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Regulated Demethylation of the *myoD* Distal

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myoD is one of a family of four related basic helix-loop-helix transcription factors involved in the specification and differentiation of skeletal muscle. We previously identified a 258-bp distal enhancer that is sufficient for embryonic activation of *myoD* and is highly conserved between humans and mice. In this paper, we show using a modified bisulfite deamination/PCR amplification method that the distal *myoD* enhancer is completely unmethylated at all the CpG sites tested in myogenic cells and a subpopulation of somite cells. Conversely, the distal enhancer in nonmuscle cells and tissues is methylated to an average level of >50% and we find no chromosomes in these tissues with a completely unmethylated enhancer. We present evidence that demethylation of the distal enhancer in somites of mouse embryos precedes *myoD* transcription, suggesting that demethylation of the distal enhancer is an active, regulated process that is essential for *myoD* activation. We also show by analysis of transgenic mice carrying a human distal enhancer/reporter construct in which the three enhancer CpG sites have been mutated that methylation of the distal enhancer is not required to prevent precocious or ectopic embryonic *myoD* expression. We propose that a subset of somite cells demethylate the distal enhancer in response to specific developmental signals, thus making the enhancer accessible and able to respond to subsequent signals to activate the *myoD* gene. © 1996 Academic Press, Inc.

INTRODUCTION

DNA methylation in vertebrates is associated with a variety of gene regulatory processes including X-chromosome inactivation, genomic imprinting, and transcriptional regulation (reviewed by Bestor *et al.,* 1994; Razin and Kafri, 1994; Martienssen and Richards, 1995). Altering the DNA methylation level of tissue culture cells can have dramatic effects on cellular phenotypes (Michalowsky and Jones, 1989). 10T1/2 cells treated with 5-azacytidine (an unmethylatable analog of cytidine that results in a decrease in the amount of CpG methylation) convert at high frequency to muscle and at lower frequencies to fat or cartilage phenotypes (Taylor and Jones, 1982). Konieczny and Emerson showed that this conversion event is clonal and demethylation of one or a few loci appears sufficient for determination of 10T1/2 cells to a stably committed muscle lineage (Konieczny and Emerson, 1984). Independent evidence of this phenomenon was obtained by transfection of 10T1/2 cells with an antisense methyltransferase expression construct that also results in DNA demethylation and conversion of the transfected cells to muscle (Szyf *et al.,* 1992). Taken together, these experiments suggest that myogenic cell determination can be accomplished by demethylating the sequences of one or a few regulatory genes.

myoD is one of four related myogenic regulatory factors (MRFs) that control skeletal myogenesis and are first expressed in somites specifically in those cells which will give rise to skeletal muscle (reviewed by Buckingham, 1994; Rudnicki and Jaenisch, 1995). *myoD* and a second MRF (*myf5*) together are required to specify and/or maintain cells in the myogenic lineage as mice doubly mutant for these two regulatory factors make no distinguishable myoblasts or muscle (Rudnicki *et al.,* 1993). Establishing how these MRFs are developmentally regulated will be fundamental in understanding how somitic cells become determined to the skeletal muscle lineage. Jones *et al.* (1990a) have shown that the *myoD* gene is expressed and the *myoD* CpG island demethylated in myogenic cells derived from 10T1/2 cells by 5-azacytidine treatment. However, the CpG island is not methylated *in vivo.* Thus, while methylation of *myoD* sequences could play a regulatory role in normal embryonic muscle development, the target must be sequences other than the *myoD* promoter.

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We have identified a distal enhancer 20 kb upstream of the *myoD* gene that is sufficient to recapitulate the entire program of embryonic *myoD* activation (Goldhamer *et al.,* 1992, 1995; Faerman *et al.,* 1995). This distal enhancer has some interesting properties that suggest it could be a target for epigenetic regulation. The distal enhancer can direct appropriate expression of lacZ reporter constructs in transgenic mice, while those same constructs are expressed promiscuously in tissue culture cell transfection assays (transient or stable) in nonmuscle cells that do not express the endogenous *myoD* gene (Goldhamer *et al.,* 1992, 1995; Faerman *et al.,* 1995). In addition, the distal enhancer is hypersensitive to DNaseI in tissue culture cells (both primary cells and established cell lines) that express the *myoD* gene but is not hypersensitive in cell lines in which the gene is not expressed (Goldhamer *et al.,* 1995). These observations suggest that the distal *myoD* enhancer is a target for epigenetic regulation that is established in the embryo and, while maintained in tissue culture cells at the endogenous *myoD* locus, is not established *de novo* in these cells. Taken together with 5-azacytidine activation of the *myoD* gene in 10T1/2 cells, these data suggest that the distal enhancer is a good candidate target for methylation regulation. In this paper, we show that an unmethylated distal enhancer correlates with *myoD* gene expression. Additionally we present evidence showing that the distal enhancer becomes demethylated prior to detectable expression of the *myoD* gene in a subpopulation of somite cells that are presumably in the myogenic lineage. We propose that the *myoD* enhancer is demethylated by an active, regulated process in response to specific developmental signals and, while demethylation is necessary for *myoD* expression, additional signals are required to activate transcription of the *myoD* gene in somites during embryonic development.

MATERIALS AND METHODS

Tissue Culture

Cell lines were obtained from ATCC except 23A2 muscle cells (Konieczny and Emerson, 1984) and IB1 fat cells (I. Akerblom and B. Brunk, unpublished). NB41A3 and BNL cells were grown in 90% DMEM/10% FBS, C2C12 cells in 85% DMEM/15% FBS, 10T1/2 and IB1 cells in 90% BME/10% FBS, and 23A2 cells in 85% BME/15% FBS. Proliferating cell cultures were harvested for DNA by scraping in DNA lysis buffer (150 m*M* NaCl, 2% SDS, 15 m*M* EDTA, 400 μg/ml Proteinase K (Merck), 50 m*M* Tris–HCl, pH 7.5) and incubated for 2 hr at 55°C or overnight at 37°C. Samples were then extracted with phenol, phenol/chloroform, ethanol precipitated, and resuspended in TE.

Embryo Dissections and DNA Isolation

Embryos were derived from ICR (Fox Chase Cancer Center) or CD-1 (Charles River) mice. Noon of the day of the vaginal plug was considered 0.5 dpc. Embryos were dissected in PBS with or without 10% dispase (Boehringer-Mannheim) using sharpened tungsten needles and roughly dissected tissues moved into dispase-free PBS for final removal of adherent membranes. Somite and presomitic mesoderm samples were dissected free of axial structures and lateral mesoderm. Presomitic mesoderm samples included all the unsegmented paraxial mesoderm which is the equivalent of 3–4 somites in length in a 9.5-dpc embryo. Tissue was then placed in 100 μl DNA lysis buffer (see above) containing 0.02 μg/μl plasmid DNA carrier and vortexed vigorously. Samples were then processed as above and resuspended in 20 μl of TE.

Bisulfite Treatment

The bisulfite treatment protocol was modified from Frommer *et al.* (1992) and Clark *et al.* (1994). Ten microliters of DNA (1 μg including plasmid carrier) was denatured by addition of 0.2 *M* sodium hydroxide and incubation at 65°C for 10 min, neutralized by addition of 0.1 vol 3 *M* sodium acetate, pH 5.2, and precipitated with 3 vol of ethanol. Alternatively, the DNA was denatured in a total volume of 12.5 μl in 0.2 *M* sodium hydroxide for 10 min at 65°C and then used directly in the bisulfite reaction without precipitating. DNA was resuspended in 22.5 μl sterile water and 77.5 μl 4 *M* sodium bisulfite, pH 5.0 (0.43 g sodium bisulfite, 0.03 g sodium sulfite, 860 μl sterile water, and 10 μl 50 m*M* hydroxyquinone) was added. Samples were mixed, overlaid with mineral oil, and incubated at 50°C in the dark overnight. The sodium bisulfite was removed by chromatography over a 1-ml Sephadex G50-80 (Sigma) spin column. Forty microliters of 1 *M* sodium hydroxide was then added and the samples incubated at 37°C for 30 min followed by chromatography through a second spin column to remove the base. Samples were stored at −20°C until PCR amplification.

