

5-Methylcytosine DNA glycosylase participates in the genome-wide loss of DNA methylation occurring during mouse myoblast differentiation

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Received June 20, 2001; Revised and Accepted September 7, 2001

ABSTRACT

Changes in gene expression during mouse myoblast differentiation were monitored by DNA microarray hybridisation. Four days after the onset of differentiation 2.37% of the genes increased in activity from a value of zero, whereas during the same time 1.68% of total genes had decreased expression. During the first 24 h of differentiation an average of 700 000 CpG sites per haploid genome were demethylated. Maximal loss of DNA methylation is attained after 2 days of differentiation, followed by a gradual remethylation. The highest demethylation is observed in highly repeated DNA sequences, followed by single copy sequences. When DNA replication is inhibited by aphidicolin or L-mimosine this genome-wide demethylation is still observed. During the first 3 h of differentiation there is an increase in the number of hemimethylated CpG sites, which disappear rapidly during the course of genome-wide hypomethylation. Transfection of cells with an antisense morpholino oligonucleotide to 5-methylcytosine DNA glycosylase (G/T mismatch DNA glycosylase) decreases both the activity of the enzyme and genome-wide demethylation. It is concluded that the genome-wide loss of DNA methylation in differentiating mouse myoblasts occurs in part by formation of hemimethylated CpG sites, which can serve as the substrate for 5-methylcytosine-DNA glycosylase.

INTRODUCTION

Genome-wide loss of DNA methylation is usually associated with cellular differentiation. This has been documented for the early development of mouse embryos (1–8), differentiating mouse myoblasts (9,10), Friend erythroleukemia cells (11–13) and teratocarcinoma cells (13–15). In spite of intensive investigations, the mechanism(s) responsible for transient genome-wide demethylation has not yet been completely elucidated. In the case of differentiating erythroleukemia cells, Adams *et al.* (11) proposed that a delay in methylation of DNA synthesised in the early period following the onset of differentiation may

lead to undermethylation of DNA. As an alternative explanation, the results of the group of Razin *et al.* (16) suggest that genome-wide loss of DNA methylation preceding the differentiation of erythroleukemia cells occurs by enzymatic replacement of methylcytosine by cytosine. Similarly, we have previously shown (9) that maximal *ex vivo* genome-wide loss of DNA methylation in differentiating mouse myoblasts correlates with maximal activity of 5-methyldeoxycytidine excision repair. In addition, inhibition of ADP ribosylation in these cells by 3-aminobenzamide simultaneously prevented differentiation of myoblasts and genome-wide demethylation (9). At the moment it cannot be excluded that the demethylation phenomenon results from a combination of passive and active mechanisms (17–20). Here we show that genome-wide demethylation in differentiating mouse myoblasts starts with the generation of hemimethylated sites which become the substrate of 5-methylcytosine DNA glycosylase (5-MCDG).

MATERIALS AND METHODS

Cell culture and treatments

Mouse G8 myoblasts were cloned and selected for the highest baseline levels of 5-MCDG. All experiments presented here were carried out with the same clone. Cells were grown on collagen-coated plates in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) horse serum, 10% (v/v) heat-inactivated fetal calf serum and 4.5 g/l glucose (9). Before adding the differentiation medium, cells were rinsed once with phosphate-buffered saline. Differentiation was initiated at 80–90% cell confluency by changing to a serum-poor medium consisting of Dulbecco's modified Eagle's medium supplemented with 2% (v/v) heat-inactivated fetal calf serum and 4.5 g/l glucose. Pulse labelling of cells with [³H]thymidine was carried out in microwell plates for 3 h in the presence of 2 μCi/ml [³H]thymidine in culture medium. L-Mimosine (stock 10 mM) was dissolved in 10 mM HCl. Aphidicolin (stock 5 mg/ml) was dissolved in dimethylsulphoxide. The maximal final concentration of dimethylsulphoxide in cell culture was 0.5% (v/v). For each drug a dose–response curve was established by incorporation of [³H]thymidine. Similarly, each drug was also tested *in vitro* for its effect on 5-MCDG and DNA methyltransferase activities.

