The Dynamics of Myogenin Site-specific Demethylation Is Strongly Correlated with its Expression and with Muscle Differentiation*

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SUMMARY

The molecular mechanisms underlying the activation of tissue-specific genes have not yet been fully clarified. We analyzed the methylation status of specific CCGG sites in the 5'-flanking region and exon 1 of *myogenin*, a very important myogenic differentiation factor. We demonstrated a loss of methylation, at the onset of C2C12 muscle cell line differentiation, limited to the CCGG site of *myogenin* 5'-flanking region, which was strongly correlated with the transcriptional activation of this gene and with myogenic differentiation. The same CCGG site was also found to be hypomethylated, *in vivo*, in embryonic mouse muscle (a *myogenin*-expressing tissue), as opposed to non-muscle (non-expressing) tissues which had a fully methylated site. In a C2C12 derived clone with enhanced myogenic ability, demethylation occurred within 2 hours of induction of differentiation, suggesting the involvement of some active demethylation mechanism(s) which occur in the absence of DNA replication. Exposure to drugs which inhibit DNA methylation by acting on the S-adenosylmethionine metabolism produced a further reduction, to a few minutes, in the duration of the demethylation dynamics. These effects suggest that the final site-specific DNA methylation pattern of tissue-specific genes is defined through a continuous, relatively fast interplay between active DNA demethylation and re-methylation mechanisms.
INTRODUCTION

Cytosine methylation is, in eukaryotic nuclear DNA, a well-established epigenetic mechanism which controls the expression of housekeeping, and possibly also tissue-specific genes (1-3), as well as several important cellular functions, such as X chromosome inactivation and genomic imprinting (4-6), mutagenesis and tumorigenesis (7-9), senescence and virus latency (10-12). Developmental changes in the methylation pattern are particularly evident (13-16). In fact, during early embryogenesis, the original gamete methylation pattern is erased and most of the DNA in the blastocyst becomes demethylated. After implantation, a de novo methylation activity produces, in the gastrula, a methylation pattern characteristic of the adult animal. During the subsequent development, tissue-specific genes undergo specific demethylation events required for their transcriptional activation, according to the general paradigm of an inverse correlation between DNA methylation and gene expression. Knock-out experiments have highlighted the lethality of even modest abnormal methylation patterns in the embryo (17). There are, in addition, several lines of evidence indicating that endogenous genes can be activated by demethylating agents and that exogenous methylated genes are not expressed when transfected into cells, but that their expression is re-activated by demethylating agents (18-21).

Despite many years of intense studies on DNA methylation, neither the mechanism that regulates this process nor its exact functional role in the activation of genes have been fully clarified. Two steps need to be considered: 1) the creation of a methylation pattern in the DNA control region of a gene and 2) the effect of this methylation pattern on the activity of that gene. As regards the latter step, new light on the role of DNA methylation in gene transcription has been shed by the three-way connection between DNA methylation, chromatin structure and gene activity (22), resulting from the methyl-CpG binding proteins and a repression multiprotein complex that includes histone deacetylases HDAC1 and HDAC2 (23-27): a pivotal role of methyl-cytosine as primary modification in establishing and maintaining several genes in an inactive state has thus been demonstrated. The former step is somewhat more complex. Two main mechanisms have been proposed for the creation and maintenance of methylation patterns. There is, on the one hand, a methylating activity based on DNA methyltransferase enzymes which can methylate the cytosine either in a process of reproduction of previously established methylation patterns (maintenance methylation) or in a process of creation of new methylation patterns (de novo methylation) (28-30). Some distinction between several DNA methyltransferases specific for either maintenance or de novo methylation has emerged (31, 32). In all these methylation reactions, S-adenosylmethionine (AdoMet) has a pivotal role as a methyl-donor (33). Hypomethylation can thus arise from the absence of methylation activity after DNA replication (passive demethylation). On the other hand, some experimental results point, as an alternative pathway, to some active shaping of methylation patterns, and in particular to active mechanisms of removal of methyl moiety (active demethylation) that do not require DNA replication. So far three
kinds of such mechanisms have been proposed: 1) a dinucleotide-exchange reaction (34); 2) a glycosylase-based mechanism that removes the 5-methyl-cytosine moiety, the overall demethylation process then being completed through the involvement of mismatch repair enzymes (35); 3) the direct removal of the methyl group from 5-methyl-cytosine residues in DNA, by a reaction catalyzed by a real DNA demethylase (36), which unlike the other mechanisms, would be reversible (37).

