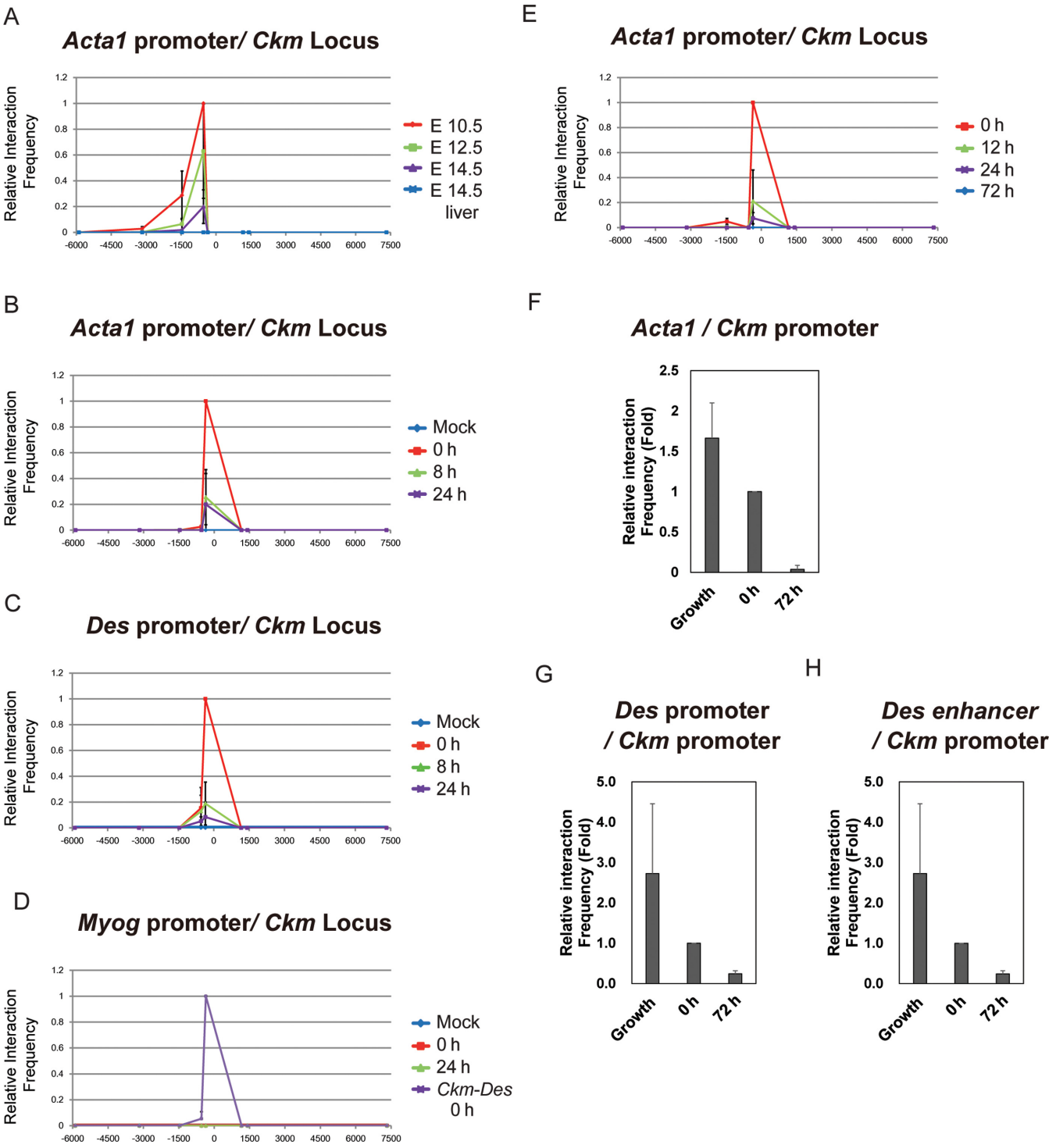


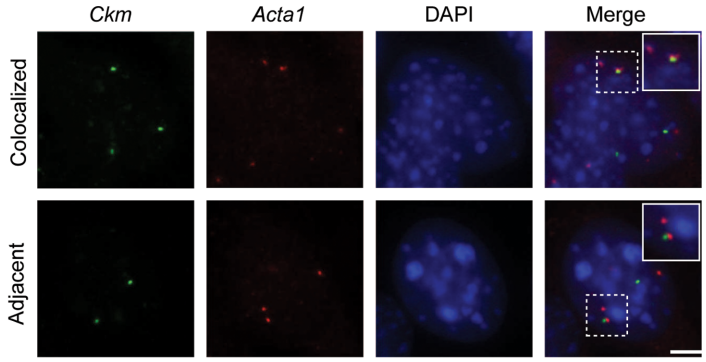
Figure 1. 3C analyses of the indicated gene promoter sequences with the *Ckm* locus. (**A**) Interactions as a function of time of embryonic development. (**B**–**D**) Interactions in MyoD-differentiated fibroblasts. (**E**) Interactions in C2C12 cells. Numbers along the x-axis represent *Ckm* sequences relative to the start site of transcription. Interaction frequencies were based on ΔCt values and normalized based on primer set amplification efficiencies. The maximum interaction frequency value was normalized to 1 and all other values are shown relative to that value. Values represent the average plus/minus the standard deviation from three independent experiments. In (D), interactions were measured relative to the frequency of *Des*–*Ckm* interactions at time 0. (**F**–**G**) Interactions between the indicated gene sequences and the *Ckm* promoter sequence. The interaction frequency at time 0 was normalized to 1 in each experiment and all other values are shown relative to that value. Values represent the average ± the standard deviation from three independent experiments.