PCR Amplification and Quantitation of Methylation

The coding strand relative to the *myoD* gene of all samples was PCR amplified for 25 cycles of amplification (94°C 1 min, 51°C 1.5 min, 74°C 1 min) with oligos 632f (GTTTTATAGTATTTGGGG-GTAT) and 966fc (AAAACCCTAAAACTAACCTTC). The numbering in oligo naming is relative to a 1.6-kb *Rsa*I fragment that contains the mouse core enhancer (Goldhamer *et al.,* 1995) starting at base 634. Two microliters of this reaction was then amplified 5 cycles (94°C 1 min, 54°C 1.5 min, 74°C 1 min) followed by 25 cycles (94°C 1 min, 64°C 1.5 min, 74°C 1 min) with oligos 668f (GGAATTCGGGGTTTTTTTATAAATTTTTGAGATAGT) and 939fc (GGTCGACAAAATACTAACCTCTCATACCT). Products were gel purified using the GeneClean kit (Bio101). Ten percent of the purified band was sequenced by thermal cycle sequencing (Circumvent, New England Biolabs) using primer 668f and electrophoresed on an 85-cm sequencing gel to facilitate band separation. Dried gels were exposed for 4 hr to overnight on a phosphorimager (Fuji Bas1000 or Molecular Dynamics 445si). Band intensities were quantitated using either MacBas (Fuji) or IPLabGel (Signal Analytics) software and the data analyzed and graphed using Microsoft Excel. Averages for all samples are from at least three independent experiments and error bars represent one standard deviation above and below the mean.

Subcloning and Sequencing PCR Products

PCR products were phenol/chloroform extracted and precipitated then digested with *Eco*RI and *Sal*I, gel purified using GeneClean

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(Bio 101), and ligated into a *Sal*I/*Eco*RI-digested bluescript (KSII$^+$) vector (Stratagene). Subcloned plasmid DNA was prepared using the Wizard kit (Promega) and sequenced using sequenase 2.0 (USB) and standard protocols.

Genomic Southern Methylation Analysis

Twenty micrograms of genomic DNA was digested to completion with *Rsa*I and *Hpa*II or *Msp*I, phenol/chloroform extracted, precipitated, and electrophoresed in a 1% agarose gel. The DNA was transferred to a nytran membrane (Schleicher & Schuell) via vacublotting (Hoeffer) and cross-linked in a stratalinker (Stratagene) following manufacturers' recommendations. The filter was hybridized following the protocol of Church and Gilbert (1984) with a ^{32}P-labeled 1.6-kb *Rsa*I fragment containing the mouse core enhancer.

Mutagenesis

CpG mutations were created by the PCR-based overlap extension method as previously described (Ho *et al.*, 1989), using Vent polymerase, and the core enhancer cloned in pBluescript (KS$^+$) as the template (Goldhamer *et al.*, 1995). By using mutant derivatives as the template for subsequent rounds of mutagenesis, a construct was obtained in which all three CpG dinucleotides were mutated to TpG. Mutations were confirmed by sequencing both strands of the core enhancer.

Transgenic Mice

The CpG mutant construct 258(CpG1-3)/-2.5lacZ was made by excising the mutagenized core enhancer from pBluescript (KS$^+$) with *Sal*I and *Xba*I and cloning the fragment upstream of the *myoD* promoter in the lacZ construct -2.5lacZ (Goldhamer *et al.*, 1995) utilizing unique *Sal*I and *Spe*I sites. The *myoD* mutant enhancer/promoter lacZ fusion was liberated from vector sequences and fragments purified as previously described (Goldhamer *et al.*, 1992). Transgenic mice were produced by pronuclear injection of B6/D2 F1 hybrid one cell stage embryos by the Cancer Center Transgenic Facility of the University of Pennsylvania Medical School. Three founder mice were identified by slot-blot analysis of tail DNA using a probe specific to lacZ sequences. Stable transgenic lines were produced by mating founder mice to B6/D2 F1 hybrid mice. For embryo analysis, transgenic mice were mated to either B6/D2 F1 hybrid mice or FVB/N mice with similar results. Histochemical staining for β-galactosidase was done as previously described (Goldhamer *et al.*, 1992).

RESULTS

Three regulatory regions that have been identified upstream of the *myoD* gene are depicted in Fig. 1A. We identified a distal enhancer located 20 kb upstream of the human gene that is sufficient for embryonic activation of *myoD* in all muscle forming regions of the embryo (Goldhamer *et al.*, 1992, 1995; Faerman *et al.*, 1995) recapitulating *myoD* embryonic expression as determined by *in situ* hybridization analyses. Two additional regulatory elements have been identified in mouse sequences; the promoter (Zingg *et al.*, 1991) also termed the proximal regulatory region (PRR) (Tapscott *et al.*, 1992) and an enhancer approximately 5 kb upstream named the distal regulatory region (DRR) (Tapscott *et al.*, 1992). These more proximal elements together recapitulate part of the developmental program of *myoD* expression including autoregulation (Tapscott *et al.*, 1992; Zingg *et al.*, 1994), fiber type-specific expression (Hughes *et al.*, 1993), and some aspects of embryonic expression (Asakura *et al.*, 1995). We localized the distal enhancer to a 258-bp core sequence that is highly conserved in mice (Goldhamer *et al.*, 1995) and also is located distally 20 kb upstream of the mouse *myoD* gene (Fig. 1A). There are six CpG sites in the mouse core enhancer (Fig. 1B) that lie in regions of the enhancer where proteins interact as shown both by *in vitro* footprinting assays (Goldhamer *et al.*, 1995) and by gel electromobility shift analysis (unpublished data). Three of these sites (CpG 2, 4, and 5) are conserved in the human enhancer. We used the bisulfite deamination method (Frommer *et al.*, 1992; Clark *et al.*, 1994) to determine the methylation status of each of the conserved sites in DNA isolated from mouse tissue culture cell lines, neonates, and embryos.

The Bisulfite Method Is a Reliable Assay for Site-Specific DNA Methylation

We examined whether the bisulfite deamination method combined with phosphorimager quantitation provides a consistent and accurate measure of the methylation state of specific CpG residues. Incubation of DNA in sodium bisulfite at low pH followed by a high pH incubation results in deamination of cytosine to uracil. Methylated cytosines are protected from this deamination reaction. Thus, following PCR amplification of bisulfite-treated DNA and subsequent cycle sequencing of the product, the intensity of the bands on a sequencing gel in the C lane relative to the T lane quantifies the level of methylation at any C residue subject to methylation. In order to determine by independent means the methylation levels of the C2C12 and NB41A3 DNAs that we used in the control experiment, we performed a genomic Southern analysis using the methylation-sensitive enzyme *Hpa*II and its isoschizomer *Msp*I, which cut at CpG 6 and at a second site outside the core enhancer. *Hpa*II digested the C2C12 DNA to completion and the NB41A3 DNA only slightly (Fig. 2B), demonstrating that these two *Hpa*II sites are unmethylated in C2C12 DNA and greater than 90% methylated in NB41A3 DNA. We then conducted a mixing experiment using C2C12 DNA and NB41A3 DNA. As an internal control for the bisulfite deamination and subsequent sequencing reactions, the DNA was methylated to completion *in vitro* with *Alu*I methylase prior to the bisulfite treatment. Figure 2A shows the raw data from this mixing experiment. In the 0% NB41A3 DNA sample, there are very faint if any signals in the C lane and correspondingly strong signals in the T lane, indicating a very low level of methylation. The exceptions are the control *Alu*I-methylated residues which have a