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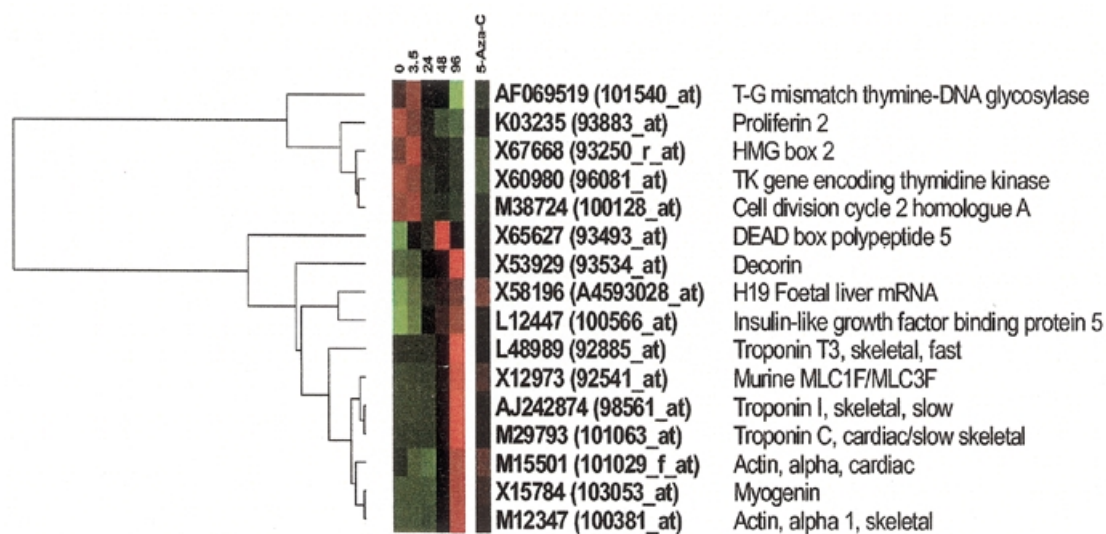


Figure 1. Hierarchical tree cluster of muscle-related gene expression changes during myoblast differentiation. This tree cluster was generated using the dChip program (23). For each gene the colour black indicates the median expression values across the arrays, green indicates lower than the median expression and red indicates higher than median expression. The code numbers represent the GenBank accession numbers and the Affymetrix identifiers (in parentheses) for each gene.

Assay for hemimethylated DNA

This assay was carried out by a modification of the procedure described by Paroush *et al.* (25). In a total volume of 100 μ l containing 10 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 25 mM NaCl and 10 μ g BSA, 10 μ g genomic DNA was digested with 20 U *Nci*I (2 U/ μ g DNA) for 30 min at 37°C. The labelled oligonucleotide substrate 5'-ATCAGGCCATCAATGTGTTCTTTCACCCGGTCAC-GCTGACCAGGAAATA-3' (only the upper strand is shown) was diluted with genomic DNA (5 ng labelled oligonucleotide in 1 μ g total genomic DNA). Under our incubation conditions 2 U of *Nci*I/ μ g DNA gave 100% cleavage of non-methylated and hemimethylated DNA. Under the same incubation conditions the fully methylated oligonucleotide gave only 3% cleavage (see Fig. 4). The fully methylated oligonucleotide incubated with 2 U/ μ g DNA gave, after 3 h incubation at 37°C, 6% cleavage (data not shown). In order to decrease the high background of non-methylated CCCGG or CCGGG, DNA was first digested with *Hpa*II (CCGG). The receding 3'-ends were filled-in with dXTPs using Klenow enzyme. Upon removal of unincorporated nucleotides the DNA was digested with *Nci*I. The DNA fragments were labelled at the 3'-end with radiolabelled dCTP and dGTP with Klenow fragment. Unincorporated nucleotides were separated by gel filtration (spin column) and the specific radioactivity of the DNA was determined.

As an alternative procedure we used DNA methyltransferase purified from HeLa cells (13). The incubation conditions were the same as for the *Sss*I methyl-accepting assay described above except that the incubation time was only 15 min. As tested with synthetic hemimethylated and non-methylated oligonucleotides, a short incubation of these substrates with Dnmt1 of 15 min resulted in >90% of maintenance methylation activity (methylation of hemimethylated DNA), the rest being *de novo* methylation. Commercial recombinant DNA methyltransferase 1 produced in insect cells did not give any

incorporation under the present incubation conditions. Only very weak incorporation was observed with genomic DNA incubated for several hours.

Fractionation of genomic DNA

Total genomic DNA was fractionated into highly repeated, moderately repeated, low copy and single copy genes by means of reassociation kinetics (24).