Despite this variety of demethylating pathways, a number of aspects are still somewhat obscure. It is not in fact clear whether these mechanisms, taken together or individually, can mediate not only a stable repression of genes which have to be silenced permanently, but also a transient repression of other genes which need to be switched on or off in response to variations in physiological conditions (e.g. to developmental and/or environmental stimuli) and to be expressed only in specific tissue(s) at the right time. In approximately 98% of a mammalian genome, CpG dinucleotides are less frequent than would be expected and their cytosine moiety is almost constantly methylated. The remaining 2% of the genome, however, contains the so-called CpG islands (2, 38), where these dinucleotides are densely clustered, more frequent than expected and unmethylated. It is not yet clear whether the presence or absence of a CpG island in the control region of genes may influence any of the previously described mechanisms. Approximately one half of mammalian genes, comprising the totality of housekeeping genes and a minority of tissue-specific genes, are reported to have a CpG island at their 5' end; the majority of tissue-specific genes, however, are not associated with an island (39, 40). The housekeeping genes associated with unmethylated islands are transcriptionally active, while in most cell types the tissue-specific genes associated with unmethylated islands are, as a rule, transcriptionally inactive. These results raise questions as to the mechanism activating the transcription of genes in a tissue-specific manner and to how CpG sites are methylated to affect stable and/or transient repression. The relative contribution to the final methylation pattern of each of the aforementioned mechanisms, and their possible interconnection, which lead to the final expression pattern, has yet to be addressed. A crucial point is whether these mechanisms provide the high level of plasticity required for the regulation of the expression of tissue-specific genes.

Of the several tissue-specific transcription factors that have been identified in mammalian cell types, the muscle regulatory factors (MRFs), which belong to the basic helix-loop-helix family (41-43) and are involved in the commitment to myogenic fate and in muscle terminal differentiation, are unique in terms of their ability to control a very complex array of tissue-specific genes. The high level of structural and functional characterization of these genes and their highly integrated auto- and cross-activating network define the muscle as one of the most useful cellular systems for the clarification of mechanisms of tissue-specific gene regulation during differentiation. Of particular interest, from this point of view, are those genes which, at the onset of cell differentiation, show a definite on/off switch closely connected with strict transcriptional control: a typical example is *myogenin* (44, 45), which plays a central role in myogenesis. Several studies, besides dealing with the methylation patterns of
single muscle genes (46-50), have also demonstrated a general role of hypomethylation in the induction of muscle differentiation (51-54).

The aim of this work was to study, during muscle differentiation, the structural and temporal variation of the methylation pattern of *myogenin*. We used, for this purpose, the Hpa II / PCR technique (55, 56), optimized as a multiplex assay, to study the methylation status of a single CpG site of *myogenin* 5'-flanking region and of the three CpG sites of exon 1, and relate our findings to the transcriptional activation of this gene. We demonstrated a strong correlation between the temporal dynamics of demethylation of the sole CpG site of the 5'-flanking region and *myogenin* expression. *In vivo*, the *myogenin* 5'-flanking region was found to be fully methylated in non-muscle tissues, partially methylated in adult muscle (where no *myogenin* expression was detected), and demethylated in fetal muscle where, by contrast, *myogenin* was expressed at high levels. The fact that the dynamics of hypomethylation induction *in vitro* did not last nearly as long as the cell cycle supports the idea of active demethylation mechanisms. However, the presence of 3-deaza-adenosine, an inhibitor of biological methylation reactions based on the AdoMet metabolism (33, 57), together with homocysteine to enhance its inhibitory effect, further shortened the duration of the dynamics of demethylation, which suggests that re-methylation mechanisms are also involved. These findings point, as far as *myogenin* is concerned, to: 1) an interplay between active demethylation and re-methylation mechanisms in the definition of the final methylation pattern of the gene and 2) a physiological role, in the definition of the transcriptional status of this gene, of the demethylation dynamics of the CpG site present in its 5'-flanking region.
EXPERIMENTAL PROCEDURES

Chemicals – Restriction enzymes were purchased from New England Biolabs, Inc. (Mississauga, Ontario, Canada) and from Boehringer Mannheim GmbH (Mannheim, Germany). The Megaprime DNA labeling system was obtained from Amersham International (Little Chalfont, Buckinghamshire, United Kingdom). Radiochemicals were purchased from DuPont-NEN (Bad Homburg, Germany). Centri-Sep columns were from Princeton Separations (Adelphia, NJ, USA). DH5α bacteria were from Life Technologies, Inc. (Gaithersburg, MD). The CK-NAC kit was from Abbott Laboratories (Irving, TX). Oligo-d(T)16 were from Boehringer Mannheim GmbH (Mannheim, Germany). M-MuLV (Moloney murine leukemia virus) reverse transcriptase (cloned), Super Taq (Thermus thermophilus DNA polymerase, licensed Hoffmann-La Roche), human placental ribonuclease inhibitor, buffer for reverse transcription, buffer for PCR and ΦX/HaeIII marker were obtained from HT Biotechnology Ltd (Cambridge, UK). Oligonucleotides used as primers were synthesized by M-Medical GENENCO (Firenze, Italy). 3-deaza-adenosine was a kind gift from Bioresearch Co. (Milan, Italy). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Cell Cultures – The experiments were performed on the C2C12 mouse muscle cell line (58) and on the clone C2T18, the latter being selected, as previously described (59), on account of its enhanced differentiative ability (see results section). Cells were cultured in F14 medium supplemented with 50 µg/ml neomycin and 10% fetal calf serum, which favors cell growth, with only a limited amount of differentiation (growth medium, GM), or with 1% fetal calf serum (differentiation medium, DM), which induces differentiation with the appearance of myotubes and of creatine kinase (CK) activity after a limited number of cell divisions (59). In the differentiation experiments, flasks and multiwells were coated with 0.2% gelatin. 24 hrs after plating, the cells were either shifted to DM, or re-fed with GM according to the experimental design; the time at which this medium shift occurred was indicated as day 0. The demethylating drugs 3-deaza-adenosine 3 µM and homocysteine 50 µM (jointly indicated as DH) were added to DM according to the experimental design. Cultures were re-fed every second day with the appropriate medium (with or without drugs).