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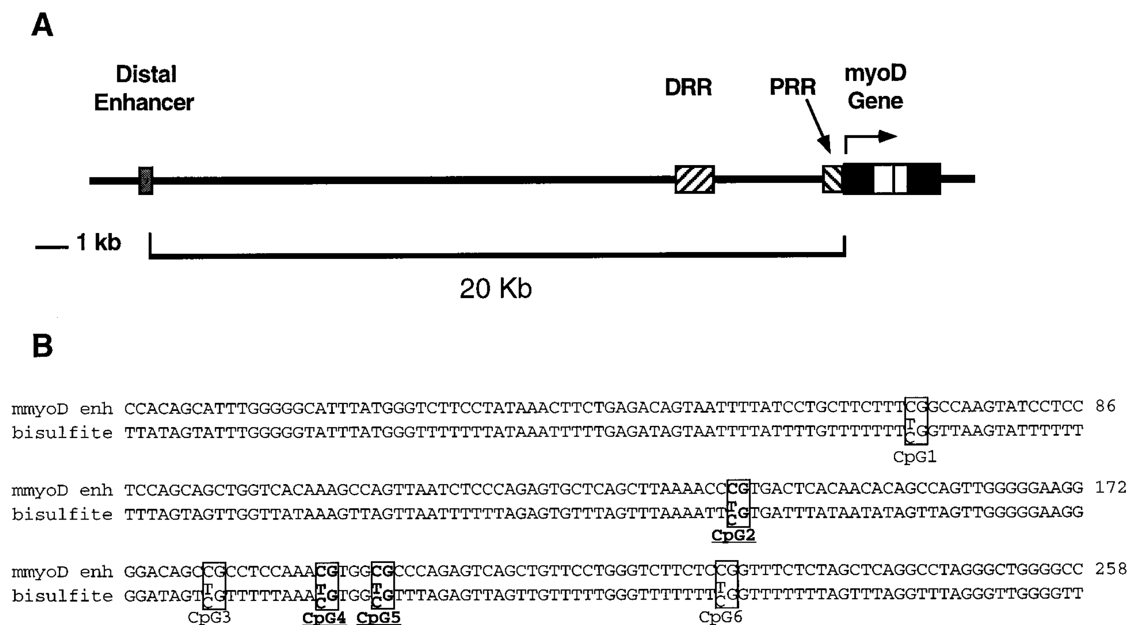

FIG. 1. Structure of the *myoD* gene and sequence of the distal enhancer. (A) The *myoD* gene structure derived from restriction mapping a cosmid clone and published sequence (Zingg *et al.,* 1991). The distal regulatory region (DRR) and proximal regulatory region (PRR) were identified by Tapscott *et al.* (1992) and Zingg *et al.* (1991). (B) The sequence of the mouse distal core enhancer which begins at base 634 relative to the beginning of the 1.6-kb *Rsa*I fragment containing the enhancer. The bisulfite-treated forward strand is shown aligned with the mouse sequence. All CpG dinucleotides are marked with a box and those that are conserved between mice and humans are indicated in bold. The three CpG sites that are the focus of this analysis are indicated by underlining.

strong C signal and a weak T signal. In contrast, in the 100% NB41A3 DNA sample, there are strong signals in the C lane and relatively weak signals in the T lane, indicating extensive methylation of the five CpG sites visible on the gel. CpG 1 migrates far ahead of the other five sites and was not included in our analysis.

Figures 2C and 2D show the corresponding graphs using the phosphorimager quantitated data from the control gel shown in Fig. 2A. In Fig. 2C, the data are graphed to show the relationship between the percent NB41A3 DNA in the sample and the percent C as calculated from the gel. Values for sites 2, 4, and 5 are similar and, more importantly, follow the linear increase of NB41A3 DNA, demonstrating the validity of the bisulfite reaction and phosphorimager quantitation for comparing relative methylation levels between samples for these three sites. In contrast, sites 3 and 6 deviate from this linear relationship and even at 0% methylated DNA, where there are no methylated CpG residues in the enhancer, there is a wide disparity between the percent C of CpG sites 3 and 6 compared to sites 2, 4, and 5. The variation in the phosphorimager data seems to be due to higher general background labeling in the region of the sequencing gels where sites 3 and 6 migrate. This particular gel shows lower background than many. Thus, the values for sites 3 and 6 can not be reliably quantitated using the phosphorimager. We have no evidence that the methylation levels of sites 3 and 6 differ substantially from sites 2, 4, and 5. In this study, therefore, we have limited our analysis to the conserved CpG sites 2, 4, and 5 unless otherwise noted. In Fig. 2D, the data are presented as a bar graph for each sample showing the percent C of CpG sites 2, 4, and 5 and the average of these three sites to provide an overall value reflecting the relative level of *myoD* enhancer methylation.

To verify the accuracy of the phosphorimager quantitation, we sequenced subcloned products of PCR amplifications and found that the percent C was consistent with the percent C as determined by phosphorimager quantitation (Table 1). These data together demonstrate the efficacy of using bisulfite deamination and phosphorimager quantitation to detect differences in the CpG methylation level between DNA samples, providing the means to quantitate the methylation state of the *myoD* distal enhancer not only in cell lines but also in somites of the developing embryo.

The myoD Enhancer Is Unmethylated in Muscle Cells in Culture

We first tested the CpG methylation status of the distal *myoD* enhancer in established cell lines. Myogenic C2C12 cells, which express the *myoD* gene at high levels (Tapscott *et al.,* 1988), show a very low level of enhancer methylation (average for the three CpG sites of 8% C, Fig. 3A). In contrast, the *myoD* enhancer in nonmuscle cell lines, which

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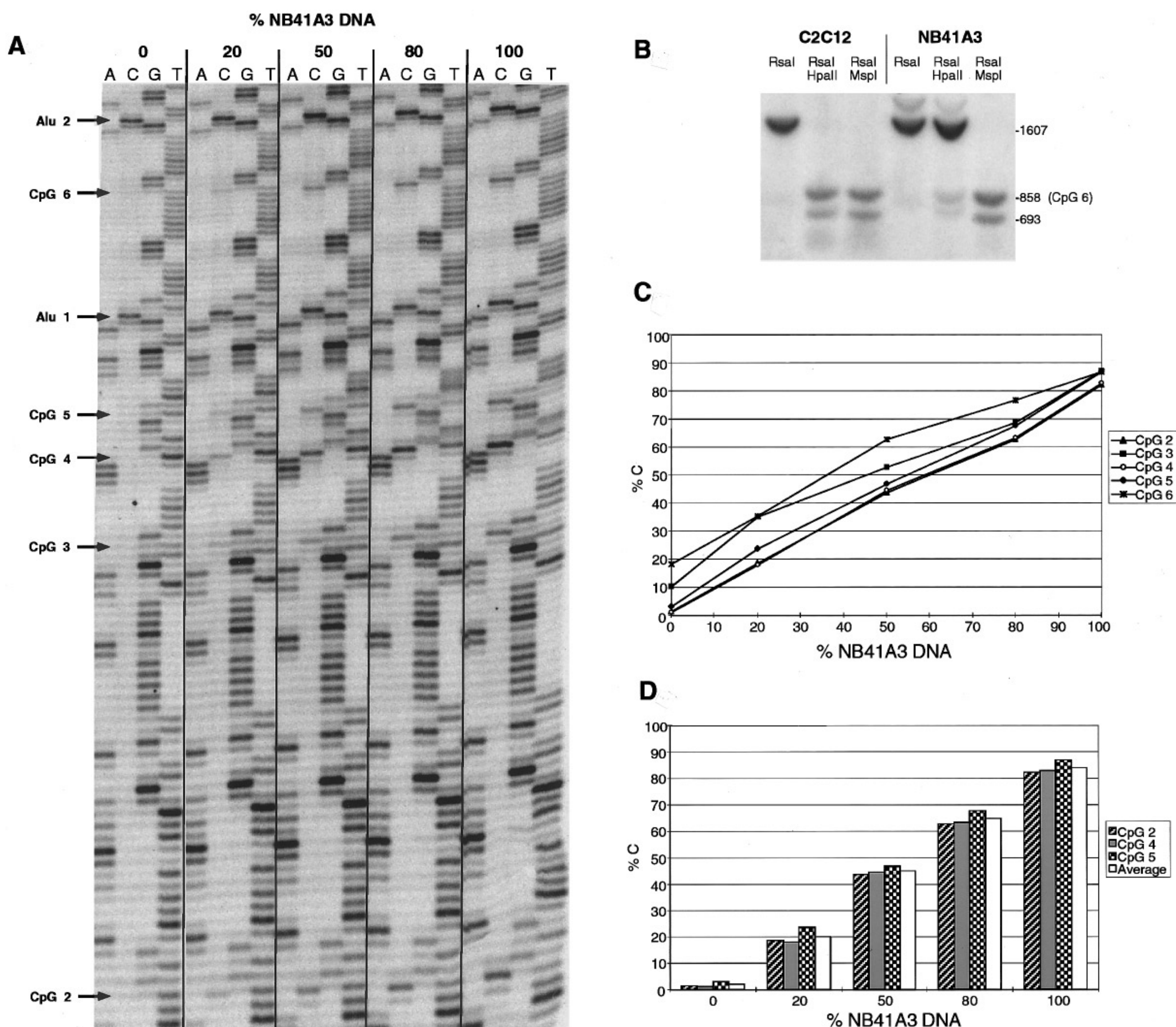