A

C2C12 cells

	experiment 1	experiment 2	experiment 3
0 h	7.9%(*n*=318)	8.5%(*n*=352)	10.6%(*n*=397)
24 h	0.6%(*n*=314)	0.9%(*n*=344)	0.3%(*n*=373)
72 h	0.0%(*n*=352)	0.3%(*n*=334)	0.5%(*n*=366)

B



C

MyoD-differentiated fibroblasts

+Tetracycline		experiment 1	experiment 2	experiment 3
MyoD	0 h	7.7%(*n*=323)	9.3%(*n*=344)	10.0%(*n*=360)
Empty Vector	0 h	0.6%(*n*=323)	0.6%(*n*=337)	0.5%(*n*=378)
MyoD	24 h	0.7%(*n*=306)	1.2%(*n*=346)	0.3%(*n*=365)
Empty Vector	24 h	0.3%(*n*=300)	0.6%(*n*=365)	0.8%(*n*=372)

-Tetracycline		experiment 1	experiment 2
MyoD	0 h	1.4%(*n*=216)	1.5%(*n*=258)
Empty Vector	0 h	0.9%(*n*=234)	1.1%(*n*=267)
MyoD	24 h	0.8%(n=245)	1.7%(*n*=240)
Empty Vector	24 h	0.4%(*n*=266)	0.8%(*n*=245)

D

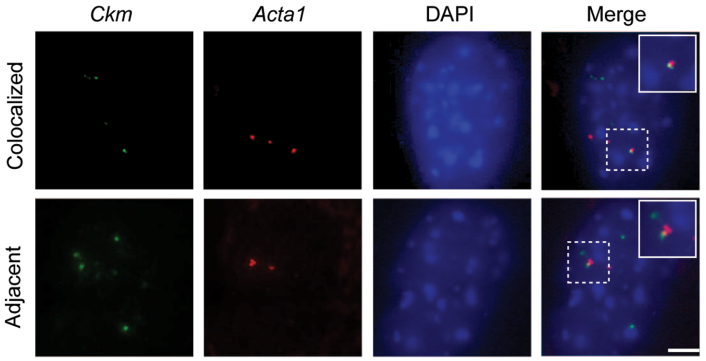


Figure 2. FISH experiments reveal myogenic late gene associations in C2C12 and in MyoD differentiated B22 cells. Fluorescently labeled BAC DNAs including the *Ckm* or *Acta1* loci or DAPI were used for labeling fixed cells. (**A**, **C**) Gene association frequencies for each condition tested. Representative images are shown to demonstrate (**B**) co-localized or (**D**) adjacent signals. Size bar = 5 μm.

Inter-chromosomal interactions between myogenic loci are corroborated by fluorescence *in situ* hybridization and require functional SWI/SNF chromatin remodeling enzymes

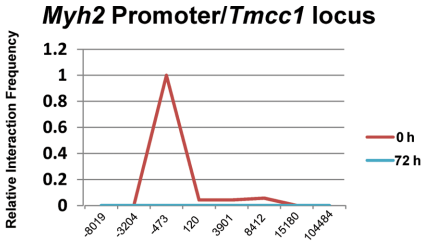
FISH was used to corroborate inter-chromosomal interactions between *Ckm* and *Acta1*. The frequency of any alleles of the genes being co-localized or adjacent and in contact in C2C12 cells, which were previously reported to be tetraploid (42,43), at time 0 was ∼10% ($P < 0.0002$), whereas the frequency of allele association fell to <1% in cells differentiated for 72 h (Figure 2A and B), consistent with the decrease in interaction frequencies noted in the 3C experiments in Figure 1E–H.

The SWI/SNF chromatin remodeling enzymes (44–46) are required to alter myogenic gene regulatory sequences during myogenesis (20,22,47). To determine whether these enzymes also contribute to the formation of the observed myogenic gene inter-chromosomal interactions, the experiment was repeated in MyoD- or mock-differentiated B22 fibroblast cells, which are also tetraploid. B22 fibroblasts contain a tetracycline suppressible, ATPase deficient, dominant negative Brg1 allele that is competent for SWI/SNF enzyme complex but cannot activate gene expression or remodel chromatin at target loci (2,18,20,22,48,49). At time 0 in the presence of tetracycline, which suppresses dominant negative Brg1 expression, the frequency of alleles of *Ckm* and *Acta1* being co-localized or adjacent and in contact (Figure 2C and D) in MyoD differentiated cells was 8–10% ($P = 0.0011$), whereas mock-differentiated cells was ∼0.5%. At 24 h post-differentiation, the frequency of allele association was 0.3–1.2% in both mock- and MyoD-differentiated cells, consistent with the idea that interactions between these genes occur at the onset of differentiation and are less frequent as differentiation proceeds. In the absence of tetracycline, where expression of dominant negative Brg1 is induced, the frequency of allele association ranged from 0.4 to 1.7%, regardless of the presence of MyoD or the time point examined (Figure 2C). The association frequencies are comparable to those of other published inter-chromosomal FISH interactions (50–52), and we believe that the nearly absolute incidence of observing only one pair of alleles interacting per cell reflects the complexity of inter-chromosomal interactions across the genome and suggests that individual genes have multiple interacting partners. Thus, gene interactions measured by FISH are differentiation- and Brg1-dependent and are consistent with the 3C data.