Differentiation Assay – Cells to be used for the enzymatic test, either in GM or DM, were rinsed twice with phosphate buffered saline and frozen at -80°C. After thawing, cells were scraped into 1 ml of 50 mM Tris/HCl (pH 7.2) and 1 mM dithiothreitol, sonified for 15s in ice and centrifuged. Supernatant was used for CK (EC 2.7.3.2) and total protein content assay (60). The results, expressed as mU CK/µg protein + SE, are the average of at least three experiments.

Tissue Biopsies – Spleen and brain tissues were isolated from adult mice; thigh skeletal muscle (quadriceps femoris) tissue was isolated from adult mice and from 17-day-old mouse embryos.

RNA Isolation and Expression Studies – Total RNA extraction was performed by the acidified phenol procedure (61 adapted in 62).
The cDNA of rat myogenin, excised from the plasmid BSM13 MGN#11 (45), was used as a probe for in vitro expression studies by Northern blotting. Blots were normalized with an 18S ribosomal DNA mouse probe cloned in the pBR-322 plasmid (63). Probes were labeled by random priming (64, 65) using the Megaprime DNA labeling system with [\(\alpha\)-\(32\)P]dATP (3000 Ci/mmol; specific activity >1.9 x 10^9 dpm/\(\mu\)g). Radioactive probes were purified by Centri-Sep columns.

Agarose electrophoresis of total RNA, vacuum transfer on Hybond N membrane (Amersham) and crosslinking were performed according to standard procedures (62). Each membrane was prehybridized in a hybridization oven at 42°C, by shaking for 2 hrs and by rolling for an additional 2 hrs, in 10 ml of 50% Quick Hyb (Stratagene), 50% formamide and 100 \(\mu\)g/ml salmon sperm DNA (Sigma). The radioactive probe was added to the prehybridization solution and the membranes incubated for 18-20 hrs at 42°C in the same oven. Washes were performed with 50 ml of the following buffers: 2x SSC (20x: 175.3 g/l NaCl, 88.2 g/l NaCitrate, pH 7.0), 0.1 % SDS at room temperature; 2x SSC, 0.1 % SDS at 50°C twice; 0.2x SSC, 0.1 % SDS at 55°C twice. Autoradiographs were quantified using a computerized densitometer (BioImage, Genomic Solutions Inc., Ann Arbor, Michigan, USA).

The expression studies in biopsies in vivo were performed by RT-PCR. For this purpose, reverse transcription was performed on 1 \(\mu\)g of total RNA by using, in each 20 \(\mu\)l sample, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl_2 and 300 \(\mu\)M each of dGTP, dATP, dCTP and dTTP, 20 units of human placental ribonuclease inhibitor, 50 pmol of oligo-d(T)_{16} and 50 units of M-MuLV reverse transcriptase at 42°C for 1 hr, followed by heat inactivation at 94°C for 5 min.

In the subsequent amplification reactions, 2 \(\mu\)l of each sample were mixed in a final volume of 50 \(\mu\)l that contained 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl_2, 0.1% Triton X-100, 0.01% gelatin and 175 \(\mu\)M each of dGTP, dATP, dCTP and dTTP, with 1 unit of Super Taq DNA polymerase and 20 pmol of each specific primer. For mouse myogenin (GenBank, accession number M95800) and for mouse \(\gamma\)-actin (GenBank, accession number L21996) genes, we performed, using a Perkin Elmer Cetus Thermal Cycler model 480, respectively 30 and 25 cycles of 1 min at 94°C, 1 min at 62°C, 1.5 min at 72°C, followed by 7 min at 72°C. For the amplification of myogenin cDNA, we used MyoP3 (exon 1, 5'-TTTCTGTCCACTCTCAGGGCTTCG-3', nt 1686 - 1709) as forward primer and MyoM2 (exon 3, 5'-AAAGTCAGCTAAATCCCTCGC-3', nt 3507 - 3531) as backward primer, obtaining an amplified fragment of 808 bp. For the amplification of \(\gamma\)-actin cDNA, the forward primer used was MMGACTP3 (exon 5, 5'-ACCCAGGCATTGCGACAGGATGC-3', nt 2753 - 2776) and the backward primer was MMGACTM2 (exon 6, 5'-CCATCTAGAAGCATTTCGCTGGACG-3', nt 3046 - 3071); an amplified fragment of 216 bp was obtained. The amplified fragments were analyzed by standard electrophoretic procedures and ethidium bromide staining. The specificity of amplified products was assessed by restriction analysis and/or
Myogenin demethylation sequencing; γ-actin was used as an internal standard to ensure that the quantity of samples was always the same.