FIG. 2. Control gel establishing the efficacy of phosphorimager quantitation of CpG methylation. (A) An example of a gel from the methylation analysis. This gel is a mixing experiment using unmethylated DNA from C2C12 muscle cells and heavily methylated DNA from NB41A3 neuroblastoma cells. The two *Alu*I sites were used as internal controls for some experiments including the one shown here. (B) An independent verification using standard *Hpa*II and *Msp*I digests showing that C2C12 DNA is unmethylated and NB41A3 DNA is heavily methylated. All samples were digested with *Rsa*I to give a 1.6-kb band. *Hpa*II and *Msp*I cut within this band at base 858, which is CpG site 6, and an additional CpG outside the core enhancer 53 bp closer to the *myoD* gene to yield two bands of 858 and 693 bp. (C and D) The data generated from quantitating the gel in A using a phosphorimager and plotting the values using Microsoft Excel. In C the value for the % C (C signal divided by the C plus T signal) for each CpG 2–6 is plotted versus the percent methylated NB41A3 DNA in the sample. (D) The data as they will be presented in the rest of this paper. The average is the average % C of sites 2, 4, and 5 and thus represents the average methylation of the enhancer for any particular sample.

do not express the *myoD* gene, shows much higher levels of methylation (57% C for BNL liver cells and 82% C for NB41A3 neuroblastoma cells). We subcloned and sequenced five PCR products representing individual chromosomes for BNL cells and six for C2C12 cells and all subclones for C2C12 and none for BNL were completely unmethylated (Table 1). Thus, there is a positive correlation between an unmethylated *myoD* distal enhancer and expression of *myoD* in these cell lines.

We also tested *myoD* enhancer methylation in muscle

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TABLE 1
Average Percentage of Cytosine Residues at CpG Sites 2, 4, and 5 Determined by Subcloning and Sequencing PCR Products Representing Individual Chromosomes Compared to the Data from Phosphorimager Quantitation

Cell line	% C from phosphorimager	% C from subcloning	% Unmethylated chromosomes
10T1/2 fibroblasts	58% ± 4%*	53%	0% (0/5)
23A2 myoblasts	12% ± 6%	7%	80% (4/5)
BNL liver cells	57% ± 5%	60%	0% (0/5)
C2C12 myoblasts	8% ± 4%	0%	100% (6/6)

Note. Column three shows the percentage of subclones that were completely unmethylated.
* Standard deviation is from four experiments.

and fat cell lines derived from 10T1/2 fibroblast cells by treatment with 5-azacytidine (an unmethylatable analog of cytidine that blocks DNA methylation). The enhancer in 10T1/2 cells and IB1 cells (a 5-azacytidine-derived fat cell line) shows a high level of methylation (Fig. 3B). The level of *myoD* enhancer methylation in IB1 cells appears to be slightly reduced relative to 10T1/2 cells, perhaps reflecting a general 5-azacytidine-induced decrease in methyltransferase activity; however, enhancer methylation is dramatically decreased in 23A2 cells, a 5-azacytidine-derived muscle line (Konieczny and Emerson, 1984) that expresses the *myoD* gene at a high level (Konieczny *et al.,* 1989). The average level of *myoD* enhancer methylation in 23A2 muscle cells is 12% C compared to 58% C in the parental 10T1/2 cell line and 48% C in the IB1 fat cell line (Fig. 3B). We subcloned and sequenced five PCR products representing individual chromosomes for 10T1/2 and 23A2 DNA and 80% of 23A2 and 0% of 10T1/2 subclones are entirely unmethylated (Table 1). 10T1/2 cells and their derivatives are hypotetraploid; thus, as many as four copies of the *myoD* gene could be present in 23A2 cells. We expect that the *myoD* gene is only expressed from those chromosomes (likely three of the potential four) that have an unmethylated distal enhancer; however, we cannot test this hypothesis. These data establish a strong correlation between an unmethylated *myoD* enhancer and expression of the *myoD* gene, suggesting that *myoD* enhancer demethylation by 5-azacytidine treatment is pivotal for the determination of 10T1/2 cells to the myogenic lineage.

The myoD Distal Enhancer Is Unmethylated in Muscles of Newborn Mice

The methylation status of the *myoD* distal enhancer in muscle and nonmuscle tissues of newborn mice was examined to establish *in vivo* relevance for the tissue culture data. We found that the level of enhancer methylation is much lower in muscle tissue compared to nonmuscle tissue (Fig. 3C). DNA isolated from forelimb or hindlimb muscle shows a low level of enhancer methylation (17% C) compared to DNA isolated from nonmuscle tissue (liver, 60% C; heart, 48% C; and brain, 52% C). The low level of methylation in the muscle samples is likely due to contamination of these samples with methylated supporting structures such as connective tissue. Thus, it appears that the *myoD* distal enhancer is unmethylated in muscle tissues in newborn pups, while the nonmuscle tissues we tested show a methylation level of 48–60% C, demonstrating a correlation between demethylation of the *myoD* enhancer and myogenesis *in vivo.* Thus, the enhancer is in a demethylated state in muscles both *in vivo* and in myogenic tissue culture cells.

It has been reported that a MLC1-CAT transgene shows a gradient of methylation along the anterior/posterior axis in transgenic mice (Donoghue *et al.,* 1992; Grieshammer *et al.,* 1995), suggesting the existence of a system for methylating regulatory sequences in muscle in a graded fashion. We do not detect differences in the methylation level of the endogenous *myoD* enhancer in hindlimb and forelimb muscle of newborn mice. This suggests that the mechanism regulating endogenous *myoD* enhancer methylation levels must differ from the mechanism regulating differential methylation of the MLC1-CAT transgene in muscles along the A/P axis.

The myoD Distal Enhancer Becomes Demethylated in Somites during Embryonic Development

To determine the CpG methylation level of the enhancer in relation to embryonic activation of the *myoD* gene, we first examined the enhancer methylation status in somites and nonmuscle tissues of 11.5-day embryos when the *myoD* gene is expressed at high levels in subpopulations of somite cells (Sassoon *et al.,* 1989; Goldhamer *et al.,* 1992; Faerman *et al.,* 1995). Somites develop in an anterior to posterior progression with new somites condensing from the paraxial mesoderm every 2–2.5 hr (Rugh, 1968). Thus, the timing of regulatory events during somitogenesis can easily be assessed as the embryo contains somites representing a broad spectrum of developmental ages. Again, we observe a positive correlation between decreased enhancer methylation and myogenesis (Fig. 4A). The highest levels of enhancer methylation are observed in the presomitic mesoderm (65% C) and the neural tube (73% C). In contrast, somites, including the three most newly formed somites, show lower levels of methylation (35–42% C). These data indicate that the enhancer becomes demethylated in 11.5-dpc embryos less than 6 hr following condensation of the presomitic mesoderm to form somites. The forelimb bud, which expresses *myoD* in some cells, and the heart, which does not express *myoD,* show intermediate levels of methylation (52–56% C). These percentages represent the average enhancer methylation for all cells in a particular sample. Data presented below show that the enhancer becomes demethylated in a subpopulation of somite cells

