The methylation state of 2 muscle-specific genes: restriction enzyme analysis did not detect a correlation with expression

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ABSTRACT

To examine the possible role of DNA methylation in the modulation of expression of genes involved in the differentiation of muscle cells, we compared the methylation state of a number of CpG sites in the rat skeletal muscle actin and myosin light chain 2 genes, in muscle and nonmuscle cells, and in proliferating myoblasts and differentiated myotubes of the myogenic cell line L8. No correlation was detected between the state of methylation of these sites and the expression of the two genes. Essentially the same pattern of DNA methylation was observed, in the sites examined, in DNA from muscle, kidney and stomach. In DNA extracted from cultures of proliferating mononucleated myoblasts, as well as from differentiated multinucleated fibers of the myogenic cell line L8, the two genes were more methylated than in other tissues.

INTRODUCTION

Vertebrate cells are relatively poor in the sequence CpG (1). The sequence is only 20 to 40% as frequent as would be expected for DNA with a random distribution of the 4 bases (2). The fact that DNA methylation is restricted mainly to the CpG sequence (2-5), and that DNA methylation could be a self-replicating modification (6), raised the possibility that methylation at this sequence may play a role in the programmed expression of genes in eukaryotic cells (7, 8) and in the maintenance of these programs during cell proliferation and differentiation. Application of 5-aza-cytidine, which inhibits DNA methylation during cell replication, resulted in several cases in the de novo activation of genes (9-13). For example, treatment of C3H/10T1/2 cell cultures with this drug resulted in the appearance of foci of differentiated muscle fibers (10). These results suggested that interfering with DNA methylation changed the program of gene expression. Several investigations have demonstrated an inverse correlation between DNA methylation and gene expression (14-20). In the chick globin, chick ovalbumin and mouse immunoglobulin genes, a difference was observed between the level of methylation of the genes in tissues where they are active in transcription and tissues where they are

inactive (16-20). However, in several other genes no correlation between gene activity and DNA hypomethylation could be detected (21-25).

The expression of muscle-specific genes during myogenesis offers a convenient system for the analysis, at the molecular level, of the changes associated with gene activation. The formation, via cell fusion, of post-mitotic multinucleated myotubes, is associated with the accumulation of large amounts of muscle-specific mRNAs and proteins (26). We have shown previously that the expression of skeletal muscle actin and myosin light chain 2 genes is associated with changes in their chromatin organization, as measured by DNAase I sensitivity (27). This indicates that the activation of the transcription of these genes occurs during the transition to the stage of cell fusion. In the present study we investigated the pattern of methylation of two genes expressed during myogenesis, aiming at determining whether changes in DNA methylation are correlated with gene expression during terminal differentiation and/or with the determination and maintenance of the latent program of expression of muscle-specific genes during development. Using the methylation sensitive enzymes HpaII, AvaI and HhaI, we determined the state of methylation of specific CpG sites in the rat skeletal muscle actin gene and myosin light chain 2 genes in proliferating myoblasts and differentiated myotubes, as well as in nonmuscle tissues. In the CpG sites examined, we did not detect changes in DNA methylation which could be correlated with the maintenance of the myogenic program or with the activation of these genes.

MATERIALS AND METHODS

Cell cultures

Proliferating mononucleated cells of the rat myogenic cell line L8 (28) were grown as described (26). To induce differentiation, the medium was changed, in cultures approaching confluency, to Eagle's medium supplemented with 2% horse serum and 0.1 ug/ml insulin. A phase of rapid cell fusion started about 30 h after the change of medium. Differentiated cultures, consisting mostly of multinucleated fibers, were harvested 100 h after the change of medium.

Hybridization probes for skeletal muscle actin and myosin light chain 2 (MLC2) genes

Plasmid pAC15-2 carries a 3.7 Kb DNA fragment containing the entire coding region and the 5' untranslated region of the rat skeletal muscle actin gene (29). The plasmid was digested with the restriction enzyme SacI and a 577 bp DNA fragment from the 5' end of the gene was isolated. This probe

hybridizes specifically with the skeletal muscle actin gene.

Plasmid p103 contains a cDNA insert of 288 nucleotides, 229 nucleotides coding for the 76 amino acids at the C-terminal end of the protein and 59 nucleotides of the 3' untranslated region of the mRNA coding for the fast skeletal muscle MLC2 (Nudel et al., in preparation).