DNA Isolation and Methylation Studies by Multiplex Hpa II / PCR – Genomic DNA was extracted using a standard phenol/chloroform method followed by ethanol precipitation (62).

Genomic DNA was treated separately with the following restriction endonucleases: i) EcoR I, which has no recognition sites within the amplified fragments of myogenin gene; ii) Hpa II, which has a recognition site within the 5'-flanking region and three recognition sites within exon 1 (Fig. 1), and is methylation sensitive (i.e. it fails to cut if the CCGG recognition sequence is methylated at any C). Exon 3, which possesses no Hpa II or EcoR I recognition sites (Fig. 1), was used as an internal standard. In each case, 1.5 µg of genomic DNA were digested at 37°C, first with 5 units of enzyme overnight, then with 3 more units for an additional 6 hrs, in a total volume of 40 µl of the buffer provided by the manufacturer. The following primers were used for the amplification of the mouse myogenin gene: for the 5'-flanking region, the forward primer MyoP1 5'-TGGAGTGCTCTGAGTGGTAGTGG-3' (nt 1022 - 1046) and the backward primer MyoM8 5'-ACCCAGAGATAATATAGCCAACGC-3' (nt 1496 - 1520); for exon 1, the forward primer MyoP3 (described above) and the backward primer MyoM7 5'-CGGCTCAATGTACTGGATGCCG-3' (nt 1990 - 2011); for exon 3, the forward primer MyoP10 5'-CTCAGTGGACAGCATCAG-3' (nt 3266 - 3286) and the backward primer MyoM2 (described above). These pairs of primers were expected to produce, from uncut DNA, the following fragments: 499 bp for the 5'-flanking region, 326 bp for exon 1, and 266 bp for exon 3. PCR was performed, with 20 pmol of each primer, on 50 ng of DNA, in a total volume of 50 µl containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.01% (w/v) gelatin and 200 µM dNTPs with 1 unit of SuperTaq. After an initial 3 min denaturation at 94 °C, 30 cycles (1 min at 94°C, 1 min at 62°C, 4.5 min at 72°C) and a final extension of 7 min at 72°C were performed using a Perkin Elmer Cetus Thermal Cycler model 480. Aliquots of the PCR products (15 µl) were examined by electrophoresis in 1.5% agarose gel. Each gel was scanned by a CCD camera and acquired on the BioImage computerized densitometer. The specificity of the fragments was assessed by restriction analysis and/or sequencing.
**RESULTS**

*Differentiation* – Table I shows the results of differentiation (measured as CK activity) of C2C12 and C2T18 cells. The C2C12 cells showed, after 96 hrs in GM, a low level of CK activity, which, 96 hrs after the shift to DM, underwent a seven-fold increase. The C2T18 clone was characterized by higher CK activity in GM and a further increase after the shift to DM, thereby showing greater myogenic potentialities than C2C12. The presence of the drugs increased the degree of differentiation of both C2C12 and C2T18, the increase being greater in C2T18.

*Myogenin expression in in vitro cell cultures* – In GM, C2C12 and C2T18 showed, at early times (48 h), a very low level of *myogenin* expression, which increased at later times (96 h) (Fig. 2A and B, lanes 1 and 2). After the shift to DM, *myogenin* expression was enhanced both in C2C12 and C2T18 (Fig. 2A and B, lane 3), the final *myogenin* expression being markedly higher in C2T18 than in C2C12 (Fig. 2C). In DM, the drugs produced an increase in *myogenin* expression both in C2C12 and C2T18 (Fig. 2A and B, lane 4), with greater effects exerted on the latter clone.

In semiquantitative densitometric assays, the signal intensity was calculated within the blot of Figure 2A for C2C12 and of Figure 2B for C2T18; a densitometric comparison was obtained by scaling the relative signals using the common blot of Figure 2C. The densitometric analysis (Table II) confirmed the qualitative analysis of Northern blot shown in Figure 2. The C2T18 clone showed, in all conditions, higher signals than the corresponding ones of C2C12. In particular, the increase in *myogenin* expression of the C2T18 clone after 96 hrs in GM produced a signal comparable to that of C2C12 in DM, while its overall increase in DM, whether with or without drugs, produced the highest *myogenin* expression. In some experiments the C2T18 signal after 96 hrs in GM reached an intensity as strong as that reached in DM (data not shown). These results further stress the higher myogenic potentiality of C2T18 when compared with C2C12.

The results obtained by Northern blot analysis were confirmed by RT-PCR (data not shown).