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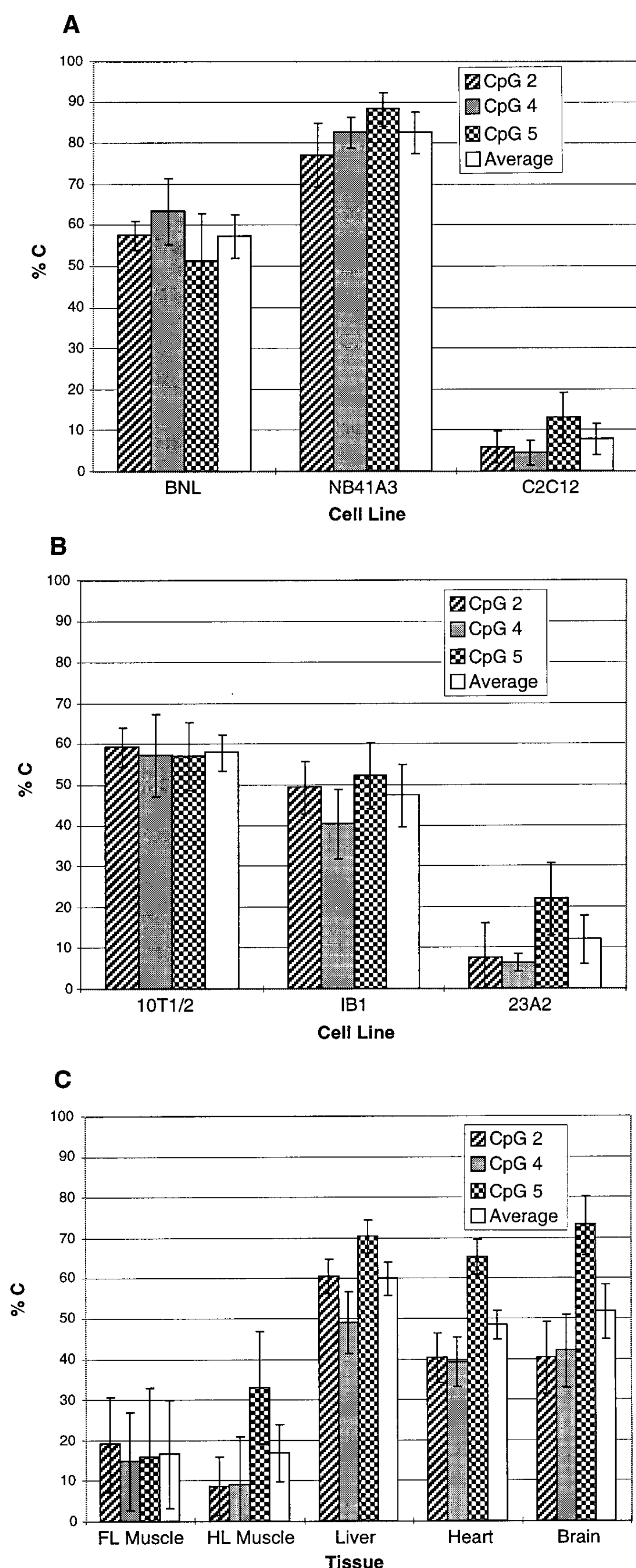

(presumably myogenic), while the majority of cells maintain a high level of enhancer methylation.

In order to determine more precisely the developmental timing of *myoD* enhancer demethylation, we examined the enhancer methylation status in somites at different positions along the anterior-posterior axis of four 9.5-dpc (23–26 somites) embryos (Fig. 4B). The methylation level of the enhancer in the presomitic mesoderm is 74% C and does not decrease significantly in somites 1–2 or somites 5–6. However, in somites 10–11, the enhancer methylation level is decreased to 50% C and remains at that level in more anterior somites. While the standard deviations for some samples from these embryos is high, somites 10–11 and somites located more anterior in each individual embryo show a reproducible decrease in enhancer methylation compared to presomitic mesoderm. As an additional test of the quantitation, we subcloned and sequenced PCR products representing individual chromosomes from a 9.75-dpc embryo (see below) and confirmed that the enhancer becomes demethylated by somite 10. These data indicate that the distal enhancer becomes demethylated during somitic development in 9.5-dpc mouse embryos between somite 6 and somite 10.

In order to distinguish whether a subpopulation of cells becomes demethylated in somites showing decreased enhancer methylation or, alternatively, the methylation level decreases for all cells, we subcloned and sequenced PCR products representing individual chromosomes from a 9.75-day (27-somite) embryo. We were able to analyze five of the CpG sites in the core enhancer in this analysis (sites 2–6) as we simply scored for the presence of a C versus a T without the need for phosphorimager quantitation. The presomitic mesoderm and somites 5–6 are heavily methylated (78–80% C) and none of the 37 sequenced subclones representing individual chromosomes from these two tissues were entirely unmethylated at all five of the sites in the core enhancer (Table 2). As seen using phosphorimager quantitation, somites 9–10 and somites 13–14 show decreased enhancer methyla-

FIG. 3. *myoD* enhancer methylation is absent in myogenic tissue culture cell lines and decreased in muscles from newborn pups. (A) A comparison of the BNL liver cell line, the NB41A3 neuroblastoma cell line, and the C2C12 myoblast cell line. (B) A comparison of three cell lines, two of which were derived from the parental 10T1/2 fibroblast cell line by treatment with 5-azacytidine. 23A2 is a 5-azacytidine-derived muscle cell line (Konieczny and Emerson, 1984) and IB1 is a 5-azacytidine-derived fat cell line. All DNA samples were isolated from proliferating cells; however, we did not detect any differences in methylation patterns when DNA was isolated from confluent, differentiated cells (data not shown). (C) Data from tissues dissected from newborn pups. FL, forelimb muscle; HL, hindlimb muscle. Error bars indicate one standard deviation above and below the mean for at least three and in most cases four experiments.

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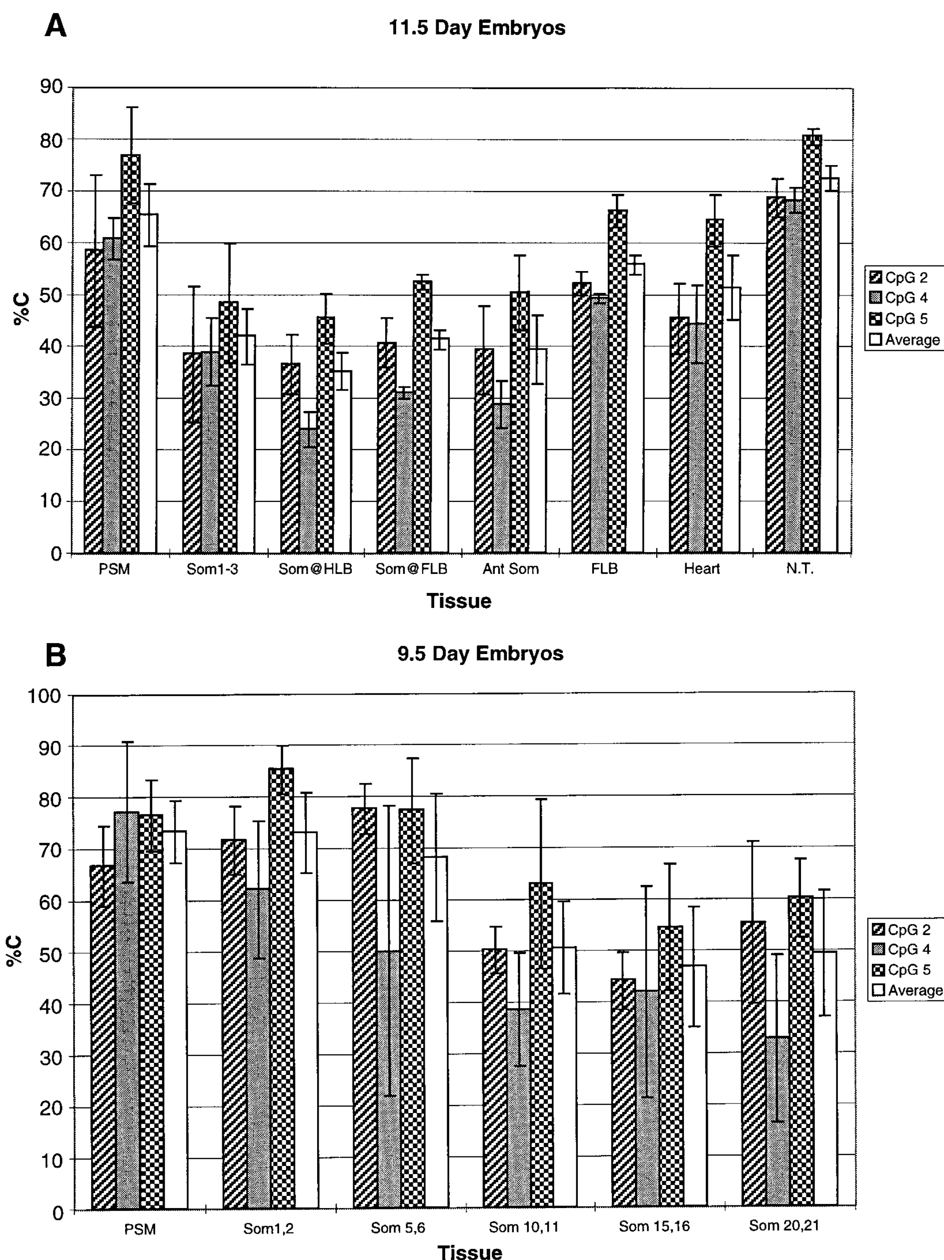