Additional corroboration of inter-chromosomal myogenic gene interactions

To provide further support for the existence of inter-chromosomal interactions between myogenic genes, we examined two additional loci on two different chromosomes. Other work in progress in our labs has identified *Tmcc1*, which encodes Transmembrane and Coiled-Coil Domain Family 1 and which has been linked to endoplasmic reticulum organization (53) and human adiposity (54), as a gene expressed in C2C12 cells with kinetics similar to those of other myogenic late genes (Supplementary Figure S3). The *Tmcc1* locus, located on chromosome 6, was tested by 3C assay for inter-chromosomal interactions with the myosin

A

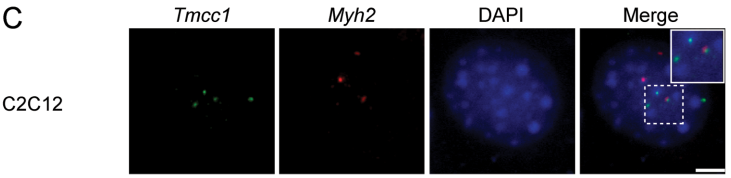


B

C2C12 cells

	experiment 1	experiment 2	experiment 3
0 h	8.0%(*n*=339)	7.4%(*n*=394)	10.1 %(*n*=308)
24 h	0.6%(*n*=333)	0.8%(*n*=375)	1.0%(*n*=290)
72 h	0.3%(*n*=325)	0.3%(*n*=365)	0.0%(*n*=310)

C



D MyoD-differentiated fibroblasts

+Tetracycline		experiment 1	experiment 2	experiment 3
MyoD	0 h	9.7%(*n*=380)	12.3%(*n*=276)	11.6%(*n*=363)
Empty Vector	0 h	0.3%(*n*=322)	1.1%(*n*=276)	0.7%(*n*=280)
MyoD	24 h	0.9%(*n*=339)	0.8%(*n*=266)	0.7%(*n*=281)
Empty Vector	24 h	0.0%(*n*=365)	0.3%(*n*=313)	0.4%(*n*=250)

-Tetracycline		experiment 1	experiment 2
MyoD	0 h	1.1%(*n*=266)	1.0%(*n*=315)
Empty Vector	0 h	1.2%(*n*=246)	1.2%(*n*=339)
MyoD	24 h	0.9%(*n*=226)	0.6%(*n*=344)
Empty Vector	24 h	0.4%(*n*=229)	0.9%(*n*=330)

E

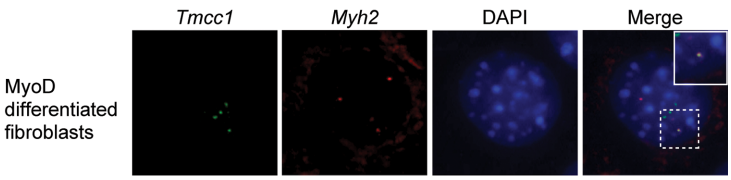


Figure 3. Additional genes show inter-chromosomal interactions by 3C and FISH. (**A**) Conventional 3C analysis indicating interactions between the *Myh2* promoter and the *Tmcc1* locus in C2C12 cells at times 0 and 72 h. PCR amplification efficiency for each primer pair was evaluated as in Supplementary Figure S1, and differences in amplification efficiency between primer sets were accounted for when inter-chromosomal interaction frequencies were determined. The maximum interaction frequency value was normalized to 1 and all other values are shown relative to that value. Values represent the average from two independent experiments. (**B**) FISH association frequencies between *Tmcc1* and *Myh2* in C2C12 cells at the indicated times. (**C**) FISH images showing association of *Myh2* and *Tmcc1* in C2C12 cells at the onset of differentiation. (**D**) FISH association frequencies between *Tmcc1* and *Myh2* in MyoD-differentiated B22 cells for each condition tested. (**E**) FISH images showing association of *Myh2* and *Tmcc1* in MyoD-differentiated B22 cells cultured in tetracycline at the onset of differentiation. Size bar = 5 μm.

heavy chain 2 (*Myh2*) promoter, which is located on chromosome 11 and is also expressed with late gene kinetics in C2C12 cells (Supplementary Figure S3). The results clearly showed that the *Myh2* promoter sequence was in close proximity to sequences just upstream of the start site of *Tmcc1* transcription at the onset of differentiation and that the interaction frequency between these sequences was greatly decreased in differentiated cells (Figure 3A). FISH experiments examining the frequency of allele associations between *Myh2* and *Tmcc1* in C2C12 and B22 cells at the onset of differentiation (Figure 3B–E) were entirely consistent with the results of the *Ckm*/*Acta1* FISH analysis ($P < 0.0002$ and $P = 0.001$, respectively). Expression of the dominant negative Brg1 in MyoD-reprogrammed B22 fibroblasts prevented inter-chromosomal interactions between the *Tmcc1* and *Myh2* promoters at the onset of differentiation (Figure 3D). These data extend the generality of the observation that myogenic late gene promoters are in close proximity to other myogenic late gene promoter sequences at the onset of differentiation and reiterate the requirement for commitment to the skeletal muscle lineage and for Brg1 in formation of these gene associations.

Interactions between late gene regulatory sequences require MyoD and functional SWI/SNF chromatin remodeling enzymes

To more definitively address the dependency of the observed inter-chromosomal interactions on commitment to the skeletal muscle lineage, C2C12 cells were treated with siRNA that targets MyoD, as previously described (21). Our prior work demonstrated that siRNA treatment inhibited myogenic gene expression (21) and ChIP analysis reiterates the previous observations by showing inhibition of MyoD binding to target genes (Supplementary Figure S4). 3C interaction frequencies between the *Acta1* and *Ckm* promoters were greatly reduced upon knockdown of MyoD (Figure 4A and B). Since both the fibroblast and myoblast based differentiation systems require cell confluence at the onset of differentiation and since previous work has demonstrated that cell cycle exit is achieved in our hands (20,21), the observed differences in inter-chromosomal interactions are more likely due to the expression of MyoD than to cell cycle state.