Preparation and hybridization of DNA

Tissues were dissected from Wistar rats, quick frozen, and stored at -70°C. High molecular weight DNA was extracted as described by Blin and Stafford (30). Cultured cells were lysed by addition of 0.5% Sarcosyl 0.25 M EDTA containing 50 µg/ml proteinase K. The lysates were incubated at 50°C for 4 h, extracted with phenol/chloroform and dialyzed extensively. DNA was ethanol precipitated and resuspended in 10 mM Tris pH 7.4 and 1 mM EDTA.

DNA was digested with restriction enzymes under conditions specified by the manufacturers. At least 5- to 10-fold excess of enzymes were used to ensure complete digestion. DNA fragments were separated by electrophoresis on 0.8% or 1% agarose gels and transferred to nitrocellulose filters, according to Southern (31). Filters were hybridized with ³²P-labeled probes, as described (27). Filters were washed in 0.1 XSSC, 0.2% SDS, at 70°C for several hours. The labeled DNA fragments were detected by fluorography.

RESULTS

The methylation of the rat skeletal muscle actin gene in muscle and nonmuscle cells

The rat skeletal muscle actin gene contains 6 introns, 5 of which are in the coding region, and one large intron located 12 nucleotides upstream from the initiator ATG codon (29). We analyzed the methylation state of 2 out of the 9 HpaII sites in this gene, using the isoschizomers HpaII and MspI. The cleavage at other HpaII sites generated fragments that were too small to be accurately analyzed by the Southern technique. Whenever the internal C-residue of the recognition sequence of these enzymes, CCGG, is methylated, HpaII cannot cut, whereas MspI does cut such sites (32). The first HpaII site is located in the 5' flanking region of the gene, 240 bp upstream from the cap site (site 2, Fig. 1c) and the second site is in the second exon (site 6, Fig. 1c). The probe for this set of experiments was a 0.6 kb SacI-SacI fragment, derived from the 5' end of the actin gene (see map in Fig. 1). When DNA from proliferating myoblasts or differentiated myotubes of the myogenic cell line L8 was digested to completion by EcoRI in conjunction with either MspI or HpaII, a single hybridizable band of about 1.5 kb was observed (Fig. 1a). The same band was formed by digestion of DNA from skeletal

FIGURE 1. Southern blot analysis of the methylation of CpG sites in the rat skeletal muscle actin gene

(a) and (b) Blots of DNA isolated from the indicated tissues and from proliferating myoblasts (L-Pre) or differentiated myotubes (L-post) of the myogenic cell line L8. The DNA was digested by EcoRI and then cleaved by HpaII or MspI (a) or by AvaI or HhaI (b). The migration of the size marker fragments is given in kilobases. The probe was the SacI-SacI fragment indicated by a solid bar above the gene map (c).

(c) Map of CpG sites in the muscle actin gene. The sites analyzed are numbered 1-9. Empty and solid bars represent untranslated and translated regions of the exons, respectively. The sites cut by the restriction enzymes HpaII (Δ), AvaI (Δ), HhaI ($\downarrow$), EcoRI (R) and SacI (S) are indicated. The lines below the map indicate the fragments detected by hybridization (kb).

muscle, heart, liver and spleen, indicating that these sites were not methylated in DNA of myogenic cells, as well as in other tissues.

Several other CpG sites were examined by digestion of the DNA with the enzymes AvaI and HhaI that recognize the sequences CPyCGPuG and GCGC, respectively. When DNA from spleen, kidney, stomach and skeletal muscle was cleaved by EcoRI and AvaI and hybridized with the SacI-SacI probe, two bands of 1.6 and 2.5 kb were obtained. This suggests that in the DNA in these tis-

FIGURE 2. The state of methylation of the rat skeletal muscle actin and myosin light chain 2 genes.