FIG. 4. The *myoD* distal enhancer becomes demethylated in somites of mouse embryos. (A) The data from three 11.5-day embryos. Psm, unsegmented presomitic mesoderm; Som1-3, the newest three somites to segment from the presomitic mesoderm; Som@HLB, three somites from the level of the hindlimb bud; Som@FLB, three somites from the level of the forelimb bud; Ant Som, the three most anterior somites; FLB, forelimb bud; N.T., neural tube from the level of the forelimb bud. (B) Data from four 9.5-day embryos. Somites are numbered from the most newly formed somite, i.e., Som1,2 indicates the two most newly formed somites.

tion levels to about 50% C. Significantly, this decrease in methylation can be accounted for almost entirely by the presence of subclones representing chromosomes that are completely unmethylated at all five of the CpG sites (15% of subclones for somites 9–10 and 37% of subclones for somites 13–14). The methylation level of subclones which contain at least one methylated site (Table 2, column 3) does not decrease significantly during somite development. These data show that the *myoD* distal enhancer is heavily methylated in the presomitic

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TABLE 2

Average %C of CpG Sites 2–6 as Determined by Subcloning and Sequencing Individual PCR Products

Tissue	% C at CpG sites 2–6	% Unmethylated chromosomes	% C of methylated chromosomes
Presomitic mesoderm	80%	0% (0/18)[a]	80%
Somites 5–6	78%	0% (0/19)	78%
Somites 9–10	54%	15% (3/20)	64%
Somites 13–14	45%	37% (7/19)	72%

Note. Tissue was dissected from a 9.75-dpc (27-somite) embryo. Somites are numbered with 1 being the most posterior. The decrease in % C of the enhancer in somites 9–10 and somites 13–14 can be accounted for by those chromosomes that are entirely unmethylated at these five CpG sites as the % C for those chromosomes which retain at least one methylated CpG at these sites remains relatively constant (column 3).

[a] The number of subclones analyzed is indicated in the parentheses.

mesoderm and somites 5–6 of 9.75-dpc mouse embryos and becomes completely demethylated between somite 6 and somite 10 in chromosomes of a subpopulation of somite cells as myogenesis progresses during somite maturation.

Methylation of the Distal Enhancer Is Not Required to Prevent Precocious or Ectopic Embryonic Expression of myoD

We tested whether the timing or skeletal muscle restriction of embryonic *myoD* expression is regulated by CpG methylation of the distal enhancer. The three CpG sites in the 258-bp human enhancer, all of which are conserved with the mouse *myoD* enhancer, were mutated to TpG, ligated to a human *myoD* promoter/lacZ reporter, and injected into mice. Three independent stable transgenic lines were generated and analyzed. There is variability in the level of expression in the three lines, probably due to site of integration effects; however, even the high expressing line does not show premature expression in muscle forming regions of whole mount (Fig. 5) or sectioned (data not shown) embryos. This is evidenced in the 11.5-day embryos in Fig. 5 as the posterior extent of expression in both the ventral and dorsal somite is the same for the wildtype embryo in Fig. 5A and the highly expressing mutant embryo in Fig. 5B. The highly expressing line does show ectopic expression in the neural tube and forebrain and broadened expression in flank somites; however, as the other two lines do not show similar expression patterns, it is likely that the aberrant expression observed in the highly expressing line is caused by site of integration effects. All three lines show specific loss of expression in the anterior muscle masses of the forelimb buds (indicated by the arrows in Fig. 5). This loss of expression is likely due to an alteration in DNA/protein interactions caused by mutation of CpG 2 as a linker–scanner mutation of this region shows a specific limb phenotype simi-

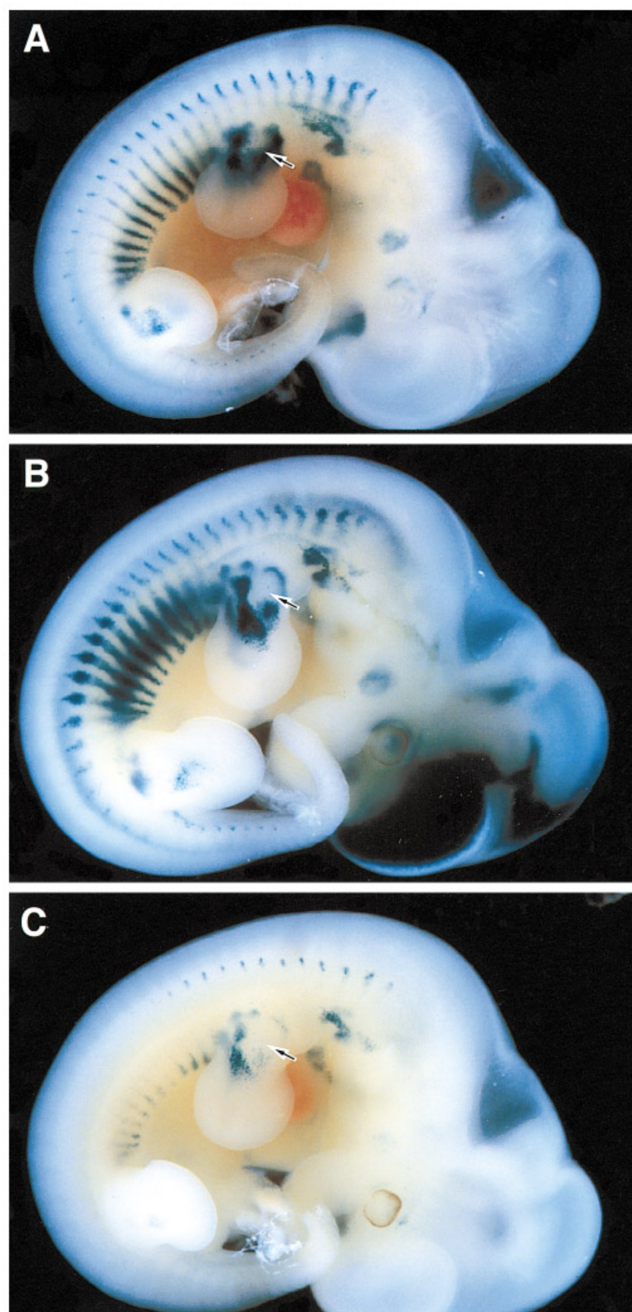

FIG. 5. 11.5-dpc transgenic mice carrying a CpG mutant, human distal enhancer reporter construct do not show precocious or ectopic transgene expression. (A) An embryo carrying the wildtype human 258/-2.5LacZ enhancer construct. (B and C) Two lines carrying a CpG mutant enhancer construct which gives expression patterns similar to the wildtype construct. B is a line that expresses very strongly and C is a line that expresses weakly. The embryo in B shows ectopic staining in the forebrain, neural tube, and broadened expression in flank somites. As the other two lines do not show similar staining (compare with C), there is no consistent misexpression that can be attributed to loss of methylation. The loss of transgene expression in the developing limb is indicated by an arrow.