The FISH experiments indicated a Brg1-dependency for inter-chromosomal myogenic gene promoter associations (Figures 2 and 3). To determine whether these enzymes also contribute to the formation of myogenic gene inter-chromosomal interactions observed by the 3C assay, we again used the B22 cells to perform experiments in the pres-

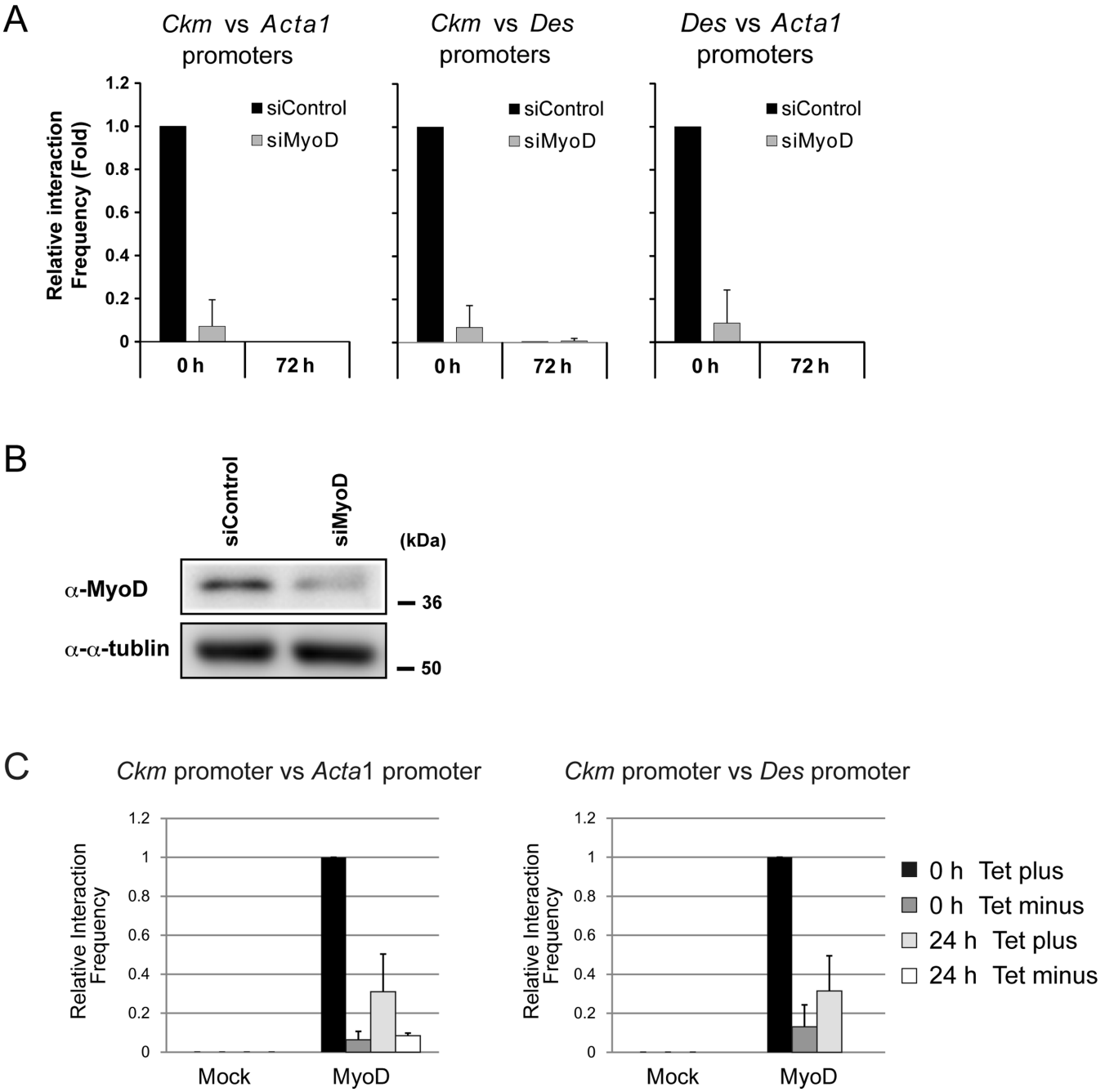


Figure 4. MyoD and Brg1 involvement in inter-chromosomal interactions at the onset of differentiation. (**A**) 3C experiments supporting the MyoD dependency of inter-chromosomal interactions between the indicated myogenic promoters. C2C12 cells were treated with MyoD siRNA or an siRNA control as described (21) and used for conventional 3C experiments at time 0 h. (**B**) Western blot indicating MyoD and tubulin levels in extracts from duplicate plates of C2C12 cells treated with MyoD siRNA or an siRNA control. (**C**) 3C interactions between the indicated promoters require functional Brg1. Interaction frequencies were based on ΔCt values. The maximum interaction frequency value was normalized to 1 and all other values are shown relative to that value. Values represent the average ± the standard deviation from three independent experiments.

ence and absence of functional SWI/SNF chromatin remodeling enzymes. Cells maintained in tetracycline showed interactions between the *Ckm* and the *Acta1* or *Des* promoters at time 0, with reduced interaction frequencies at 24 h post-differentiation (Figure 4C), consistent with data presented in Figure 1B and C. B22 cells cultured without tetracycline expressed dominant negative Brg1; 3C interactions between the *Ckm* and *Des* regulatory sequences were significantly inhibited at both 0 and 24 h post-differentiation (Figure 4C). Thus, MyoD-induced re-organization of late gene regulatory sequences requires functional Brg1.