*Myogenin expression in vivo in biopsies of various tissues* – Figure 3 shows the expression of γ-actin, used as a positive control, and of *myogenin* in adult and embryonic muscle as well as in brain and spleen. *Myogenin* expression was detected in vivo only in embryonic muscle (lane 11). Despite the large number of cycles performed in some RT-PCR experiments, there was no expression in any of the other adult tissues. The lack of *myogenin* expression found in adult muscle is in agreement with the findings of some authors (45) but in contrast with those of others (44).

*Optimization and test performance of Hpa II / multiplex PCR assay* – PCR amplification from EcoR I treated DNA produced the expected 3 bands (shown in all EcoR I panels in Figs. 4, 5 and 6); some particularly efficient PCR reactions also displayed another product, of 990 bp, which originated in a secondary, though specific reaction from MyoP1 and MyoM7 primers (often visible in the panels in Figs. 4, 5 and 6).
The same band pattern was obtained both from uncut DNA and from DNA treated with heat-inactivated Hpa II (data not shown). In preliminary experiments we also verified that demethylated PCR products, digested with Hpa II and re-amplified after the cut, failed to show any band relative to the 5'-flanking region or exon 1, but produced, by contrast, an amplified product relative to exon 3. In addition, we selected a prolonged extension time (4.5 min) in order to obtain a more efficient amplification of the largest PCR product (499 bp); the result was a signal with an intensity similar to that of the other amplified fragments, regardless of the amount of target DNA used.

Myogenin methylation patterns in in vitro cell culture, without drugs – The Hpa II / multiplex PCR assay yields an amplified product only if the DNA fragment which is to be amplified by the specific pairs of primers fails to be cut by a restriction endonuclease. The analysis of the amplified products obtained from the Hpa II-treated samples allows the determination of the methylation status of the single CCGG site in the 5'-flanking region and of the three CCGG sites in exon 1 of the myogenin gene. Exon 3 was used together with EcoR I-treated samples as a positive control. The panels in Figures 4 and 5 show the methylation patterns of the myogenin gene at different times, after either EcoR I digestions as positive controls (on the left of each panel), or Hpa II digestion in methylation-sensitive experiments (on the right of each panel).

In C2C12 grown in GM, all the CCGG myogenin sites were methylated up to 48 hrs (Fig. 4A), though in some experiments a partial loss of methylation in the 5'-flanking site occasionally occurred at 48 hrs (data not shown). In all the experiments, this site was found to be unmethylated after 72 hrs of culture in GM (Fig. 4B, lane 9), and remained unmethylated at 96 hrs and 120 hrs. In DM (Fig. 4C), C2C12 showed a demethylation of the 5'-flanking site 24 hrs after induction of differentiation (lane 12), though this site was occasionally still methylated at 24 hrs (Fig. 7A, lane 3). The 5'-flanking site was however always unmethylated 48 hrs after differentiation induction (Fig. 4C, lane 13 and Fig. 7A, lane 4). We never observed demethylation of any of the three CCGG sites of exon 1.

All the CCGG sites of the C2T18 clone were methylated in GM up to 2 hrs (Fig. 5A, lane 7). In all the experiments, the CCGG site of the 5'-flanking region was found to be demethylated after 24 hrs of culture in GM (Fig. 5A, lane 8). Demethylation was maintained for over 96 hrs in GM. In DM, the C2T18 clone showed early demethylation of the 5'-flanking site 2 hrs after differentiation induction (Fig. 5B, lane 8), this pattern being maintained for over 72 hrs. Earlier time courses in DM showed methylation of the 5'-flanking site of this clone up to 1 hr after differentiation induction (Fig. 5C, lane 10). In addition, there was no demethylation of the CCGG sites of exon 1 in C2T18.

Myogenin methylation patterns in biopsies of various tissues in vivo – Myogenin was found to be fully methylated in non-expressing tissues (spleen and brain, Fig. 6, lanes 7 and 8) and demethylated at the 5'-flanking site in myogenin-expressing embryonic muscle (Fig. 6, lane 10). Adult muscle (Fig. 6, lane 9), where no myogenin expression was found, showed partial demethylation of the 5'-flanking region site, with a band of reduced intensity visible in some preparations. Demethylation in vivo was also strictly limited to the site of the 5'-flanking region.
Myogenin demethylation patterns in in vitro cell culture, with drugs – Methylation (up to 8 hrs, Fig. 4C) of the CCGG 5′-flanking site of C2C12 in DM completely disappeared as early as 2 hrs after differentiation induction in the presence of DH (Fig. 7A, lane 6), even in experiments in which some methylation had been found up to 24 hrs after differentiation induction (Fig. 7A, lane 3); this demethylated status was maintained for over 72 hrs. These effects were observed only on the CCGG 5′-flanking site, never on the exon 1 CCGG sites.