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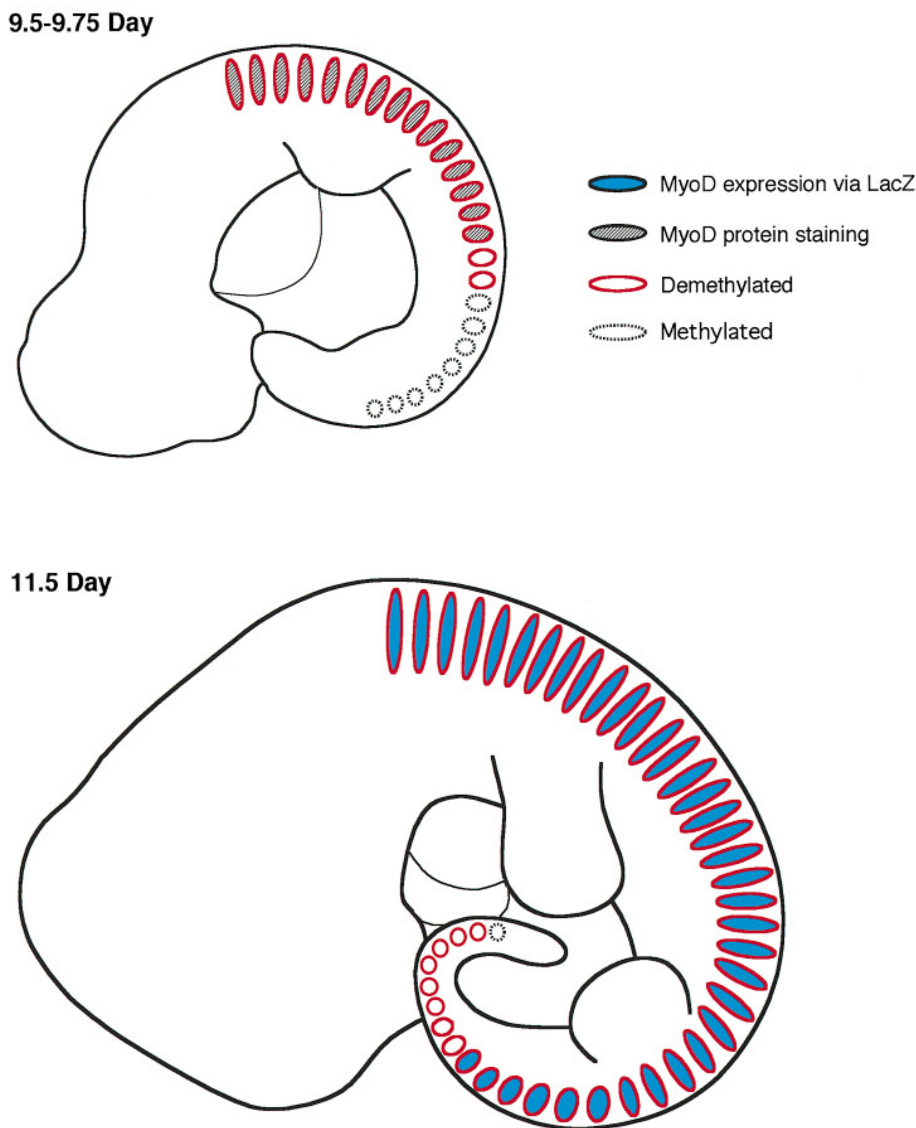

FIG. 6. Diagrammatic representation summarizing *myoD* gene expression/transgene expression, *myoD* protein staining, and somite methylation analysis data. The *myoD* enhancer/lacZ transgene staining patterns correlate well with the *in situ* data (Faerman *et al.,* 1995) (Sassoon *et al.,* 1989), so for the purposes of this figure, we only present the transgene data (Goldhamer *et al.,* 1992, 1995; Faerman *et al.,* 1995), indicated in blue. The protein staining pattern in the 9.5-dpc embryo (Smith *et al.,* 1994) is striped and the somites in which we detect demethylation relative to the presomitic mesoderm are shown outlined in red. MyoD protein staining data were not presented for 11.5-dpc embryos although 10.5-dpc embryos show staining in the fourth somite from the presomitic mesoderm (Smith *et al.,* 1994). The resolution of this representation is at the level of the entire somite. We are not attempting to distinguish subsomitic localization of expression. A somite is marked as positive if it contains a positive signal anywhere within it. Thus, at a cellular level, the differences in enhancer demethylation and MyoD protein staining are much more striking, as we see 15% of enhancer subclones representing individual chromosomes from somites 9–10 are unmethylated and Smith *et al.* (1994) report only one or two MyoD-positive cells in somite 11.

lar to the CpG mutant mice (unpublished observations). Additionally, CpG 2 is in a region of the enhancer that contains a predicted AP-1 site and is protected from DNAseI digestion in *in vitro* footprinting assays (Goldhamer *et al.,* 1995). These data suggest that methylation of the enhancer is not required to restrict the domain of expression or timing of activation of the human *myoD* enhancer transgene during early embryogenesis.

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DISCUSSION

Demethylation of the Distal Enhancer Correlates with myoD Gene Expression

We previously identified a distal enhancer 20 kb upstream of the *myoD* gene that is sufficient to recapitulate all aspects of embryonic *myoD* activation. This enhancer has properties suggesting that it is regulated in part by epigenetic means (Goldhamer *et al.,* 1992). In this paper, we show that this distal *myoD* enhancer is methylated in nonmuscle embryonic tissues and cells in culture. However, in muscle cells in culture and in subpopulations of somite cells, the core enhancer becomes completely demethylated at all five of the CpG sites we tested, providing evidence that demethylation of the distal enhancer is an important component of *myoD* gene regulation during myogenesis. We propose that complete demethylation of the *myoD* enhancer is required for *myoD* expression, both in cell culture and during embryogenesis in the mouse.

Unlike the distal enhancer, the methylation state of other regulatory regions of the *myoD* gene does not correlate with *myoD* expression. The *myoD* promoter is unmethylated in both muscle and nonmuscle tissues in 11.5-dpc embryos (data not shown), in adult mice (Jones *et al.,* 1990b), and in tissue culture cell lines (data not shown; Rideout *et al.,* 1994). The lack of methylation of the *myoD* promoter in nonmuscle cells is likely because the promoter borders a CpG island. CpG islands have been shown to be protected from methylation during normal development unless they are on the inactive X chromosome (reviewed by Bird, 1986). We have also examined the CpG methylation level of the distal regulatory region (DRR) identified by Tapscott *et al.* (1992) in cell lines and find a variable but low amount (<20% C) of methylation of this regulatory sequence; however, the level of methylation does not correlate with expression of *myoD* as parental 10T1/2 fibroblast cells show a lower level of DRR methylation than do 5-azacytidine-induced 23A2 myogenic cells (data not shown). Thus, of the identified *myoD* regulatory regions, the methylation state of the distal enhancer alone correlates with *myoD* gene expression and myogenesis.