The dependency of late gene regulatory sequence associations on functional Brg1 was initially puzzling because the interactions had already occurred by start of differentiation, while previous experiments demonstrated that Brg1 bound to late gene regulatory sequences at 8 h post-

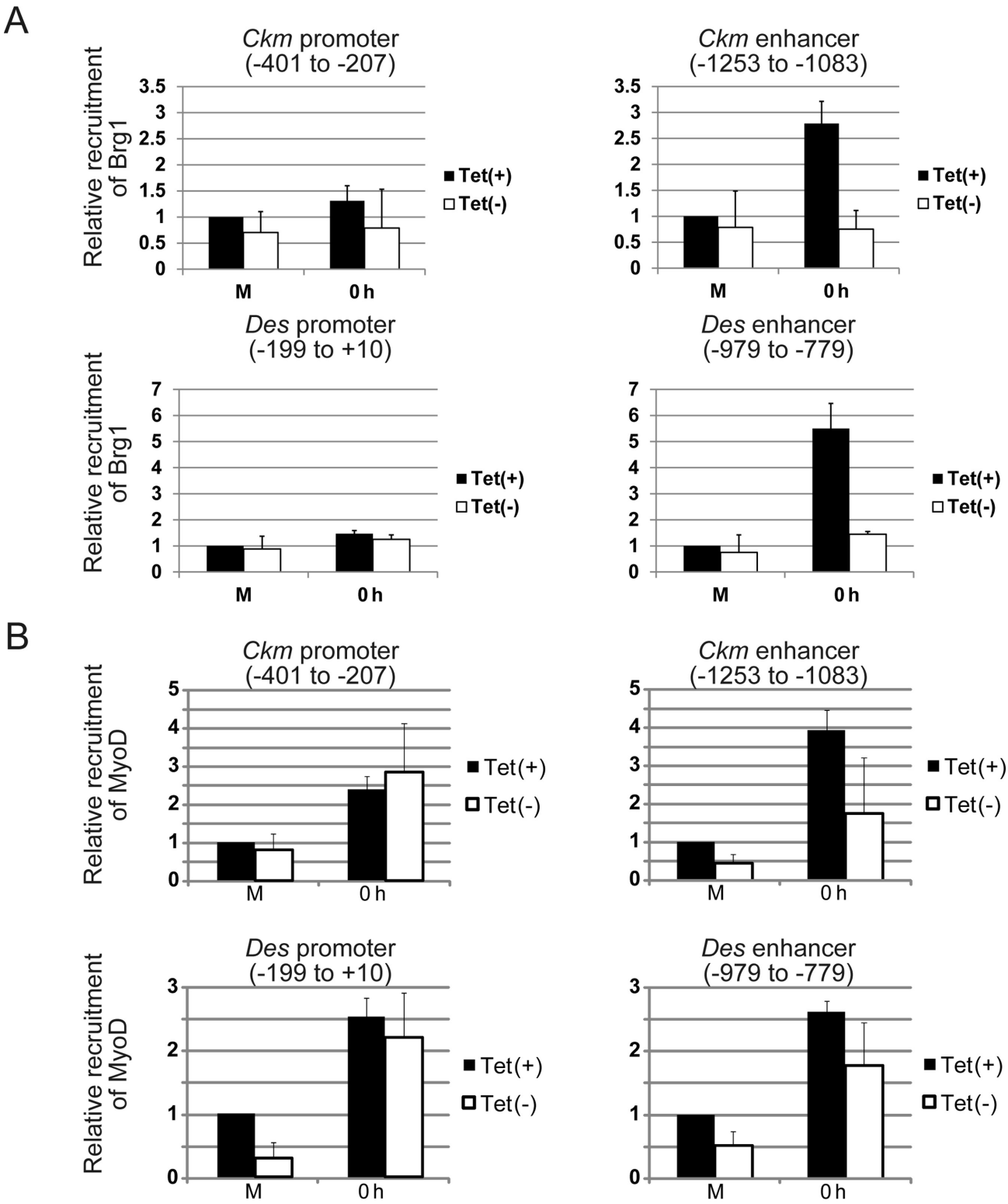


Figure 5. BRG1 binding to myogenic late gene enhancer and promoter sequences. (**A**) Brg1 binding to the enhancer, but not to the promoter sequences, can be detected at the onset of differentiation (time 0) by ChIP. The lack of dominant negative Brg1 binding is consistent with previously published data (2). (**B**) ChIP assays demonstrating that MyoD binds to both enhancer and promoter sequences in a Brg1 independent manner at the onset of differentiation. Data in (A and B) represent the average plus/minus the standard deviation from three independent experiments. For each data set, the value of the mock-differentiated, plus tet sample was set at 1.0. M; mock-differentiated, where cells were infected with an empty, instead of a MyoD encoding, retrovirus.