Clone C2T18 showed a completely demethylated 5′-flanking site from 2 to 72 hrs, whether with (data not shown) or without (as previously shown in Fig. 5B) DH. An earlier time course (Fig. 7B) showed demethylation of this site after only 20 min, in DM in the presence of DH, whereas controls remained methylated up to 1 hr. The effect was, also in these experiments, strictly limited to the 5′-flanking site.
DISCUSSION

The DNA methylation patterns of MRFs have seldom been studied. MyoD1 plays a fundamental regulatory role in the commitment of the cell to muscle fate; it is expressed both in myoblasts and in myofibers and acts upstream of myogenin which, given that its expression is restricted to terminally differentiated muscle, is instead devoted to the control of final differentiation. This functional distinction is associated with structural differences in the control region of the two genes, since MyoD1 is associated with a CpG island (48, 66), whereas myogenin is not. Some authors (67) have shown that the density of methylated sites in the MyoD1 promoter is inversely correlated with the ability to undergo auto- and cross-activation and that, in vivo, the MyoD1 promoter is partially methylated in non-muscle tissues but is unmethylated in skeletal muscle. Other studies have indicated, however, that the CpG island of MyoD1 is unmethylated also in normal non-muscle mouse tissues, that it becomes methylated during immortalization of cell lines and in vitro transformation (48), as well as during in vivo carcinogenesis, and that this methylation is also correlated with the heterochromatinization of this gene (66). The generalized in vivo hypomethylation of the MyoD CpG island has stimulated the search for the real target sequences with regulatory functions on the expression of this gene, leading to the identification of a distal enhancer which is completely unmethylated in myogenic cells and in somite cells but is, on the contrary, methylated in non-muscle cells (46, 47). Previous work from this laboratory (52) provided evidence of the existence of a link, following a differentiative stimulus or exposure to demethylating agents, between earlier overall DNA hypomethylation, enhanced muscle differentiation and myogenin expression. The dynamics of changes in the methylation pattern during differentiation, however, is not yet fully understood for either MyoD1 or myogenin, nor is the site-specific pattern of methylation yet known of the latter gene.

In this work we show that the myogenin expression pattern of C2C12 and C2T18 is strongly correlated with the myogenic potentialities of these cells. The final differentiation levels (evaluated as CK activity) were in fact closely correlated with the final expression levels of myogenin in DM and with the partial induction of myogenin in GM, as well as with its enhanced expression in C2T18. The drugs produced enhanced myogenin expression and a concomitant increase in terminal differentiation, both effects being highest in the C2T18 clone.

Demethylation of myogenin always preceded its activation. The dynamics of the demethylation displayed, in both C2C12 and C2T18, a positive correlation between early demethylation on the one hand, and the levels of myogenin expression and muscle differentiation on the other. After the shift to DM, the 5'-flanking site of myogenin in C2T18, i.e. in the clone with the highest expression of myogenin and the best differentiative ability, was methylated up to 1 hr, but demethylated after 2 hrs, whereas in C2C12 it became demethylated only after 24 hrs. Although GM allowed only limited myogenin expression and muscle differentiation, there was also in this culture condition a good correlation between myogenin demethylation, its expression and muscle
Myogenin demethylation
differentiation. In fact, higher myogenin expression and muscle differentiation were shown in the C2T18 clone, whose 5'-'flanking site was methylated at 2 hrs in GM but demethylated at 24 hrs, than in C2C12, which showed the lowest level of myogenin expression and muscle differentiation in GM combined with a very late methylated status (up to 48 h). However, hypomethylation eventually occurred also in this condition (at 72 h), accounting for the limited effect on myogenin expression and muscle differentiation. By comparing myogenin methylation in DM vs. GM, the demethylation of both C2C12 and C2T18 was found to be anticipated after the shift to DM. Demethylating agents (DH) were able to further anticipate demethylation of the 5'-flanking region site in both clones, concomitantly producing enhanced myogenin expression and terminal differentiation.

Taken together, these findings indicate that the demethylation of myogenin follows a highly specific time pattern; myogenin expression and terminal differentiation were strongly correlated with the timing, rather than with the extent, of 5'-flanking region demethylation, since the clone and conditions showing greatest differentiation and myogenin expression also exhibit earlier demethylation. The evidence of demethylation dynamics occurring much faster than the cell cycle (which lasts approximately 24 hrs in these cells), i.e. within a few hours without drugs, or even in minutes in the presence of the drugs, strongly points to the involvement of an active demethylation mechanism. No definite answer can yet be given as to which of the mechanisms so far proposed (34, 35, 37) is most likely to be involved in the control of myogenin expression; nevertheless, the myogenin/muscle system appears to be a model which can be used to investigate the involvement of active demethylation processes in the transcriptional control of tissue-specific genes. The observed effect of the further anticipation of demethylation by drugs interfering with the AdoMet metabolism is intriguing given that these drugs should deal only with the methylation processes and with passive demethylation. 3-Deaza-adenosine is, in fact, both a substrate and a competitive inhibitor of S-adenosylhomocysteine (AdoHcy) hydrolase (EC 3.3.1.1). Administration of 3-deaza-adenosine, particularly in the presence of homocysteine (DH), results in intracellular accumulation of AdoHcy and 3-deaza-AdoHcy, which are both inhibitors of transmethylation processes; the accumulation of these inhibitors often also causes increased levels of AdoMet (owing to its decreased utilization) (33, 57, 68, 69). The overall effect at the DNA level, demonstrated also specifically in an in vitro muscle system (52, 70), is marked hypomethylation due to inhibition of methylating processes. Since passive demethylation mechanisms are unlikely to be involved in the dynamics of demethylation that occurs within a few minutes, some interplay between demethylation and methylation processes must be hypothesized. In our opinion, there may be a balance between active demethylation processes removing the methyl moieties with very fast dynamics on the one hand, and re-methylating mechanisms acting on the same sites, on the other. The equilibrium between these two main mechanisms would produce the final methylation pattern of the gene, thereby defining its transcriptional status. As far as myogenin is concerned, the differentiative stimulus can, by acting on active demethylation processes, lead to early demethylation which is possibly enhanced by a
concomitant inhibition of re-methylation processes achieved by drugs acting on the AdoMet metabolism.