In the schematic map of the two genes, the bars represent exons and the lines introns. Empty and solid bars represent untranslated and translated regions of the exons, respectively. The sites cut by the restriction enzymes HpaII (Δ), AvaI (Δ) and HhaI ($\downarrow$) are indicated and numbered. The extent of methylation in the actin gene (a) or the MLC2 gene (b) is indicated by the boxes below each map; methylated sites (■), unmethylated sites (□) and partially methylated sites (◻). ?, since the HpaII site at site 6 is not methylated in DNA from the tissues, it was impossible to determine the state of methylation of these two HpaII sites.

sues, the first AvaI site (site 3), 150 bp upstream from the cap site, is not methylated; that the AvaI site in the third exon (site 7) is methylated in some of the DNA molecules; and that in these molecules the AvaI site in the fifth intron (site 9) is not methylated. Digestion of the same DNA preparations with EcoRI and HhaI produced a 1.5 kb band. This suggests that the HhaI site in the large intron (site 5), which is 1.5 kb downstream from the EcoRI site, is not methylated, while the HhaI sites at the 5' end, located 0.36 kb (site 1) and 1.2 kb (site 4) downstream from the EcoRI site are methylated in most of the DNA molecules of these tissues.

Figure 2a summarizes the methylation state of 9 CpG sites in the muscle actin gene. A similar pattern was detected in DNA from muscle, stomach and kidney. The small detectable differences in the relative methylation frequency of specific sites could not be correlated with muscle actin gene expression.

Surprisingly these sites seem to be significantly more methylated in DNA isolated from differentiated as well as undifferentiated cultures of the myogenic cell line L8. As can be seen in Figs. 1a and 2a, sites 5, 7 and 9 are more methylated in DNA isolated from both stages of differentiation than in the DNA from the other tissues examined in this experiment.

FIGURE 3. Southern blot analysis of the methylation of CpG sites in the rat skeletal muscle myosin light chain 2 gene.

(a), (b) and (c) Blots of DNA isolated from the indicated tissues and from proliferating myoblasts (L-pre) or differentiated myotubes (L-post) of the myogenic cell line L8. The DNA was digested by EcoRI and then cleaved by AvaI (a), HpaII or MspI (b) or HhaI (c). The migration of the size marker fragments is given in kilobases. The probe was the insert of plasmid p103.

(d) Map of CpG sites in the skeletal muscle myosin light chain 2 gene. The sites analyzed are numbered 1-8. Empty and solid bars represent untranslated and translated regions of the exons, respectively (Nudel et al., in preparation). The sites cut by the restriction enzymes Hpa II (▲), AvaI (Δ), HhaI (↓), EcoRI (R) and HindIII (H) are indicated. The lines below the map indicate the fragments detected by hybridization (Kb).

The methylation pattern of the rat skeletal muscle myosin light chain 2 gene in muscle and in nonmuscle cells

The nucleotide sequence of the rat myosin light chain 2 gene has been determined (Nudel et al., in preparation). As shown in Fig. 3d, this gene contains 6 introns, 5 of which are in the coding region, and a large intron located downstream to the initiator ATG codon. To examine the methylation pattern of this gene, we used the plasmid p103 as a probe. This cDNA plasmid

contains 229 nucleotides coding for 76 amino acids at the c-terminal end of the protein plus 59 nucleotides of the 3' untranslated region (Nudel et al., in preparation).

DNA from several tissues and from the myogenic cell line L8 was digested with the enzyme EcoRI and with the enzymes AvaI, HpaII, MspI or HhaI. The results of hybridization of plasmid p103 with EcoRI and AvaI cleaved DNA from muscle, spleen, kidney and the myogenic cell line L8 are shown in Fig. 3a. In DNA from muscle, spleen and kidney, a single band of 1.75 kb was detected. This indicates that the AvaI site located in the fifth exon (site 2) is not methylated, while the site located in the seventh exon (site 5) is methylated in most DNA molecules. Digestion of DNA from the myogenic cell line L8, harvested at both stages of differentiation, produced the same hybridizable 1.75 kb DNA band.

Figure 3b shows the hybridization of plasmid p103 with EcoRI and HpaII or MspI cleaved DNA from muscle, kidney, spleen, stomach and the myogenic cell line. In DNA from muscle, kidney and stomach, three bands of 0.7, 1.0 and 1.7 kb were detected. This indicates that the HpaII site located at site 6 is not methylated and that the HpaII site located at site 3 is partially methylated. In DNA from the myogenic cell line L8 at both stages of differentiation and in spleen the HpaII site at site 3 is methylated in most DNA molecules, and at site 6 it is partially methylated. The appearance of the 2.3 kb band suggests that HpaII sites 0.5 kb downstream from site 6 are also methylated. Since this region has not been sequenced, we have no information on the precise number and location of these sites and therefore they are not included in Fig. 2b.