differentiation, coincident with late gene activation (2). We therefore re-examined Brg1 localization to late gene regulatory sequences with two modifications to the ChIP protocol: sonication of genomic DNA to mononucleosome size to distinguish between the promoter and enhancer regions and use of a recently developed monoclonal antibody against Brg1. At time 0, Brg1 was present at the enhancers, but not at the promoters, of the *Ckm* and *Des* genes (Figure 5A), while MyoD was present at both (Figure 5B). The mouse *Acta1* gene does not have a defined enhancer sequence, but ChIP analysis of the *Acta1* promoter was consistent with that of the *Ckm* and *Des* promoters (Supplementary Figure S5). As shown previously, the relative recruitment of Brg1 increased at later time points (not shown and (2)). Thus, the dependency of inter-chromosomal associations on functional Brg1 can be linked to the localization of Brg1 at late gene regulatory sequences when the associations are observed. However, restriction enzyme accessibility assays done to assess chromatin structural changes at the enhancers confirmed previous findings showing that changes in chromatin structure are co-incident with gene activation (not shown and (2)). Thus, Brg1 is present at myogenic late gene enhancers when re-organization of late gene regulatory sequences occurs, but its role appears distinct from its chromatin remodeling function. This suggests dual functions for Brg1 in reorganization of genome sequences and in ATP-dependent chromatin remodeling at target genes.

MyoD and HDAC2 repress late gene expression at early times of myogenic differentiation

In the presence of MyoD, late gene regulatory sequences are bound by MyoD and HDAC2 prior to the onset of late gene expression (2,55), suggesting a putative repressive role for these factors at early times of differentiation. To further address the function of late gene regulatory sequence associations, we took advantage of our previous data showing that ectopic expression of myogenin and Mef2D1b in fibroblasts can induce myogenic late genes and differentiation without activating the endogenous *Myod1* locus (2,56). We utilized this strategy to address the functional role of MyoD binding to late gene regulatory sequences at early times of differentiation before the genes are expressed. A time course of *Ckm*, *Des* and *Acta1* gene expression demonstrated that these genes were prematurely expressed when differentiation was driven by myogenin and Mef2D1b (Figure 6A). Since MyoD is not expressed under these conditions, it cannot bind to these genes; ChIP assays demonstrated that HDAC2 also was not present on the *Ckm*, *Des* and *Acta1* regulatory sequences under these conditions (Figure 6B). Therefore the presence of MyoD and HDAC2 prior to gene expression functions to repress late gene expression at early times of differentiation.

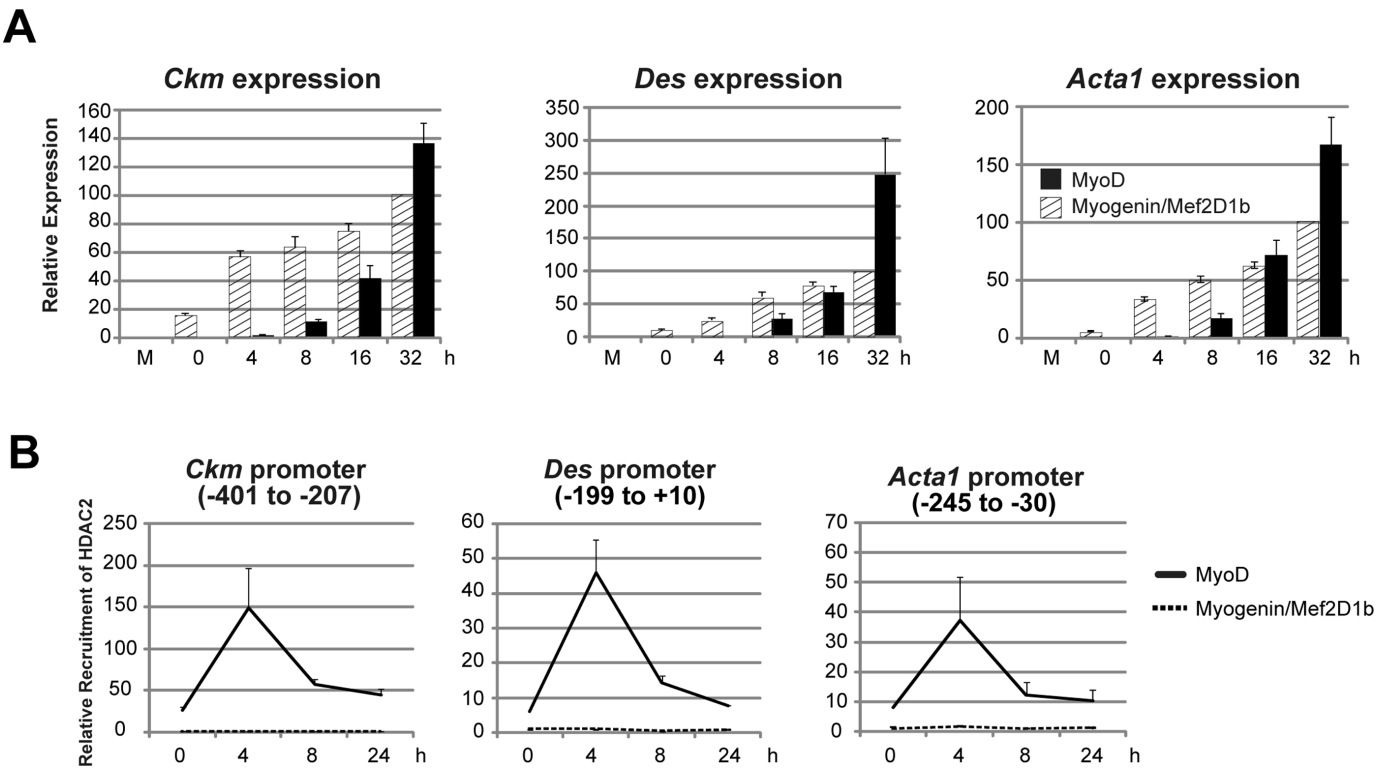


Figure 6. MyoD and HDAC2 repress myogenic late genes at early times of differentiation. (**A**) Time course of gene expression demonstrating that bypassing MyoD by differentiating B22 cells with myogenin/Mef2D1b resulted in premature late gene activation. mRNA levels were normalized to EF1α levels. (**B**) ChIP time course indicating that HDAC2 did not bind to late gene regulatory sequences in myogenin/Mef2D1b differentiated B22 cells where MyoD is not expressed. The binding in the myogenin/Mef2D expressing cells at time 0 was normalized to 1. Data are presented as the average of three or more independent experiments ± standard deviation.