Demethylation of the myoD Distal Enhancer Precedes Detectable Expression of myoD in Embryos

Comparison of the methylation data with published work reporting *myoD* expression patterns suggests that the enhancer becomes demethylated prior to *myoD* transcription. Figure 6 shows the methylation data compared with the published gene expression data as determined by *in situ* hybridization studies (Faerman *et al.,* 1995; Sassoon *et al.,* 1989), by lacZ staining of *myoD* enhancer/reporter transgenic mice (Goldhamer *et al.,* 1992, 1995; Faerman *et al.,* 1995), and by antibody staining against the MyoD protein (Smith *et al.,* 1994). In 9.5-dpc embryos, we reproducibly detect enhancer demethylation in somites 9–10 numbered from the presomitic mesoderm. MyoD protein is first detected in some 9.5-day embryos in somites 11–12 in one or a few cells per somite (depicted in Fig. 6) (Smith *et al.,* 1994). In the lacZ staining and *in situ* hybridization analyses, *myoD* expression is first detected later in 10.0- to 10.5-dpc, 30+-somite embryos. RT–PCR experiments on 9.5-dpc whole embryos detect very small amounts (<1% maximal expression) of *myoD* message (Hannon *et al.,* 1992), indicating that the *myoD* gene is transcribed at a very low level in 9.5-dpc embryos but providing no information as to the localization of expression. To detect a change in enhancer methylation level in the total somite requires an abundance of cells that have demethylated the enhancer as the majority of cells retain a high level of enhancer methylation. We find that 15% of enhancer subclones from somites 9–10 and 37% of subclones from somites 13–14 are completely unmethylated in a 9.75-dpc embryo (Table 2). This represents a much greater number of cells than are initially *myoD* positive as detected by antibody staining or later by lacZ staining of transgenic mice (see for example Fig. 5A). Thus, it is likely that the population of cells that are demethylated in these somites is much larger than the population of cells that initially activate *myoD,* providing additional evidence that enhancer demethylation precedes *myoD* expression at the cellular level during somite development in 9.5-dpc embryos and suggesting that transcriptional activation does not immediately follow enhancer demethylation. While we cannot determine from our analyses which cells in the somite undergo demethylation of the enhancer, we hypothesize, based upon the timing and pattern of activation of *myoD* expression, that demethylation is occurring in cells of the dermamyotome that are migrating into the myotome and cells of the myotome itself. We estimate that the percentage of somite cells in these populations is similar to the percent of unmethylated chromosomes that we find in somite samples.

Grieshammer *et al.* (1995) have shown that demethylation of the MLC1 enhancer sequences in a MLC1-CAT transgene is detectable at approximately somite 20 from the presomitic mesoderm in 11.5-dpc mice. They detect expression of the reporter construct in more posterior somites that have not yet demethylated the enhancer, leading to their conclusion that demethylation of these sequences is a consequence resulting from transcription of the CAT reporter construct. In contrast, we observe demethylation of the *myoD* distal enhancer in the most posterior three somites of 11.5-day embryos (depicted in Fig. 6), well before expression of the *myoD* gene is detected by lacZ staining of transgenic embryos (Goldhamer *et al.,* 1992, 1995; Faerman *et al.,* 1995). Thus, distal enhancer demethylation precedes *myoD* expression at both embryonic stages that we have analyzed. This is consistent with our finding that a mutagenized reporter construct with no CpG sites in the distal enhancer is expressed in transgenic mice with a wild-type pattern of activation in somites during embryogenesis. These data together with the finding that all cells that ex-

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press *myoD* have an unmethylated enhancer suggest that demethylation of the enhancer is necessary but not sufficient for *myoD* expression.

The Role of Distal Enhancer Methylation in the Developmental Regulation of the myoD Gene

Our findings establish that the *myoD* distal enhancer is a target of methylation regulation based on our developmental data showing demethylation of the enhancer precedes *myoD* expression and transgenic data showing the distal enhancer is sufficient for correct embryonic activation in transgenic mice (Goldhamer *et al.,* 1992, 1995; Faerman *et al.,* 1995). The CpG mutant transgene also is activated correctly, showing that methylation of the three CpG sites in the human enhancer is not essential for myoD repression or activation during early embryogenesis; however, it is possible that distal *myoD* enhancer sequences can direct the appropriate methylation of other *myoD* gene sequences that are critical for *myoD* activation or repression in nonmyogenic cells. For instance, the distal enhancer could also direct demethylation of CpG sites flanking the enhancer to open chromatin making regulatory sequences in the enhancer or promoter accessible to transcription factors. In this case, we would expect to identify additional sequences that undergo demethylation dependent on the presence of the enhancer.

Alternatively, enhancer methylation may play a repressive regulatory role later in development to ensure that the *myoD* gene remains silent in differentiated nonmuscle tissues. Such repression would be critical for a gene such as *myoD* that has dominant regulatory activity to convert cells to the muscle phenotype (Davis *et al.,* 1987; Weintraub *et al.,* 1989). Evidence for this hypothesis comes from the observation that reporter constructs containing the distal human *myoD* enhancer are expressed promiscuously in both transient and stable transfections in many tissue culture cell lines in which the chromosomal *myoD* gene is silent (Goldhamer *et al.,* 1992) and its distal enhancer methylated (this paper). These same constructs are regulated appropriately in transgenic mice (Goldhamer *et al.,* 1992, 1995; Faerman *et al.,* 1995). The simplest hypothesis to explain this dichotomy is that the distal *myoD* enhancer is regulated in part by DNA methylation that is established during embryogenesis and is required to repress the *myoD* gene in differentiated, nonmuscle cell types. While epigenetic repression is maintained in tissue culture cells at the endogenous *myoD* enhancer via maintenance methylation, repression is not set up *de novo* in these cells, allowing expression of transfected enhancer constructs that are introduced into the cells in an unmethylated state. Consistent with this interpretation, treatment of 10T1/2 cells with the demethylating agent 5-azacytidine results in demethylation of the distal *myoD* enhancer (this study) and expression of the *myoD* gene (Konieczny *et al.,* 1989). Grieshammer *et al.* (1995) have made similar observations using cell lines isolated from MLC1-CAT transgenic mice. These lines show modulated CAT activity that correlates with the methylation level of the MLC1 enhancer and must be the result of maintenance methylation because methylation cannot be extablished de novo on these same MLC1-CAT constructs directly transfected into these lines. The data presented in this paper showing that demethylation precedes myoD expression and all *myoD* expressing cells have a demethylated enhancer demonstrate the importance of enhancer demethylation for expression and are consistent with a repressive regulatory role for enhancer methylation. We do not see aberrant expression of the mutated CpG enhancer transgene in older embryos (data not shown); however, both wildtype and mutant enhancer reporters show diminished expression later in development and undetectable transgene expression in neonates and, as discussed previously, methylation of other regions of the construct could be sufficient to repress transcription in nonmuscle tissues. We can address the repressive role of enhancer methylation directly by methylating the enhancer *in vitro* and testing the effects of enhancer methylation on activity using tissue culture transfection assays.

Developmental Control of myoD Distal Enhancer Demethylation

Demethylation of the distal enhancer prior to expression of the gene suggests that *myoD* enhancer demethylation is a discreet, regulated event that is separable from *myoD* transcription. Furthermore, *myoD* expression does not immediately follow demethylation, suggesting that, while demethylation of the enhancer is necessary, it is not sufficient and additional signals/factors are required for *myoD* activation. Consistent with this interpretation, transgenic mice that carry a mutated human distal enhancer that does not contain any CpG sites and thus can not be methylated do not show precocious or ectopic embryonic expression of the transgene. These data suggest a model that is consistent with recent findings showing that multiple signals from the notochord, neural tube, and dorsal ectoderm are required for *myoD* expression (Munsterberg *et al.,* 1995; Munsterberg and Lassar, 1995; Pownall *et al.,* 1996). We propose that demethylation of the enhancer is regulated by one of these signals resulting in an open chromatin conformation that would allow the enhancer to be receptive to other signals/factors which then direct expression of the *myoD* gene. Consistent with this hypothesis, we have previously shown that the distal enhancer is DNaseI hypersensitive in myoblasts (Goldhamer *et al.,* 1995), suggesting that the chromatin at the enhancer is more open in myoblasts than in nonmuscle cell types. We can test this model by doing somite coculture experiments using inducing tissues such as notochord, neural tube, or dorsal ectoderm singly and in combinations and assaying the methylation status of the enhancer and transcriptional state of the *myoD* gene. A prediction of this model is that demethylation of the enhancer is an active process with site, or at least region, specificity. Demethylation events have been identified that do not require DNA replication, raising the possibility of site specific de-

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methylases (reviewed by Razin and Kafri, 1994). Jost and co-workers (1993, 1995) have identified and partially purified an enzyme that actively demethylates DNA which, while not sequence specific, demonstrates the possible existence of enzymes such as would be required to demethylate the *myoD* core enhancer prior to *myoD* transcription.

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