The pattern obtained after cleavage of DNA from the myogenic cell line, muscle and spleen, with EcoRI plus HhaI is shown in Fig. 3c. Digestion of DNA from the myogenic cell line and spleen generated two bands of 2.3 and 3.0 kb. This indicated that all the HhaI sites up to 2.3 kb downstream from the EcoRI site are methylated and that the site at this location is partially methylated. The band of 3.0 kb is absent from DNA from muscle, kidney and stomach; instead, a band of about 1.7 kb is generated. This indicates that the HhaI site at site 4 is methylated, that the HhaI site at site 7 is partially methylated, and that the site located 2.3 kb downstream from the EcoRI is not methylated. Figure 2b summarizes the methylation state of 8 CpG sites in the muscle myosin light chain 2 gene. The methylation state of the HpaII site at site 1 (Fig. 2b) was determined using a 5' flanking DNA probe (data not shown).

DISCUSSION

We investigated the state of methylation of several CpG sites in the skeletal muscle actin and myosin light chain 2 genes. Some tissue differences in the state of methylation of several sites were observed. However, these differences could not be correlated with the expression of these genes nor with the potentiality to express the genes. Furthermore, in the myogenic cell line L8, which expresses these genes during differentiation, some sites were found to be more methylated than in DNA from tissues in which the genes were never expressed. These results indicate that the state of methylation of at least some sites in the structural genes is not relevant to the expression of the genes. They are in accordance with several other examples, in which no correlation between DNA methylation and gene expression could be detected (21-25).

Several explanations for the present results should be considered: (a) Methylation is perhaps not involved in the modulation of expression of these genes. (b) Only specific CpG sites are involved in the control of gene expression, and those were not detected with the restriction enzymes used in this investigation. Some experiments suggest that such sites are likely to be located in the 5' flanking region of the genes. E.g., in vitro methylation of the mouse structural gene coding for gamma globin did not prevent its transcription in transfection experiments. However, methylation of the 5' flanking region of this gene inhibited transcription even when the structural gene was left unmethylated (33). The CpG sites of the MLC2 and skeletal muscle actin genes are not evenly distributed. The 5' end of both genes, including 600 bp upstream to the cap site, are relatively poor in this sequence. This is also reflected in the number and the distribution of the sites for the three restriction enzymes employed (see Fig. 2). The three restriction sites in the actin gene and the single site in the MLC2 gene that are located at the 5' flanking region, which were examined, did not reveal differences in the state of methylation that could be correlated with the expression of the genes. (c) It has been shown that the transition from muscle precursor cells to terminally differentiated myotubes is associated with only one cycle of DNA replication (34). Thus, if the demethylation necessary for gene activation occurs during that cycle of DNA replication, then the outcome will be hemimethylated DNA. However, since the restriction enzymes employed do not cleave hemimethylated DNA, hemimethylation could not be detected. The kinetics of activation of the metallothioneine I gene following the application of 5 azacytidine to cultures in which this gene was not inducible (9) suggests that, if this agent exerted its effect by inhibition of methylation of replicating

DNA, then in most cells the metallothioneine gene became expressed while the CpG sites involved in the control of expression were hemimethylated. It should be noted, however, that a recent report suggests the possibility that DNA demethylation can occur in the absence of DNA replication, possibly by active enzymatic demethylation (35). (d) It is possible that changes in DNA methylation involved in the programming or control of differentiation occur in trans or cis acting genes which regulate the activation of genes coding for muscle-specific proteins but not in the genes coding for these proteins.

We have reported elsewhere on the transfection of myogenic cells with cloned skeletal muscle actin/globin chimeric gene or with a chloramphenicol acetyl transferase (CAT) gene fused to the 5' region of the muscle actin gene. In a significant number of the transfected myogenic clones, the expression of the gene greatly increased (up to >50x) during differentiation. The temporal relation between differentiation of the cultures and the expression of the chimeric gene was very similar to that of the native actin gene (36). Differentiation-related increase in gene activity was observed also in myogenic cultures transiently transfected with muscle actin chimeric genes (unpublished). Since DNA cloned in bacteria is not expected to be methylated in the CpG sites, these results suggest that the observed increase during differentiation in expression of the cloned gene was not mediated via changes in DNA methylation of the transferred genes. It should, however, be noted that small amounts of transcripts of the transferred gene were present also in mononucleated myogenic cells. At that stage of differentiation, the native actin mRNA was undetectable. It is not known whether this difference is related to the lack of methylation of the cloned gene.

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