Functional assessment of inter-chromosomal interactions between myogenic late gene regulatory sequences

Our experiments have shown that inter-chromosomal interactions between myogenic late gene regulatory sequences occur at early times of skeletal muscle differentiation, that these interactions require MyoD, and that MyoD, in conjunction with HDAC2, serves to repress late myogenic gene expression at early times of skeletal muscle differentiation. Based on these data, we hypothesized that the formation of inter-chromosomal interactions between myogenic late genes is related to the repressed state of late myogenic genes early in the differentiation process. 3C analysis on cells differentiated by myogenin and Mef2D1b showed a significant reduction in the frequency of regulatory sequence association (Figure 7A). To complement this result, FISH was performed on myogenin/Mef2D1b-differentiated fibroblasts. The results indicate a low frequency of *Ckm*/*Acta1* association (Figure 7B) that matched the background levels seen in the previous experiments (Figure 2), and that is consistent with the 3C data. Since both *Ckm* and *Acta1* were expressed under these differentiation conditions, clearly inter-chromosomal associations are not a prerequisite for gene expression. Instead, the data implicates late gene regulatory sequence interactions as part of the mechanism by which temporal control of gene expression is mediated. Late gene expression occurred prematurely in the absence of these interactions, suggesting that these inter-genic associations are involved in the repression of late genes at early times.

In sum, our study reveals several novel findings. We conclude that higher order gene organization in differentiating skeletal muscle cells is dynamic and changes as a function of time of differentiation and that the spatial organization of the myogenic late genes is distinct from that of the myogenin gene. The physical proximity of myogenic late genes at the onset of differentiation requires both MyoD and the Brg1 ATPase of the SWI/SNF chromatin remodeling enzyme. Finally, we report that the interactions between myogenic late genes are associated with the repression of these genes at early times of differentiation, implicating higher order gene organization as a contributing mechanism to temporally regulation of gene expression during differentiation.

DISCUSSION

Higher order gene organization as a mechanism to temporally control gene expression

Molecular interactions between sequences on different chromosomes have not been functionally studied or characterized to the extent that intra-chromosomal interactions have been. There are numerous examples of links between inter-chromosomal associations and gene activation (14,15,50,52,57–62), and there is one report demonstrates inter-chromosomal interactions between silent loci are lost upon T cell activation (63). Here, we document inter-chromosomal interactions that form between differentiation-specific genes that are coordinately activated in a temporal manner. The associations between myogenic gene regulatory sequences occurred prior to gene expression, however, expression of these genes did not require the regulatory sequence associations. Since in the absence of these interactions late gene expression occurred prematurely, we propose that the associations are a part of a mechanism to keep late genes off at early times via a differentiation-dependent process that spatially organizes these genes in close proximity to each other.

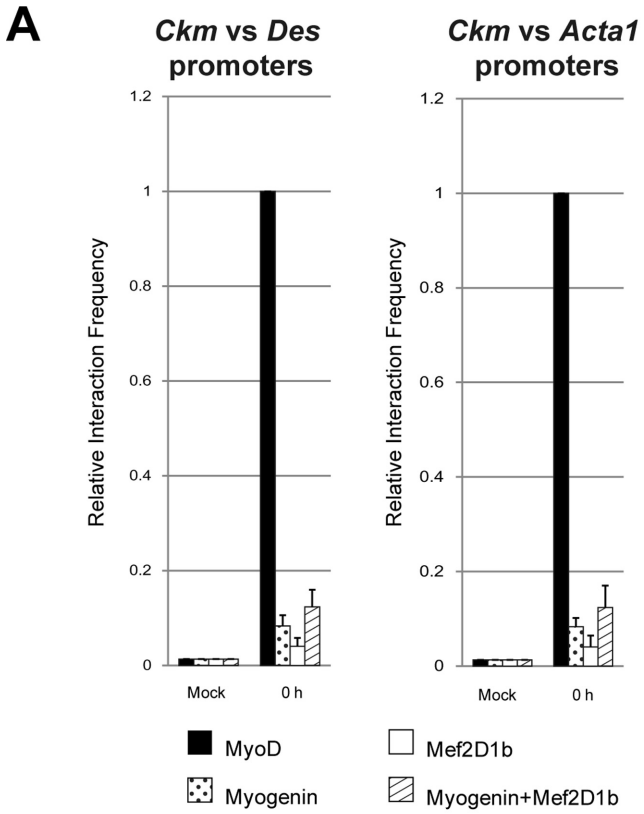
Our results indicate that the inter-chromosomal interactions we documented are MyoD-dependent. We demonstrated this dependency in the cell line systems used by knocking down MyoD in C2C12 cells and by driving differentiation in fibroblasts via ectopic expression of MyoD. While this implicates a specific requirement for the MyoD protein, *in vivo* mouse modeling indicates that the MyoD-related Myf5 regulatory factor is redundant for MyoD during embryonic development (64,65) and that ectopic expression of MyoD and Myf5 in fibroblasts result in functional reprogramming of the cells into the skeletal muscle lineage via a mechanism that requires functional SWI/SNF enzymes (66,67). We suggest that either MyoD or Myf5 may mediate the formation of inter-genic interactions between myogenic loci *in vivo*.