The drastic results of the presence or absence of an amplified product relative to the 5'-flanking site, without intermediate signal levels, obtained in almost all the experiments is indicative of a fully methylated or fully demethylated status which requires a very short time interval for transition. This supports the hypothesis according to which the mechanism involves a DNA demethylase which directly removes the methyl groups from methyl-cytosine residues in DNA (37) and is thought to have the characteristics of a processive enzyme, with the enzyme landing on a molecule of DNA and proceeding to demethylate in cis (71).

Myogenin demethylation was found to be highly controlled also in a site-specific manner. In fact, the modulation of its methylation was strictly limited to the sole CCGG site of the 5'-flanking region, while the 3 CCGG sites of exon 1 were, by contrast, always methylated. Our results suggest that one site on its own may play a role in the activation of some muscle regulatory genes. Indeed, some methyl-CpG binding proteins show distinct requirements in terms of methyl-CpG density needed for binding, spanning from greater density to single methylated CpG moiety (72-74). Interactions between the methyl-CpG density, the location of the methyl-CpG moieties and the promoter strength have also been reported (75-77).

The 5'-flanking region site was also demethylated in embryonic muscle (a myogenin-expressing tissue), but was methylated in non-expressing tissues. These findings substantiate, also in vivo, a role of methylation of the 5'-flanking site in the expression of myogenin. This site was (at least partially) demethylated in adult muscle without evidence of expression, thus confirming previous evidence that DNA demethylation is a condition which is necessary, but is not sufficient, for the complete expression of a gene.

In conclusion, our study suggests that DNA demethylation is an essential active mechanism in the transcriptional control of myogenin, both in vitro and in vivo, and that it is subject to strict site- and time-specific regulation. Demethylation dynamics results from an interplay between demethylating and re-methylating processes and is a crucial variable in the quantitative control of myogenin expression and in muscle terminal differentiation.
REFERENCES

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1 The abbreviations used are: GM, Growth medium (with 10% fetal calf serum); DM, Differentiation medium (with 1% fetal calf serum); CK, Creatine kinase; DH, 3-deaza-adenosine 3 μM + homocysteine 50 μM; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; MRFs, Muscle regulatory factors; EM, Embryonic muscle; AM, Adult muscle; S, Spleen; B, Brain; I.O.D., Integrated Optical Density; MM, Molecular weight marker; r18S, ribosomal 18S; h, hours after medium shift.
FIGURE LEGENDS

Fig. 1. **Schematic representation of myogenin gene.** Primers used are shown as gray boxes and Hpa II sites as arrows (solid if analyzed in this study, dotted if not analyzed).

Fig. 2. **Myogenin expression in in vitro cell cultures.** *A*) Expression pattern of C2C12 in GM, DM and DM +DH. *B*) Expression pattern of C2T18 in GM, DM and DM +DH. *C*) Comparison of expression in DM at 96 hrs, between C2C12 and C2T18. Because of some probing and exposure differences between blots, the only comparisons allowed are within blot. For each blot, ribosomal 18S (r18S) signals and ethidium bromide staining (EtBr) are shown as controls; h = hours after medium shift.

Fig. 3. **Myogenin expression in vivo in biopsies of various tissues.** RT-PCR signals are shown for spleen (S, lanes 2 and 8), brain (B, lanes 3 and 9), adult muscle (AM, lanes 4 and 10) and embryonic muscle (EM, lanes 5 and 11) using myogenin specific primers (from lane 8 to 12), and γ-actin specific primers as positive controls (from lanes 2 to 6); lanes 6 and 12 show negative controls; lanes 1, 7 and 13 show a φX/Hae III molecular weight marker (MM).