Speculation on the nature of long-range interactions between gene loci

The association of myogenic late gene sequences appears dynamic in that the frequency of inter-chromosomal interactions decreases as a function of myoblast differentiation, which correlates with the expression of the late genes. In addition, the data suggest that interactions within individual nuclei are also dynamic. The FISH data indicate that at any given time, any specific pair of myogenic late gene sequences can be found in association in ∼10% of the cells in a monoallelic pattern. We suggest that this reflects variability in late gene sequence interactions and that not every allele of every gene interacts with the same partner genes within the nucleus. Instead, we envision multiple networks of myogenic late gene interactions, perhaps at the borders of chromosomal territories, in keeping with our FISH-based observation that myogenic late gene associations are almost always seen as abutting signals.

It is possible that the regulatory sequences either exist within a pre-formed nuclear domain or create a new domain that spatially restricts gene expression at early times of myogenesis, though it is not a requirement that these interactions occur within or at a specific structure. At later times, when myogenin, which is required for late gene expression and terminal differentiation (68,69), has been synthesized, it and associated co-activators displace the HDAC2 co-repressor and promotes gene activation (2). Thus, we propose that the spatial arrangement and dynamic clustering of the myogenic late genes is dissolved, or, perhaps more likely, is reorganized at the time the late genes become transcriptionally competent. This hypothesis does not exclude the possibility that actively expressing myogenic late genes may be organized into different higher-order structures in which they are associated with other transcribed genes in or around the transcription factories that have been reported as sites of active polymerase II based gene expression (70,71).

The differentiation dependence of the observed interactions between myogenic late genes suggests that signal



B

+Tetracycline		experiment 1	experiment 2
Myog/Mef2D1b	0 h	0.6%(*n*=323)	0.0%(*n*=273)
Empty Vector	0 h	0.3%(*n*=312)	1.2%(*n*=234)
Myog/Mef2D1b	24 h	1.0%(*n*=286)	0.8%(*n*=247)
Empty Vector	24 h	0.7%(*n*=290)	1.3%(*n*=238)

Figure 7. Myogenic late gene interactions are associated with the timing of gene expression. (**A**) 3C analysis of late gene interaction frequencies in myogenin/Mef2D1b differentiated B22 cells where MyoD is not expressed. Data are presented as the average of three or more independent experiments ± standard deviation. (**B**) Gene association frequencies between *Ckm* and *Acta1* in myogenin/Mef2D1b differentiated B22 cells as determined by FISH.

transduction pathways regulating myogenesis may also be involved. Signaling to chromatin as a means to effect epigenetic regulation is an emerging topic (72). Recent studies on long-range interactions between adipogenic gene loci in tissue culture cells revealed that protein kinase A (PKA) signaling, which is a requirement for *in vitro* adipocyte differentiation, was required for the formation of the interactions (73). Other studies showed that TNFα induced inter- and intra-chromosomal interactions between NF-κB target genes (74). The mechanisms by which these signaling pathways contribute to higher order chromatin association between specific gene loci remain to be determined, but the studies illustrate the concept that relative gene positioning within the nucleus can be responsive to signal transduction cascades. Successful completion of myogenesis involves dozens of kinases (75), so the number of potential signaling pathways that might influence higher order gene organization is large. However, the p38 MAP kinase has long been known as an effector of myogenic gene expression and a modifier of chromatin remodeling enzymes (47,76–79). Therefore, p38 signaling may directly or indirectly affect myogenic gene positioning, perhaps at the stage when gene activation occurs and the gene associations are lost.

A chromatin remodeling enzyme is required for changes in gene positioning during myogenesis

While a model for formation of inter-chromosomal interactions may reflect global reorganization of chromatin, it is also possible that formation of these interactions reflects an accumulation of more modest, localized changes. The observation that the Brg1 ATPase of SWI/SNF chromatin remodeling enzymes is necessary for the formation of the inter-chromosomal interactions is consistent with either possibility, since it alters chromatin structure locally at specific regulatory sequences, is required for 3D structure of complex gene loci such as α- and β-globin, CTIIA,

and Th2 (80–83), and also has been proposed to be necessary for global chromatin changes (58). Additional studies postulated that Brg1 organizes chromatin near the nuclear lamina because inter-genic Brg1 binding sites coincide with lamin-associated DNA sequences (84). To date, such functions have been described only for the Brg1 ATPase. The alternate SWI/SNF ATPase protein, called Brm (46,85), has not been shown to mediate such functions, though it is unknown whether it does not or its functions are simply less well characterized. Regardless, work examining whether inter-chromosomal interactions are accompanied by global changes in chromatin/chromosome structure have provided a wide range of conclusions (50,52,57,58,86); clearly additional work by the field is necessary to provide better understanding of the structural ramifications of rapidly occurring changes in gene and/or chromosome positioning. Regardless, the demonstration of specific inter-chromosomal interactions between sequences directly upstream of differentiation-specific genes and the links to lineage determining transcription factors, chromatin remodeling enzymes, and the temporal control of gene expression should provide a platform for future investigations into the regulation of genome organization and tissue-specific gene expression.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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