Fig. 4. **Myogenin methylation patterns in C2C12, without drugs.** *A*) Methylation pattern after 1 hr to 48 hrs of culture in GM; MM = λ/Hind III + EcoR I molecular weight marker. *B*) Extended methylation pattern after 2 hrs to 120 hrs of culture in GM; MM = φX/Hae III molecular weight marker. A negative control is shown in lane 12. *C*) Methylation pattern after 1 hr to 48 hrs in DM; MM = λ/Hind III + EcoR I molecular weight marker. Each electrophoretic panel shows the EcoR I digestions, used as positive controls on the left, and the Hpa II digestions, used as methylation-sensitive experiments, on the right; h = hours after medium shift.

Fig. 5. **Myogenin methylation patterns in C2T18, without drugs.** *A*) Methylation pattern after 2 hrs to 96 hrs of culture in GM; MM = φX/Hae III molecular weight marker. A negative control is shown in lane 12. *B*) Methylation pattern after 2 hrs to 72 hrs of culture in DM; MM = λ/Hind III + EcoR I molecular weight marker. *C*) Early methylation pattern after 20 min to 8 hrs in DM; MM = φX/Hae III molecular weight marker. A negative control is shown in lane 13. Each electrophoretic panel shows the EcoR I digestions, used as positive controls on the left, and the Hpa II digestions, used as methylation-sensitive experiments, on the right; h = hours after medium shift.

Fig. 6. **Myogenin methylation patterns in vivo in various tissues.** The EcoR I panel, used as a positive control is shown on the left, while the Hpa II panel, used as methylation-sensitive experiments
Myogenin demethylation is shown on the right, in spleen (S, lanes 2 and 7), brain (B, lanes 3 and 8), adult muscle (AM, lanes 4 and 9) and embryonic muscle (EM, lanes 5 and 10); lane 11 is a negative control; lanes 1, 6 and 12 show a φX/Hae III molecular weight marker (MM).

Fig. 7. Myogenin methylation patterns in vitro with drugs. A) C2C12 methylation pattern after 2 hrs to 72 hrs of culture in DM without drugs (lanes from 2 to 5) and with DH (lanes from 6 to 9). B) C2T18 methylation pattern after 20 min to 1 hr of culture in DM without drugs (lanes from 2 to 4) and with DH (lanes from 5 to 7). Each electrophoretic panel shows Hpa II digestions, used as methylation-sensitive experiments. MM = φX/Hae III molecular weight marker; h = hours after medium shift.
TABLE I

Differentiation pattern of C2C12 and C2T18 clone. The CK activity normalized for protein content (expressed as mU CK / µg proteins ± SE), 96 hrs after induction, in cells cultured in GM, DM, or DM +DH, is shown.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Culture conditions</th>
<th>GM</th>
<th>DM</th>
<th>DM +DH</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2C12</td>
<td></td>
<td>0.18 ± 0.02</td>
<td>1.28 ± 0.03</td>
<td>1.61 ± 0.11</td>
</tr>
<tr>
<td>C2T18</td>
<td></td>
<td>0.69 ± 0.02</td>
<td>2.10 ± 0.05</td>
<td>5.60 ± 0.22</td>
</tr>
</tbody>
</table>
TABLE II

Densitometric evaluation of myogenin expression.

Densitometric values, in GM, DM and DM +DH, were normalized as described in the results section and expressed as a percentage of the highest signal (C2T18 in DM +DH, shown as 100%) ± SE.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Culture conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GM</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
</tr>
<tr>
<td>C2C12</td>
<td>2.25 ± 1.24</td>
</tr>
<tr>
<td>C2T18</td>
<td>4.27 ± 1.35</td>
</tr>
</tbody>
</table>
Fig. 2

A

myogenin

r18S

EtBr

48h 96h 96h 96h
GM DM GM DM
C2C12 C2C12

B

C

G2C12 C2T18

GM DM

DM
Fig. 3

- myogenin (800 bp)
- γ-actin (216 bp)
Fig. 5

A

GM

5'-flanking (499 bp)
exon 1 (326 bp)
exon 3 (268 bp)

1 2 3 4 5 6 7 8 9 10 11 12 13

MM 2 24 48 72 96 2 24 48 72 96 MM h

EcoR I Hpa II

B

DM

(990 bp)
5'-flanking (499 bp)
exon 1 (326 bp)
exon 3 (268 bp)

1 2 3 4 5 6 7 8 9 10 11 12 13

MM 2 8 12 24 48 72 2 8 12 24 48 72 h

EcoR I Hpa II

C

DM

(990 bp)
5'-flanking (499 bp)
exon 1 (326 bp)
exon 3 (268 bp)

1 2 3 4 5 6 7 8 9 10 11 12 13 14

MM 20 40 1h 2h 8h MM 20 40 1h 2h 8h MM

EcoR I Hpa II
Fig. 7

A

C2C12

DM

5'-flanking (499 bp)
exon 1 (326 bp)
exon 3 (266 bp)

B

C2T18

DM

5'-flanking (499 bp)
exon 1 (326 bp)
exon 3 (266 bp)
The dynamics of myogenin site-specific demethylation is strongly correlated with its expression and with muscle differentiation

Marco Lucarelli, Andrea Fuso, Roberto Strom and Sigfrido Scarpa

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