RESULTS

Changes in genome expression during mouse myoblast differentiation

Total RNA was prepared from G8 myoblasts at different stages of differentiation. Biotinylated cRNA hybridisation of Affymetrix U74A murine microarrays indicate that upon differentiation many genes are down-regulated while other genes are up-regulated. Figure 1 shows a hierarchical tree cluster of muscle-related gene expression changes during myoblast differentiation. Among the down-regulated genes are those involved in DNA synthesis and replication and among the up-regulated genes are those which are muscle specific, thus demonstrating that the selected G8 myoblast clone has retained the main myogenic functions. Among some of the genes increasing from a background of zero (silent to active) we will most likely find a change in their methylation levels. In order to test this possibility, cells grown in medium supplemented with 20% serum were treated for 4 days with 5 μ M 5-azacytidine (5-aza-C). Total RNA was extracted and tested with DNA microarrays. As can be seen, the changes in expression caused by 5-aza-C treatment are in the same direction as those observed during the differentiation time course. Results obtained with microarrays further indicate that in the presence of 5-aza-C ~2135 (6.1%, assuming a genome size of 35 000 genes)

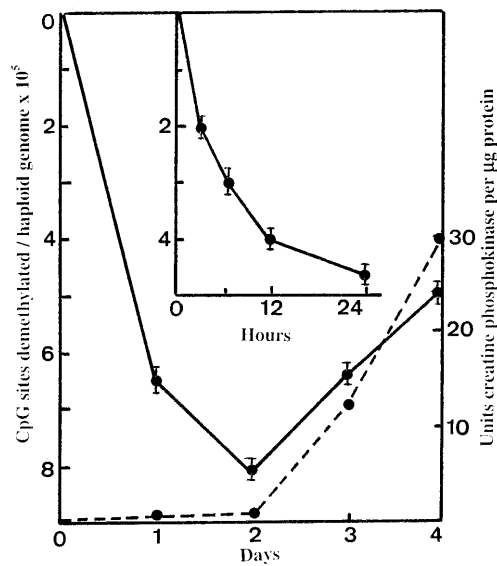


Figure 2. Kinetics of genome-wide demethylation in differentiating mouse G8 myoblasts. The inset represents the early kinetics of demethylation following addition of serum-poor medium (differentiation medium) to the cells. The solid lines represent the relative number of CpG sites demethylated per haploid genome. The dotted line is the activity of creatine phosphokinase, which is used as a marker for myogenic differentiation. Assays were run in triplicate as described in Materials and Methods. Results show the averages of triplicate measurements with the standard variation of the mean.

genes were up-regulated from zero or low values while ~ 1015 (2.9%) genes were down-regulated. During the same time 1.2×10^6 mCpG sites per haploid genome were demethylated. Among the genes up-regulated were muscle-specific genes, similar to the observation in differentiating cells. *In silico* analysis of the results of microarray RNA–DNA hybridisation also indicate that during the 4 days of differentiation 2.37% of the genes are activated (increase from zero) while 8.61% are up-regulated (increased from non-zero values) and 1.68% have a decrease in hybridisation signal. The 2.37% of activated genes (increase from zero) represent, for a mouse genome of 35 000–70 000 genes, ~ 830 –1650 up-regulated genes.

Dynamic changes in DNA methylation during mouse myoblast differentiation

It has been previously shown that a transient genome-wide demethylation takes place during mouse myoblast differentiation (9,10). In the present work we have used the less cumbersome, more reliable and sensitive *SssI* methyl-accepting assay to monitor changes in the methylation of CpG islands from total genomic DNA (24) occurring during differentiation. The results in the inset of Figure 2 show that upon onset of myoblast differentiation, there is a progressive demethylation of DNA occurring without a time lag. After 1 day of differentiation there are between 500 000 and 650 000 CpG sites demethylated per haploid genome. The variations in the number of demethylated sites observed in different experiments depends on the density of the cells at the time of change to the serum-poor medium (the optimal cell density is 250 000–500 000/cm²).

Figure 2 also shows that maximum demethylation is reached 2 days after the start of differentiation. Between days 2 and 4 there is, however, a partial remethylation of the genome. At this time a low but measurable amount of DNA methyltransferase(s) mRNA was observed (26). If we take an average of 700 000 CpG sites demethylated per haploid genome and assuming that these sites were initially symmetrically methylated, this would represent 350 000 CpG sites. For a genome size of 35 000 or 70 000 genes, the fraction of genes potentially activated from zero values by demethylation could represent 829 or 1659 genes, respectively. This would give an average of 210–420 CpG sites per activated gene. This is obviously too large a number of CpG sites per gene. As the genome is compartmentalised into CpG-rich and repeat-rich regions we determined the extent of demethylation of various fractions of DNA obtained by reassociation kinetics.

Which fraction of the genome becomes demethylated?

Studies on DNA fractionated by reassociation kinetics indicate that highly repeated sequences contain more CpG sites than the single copy fraction of the genome (27,28), suggesting that this fraction could be an important target for methylation/demethylation. Table 1 shows that the hypomethylation expressed as Δ pmol CH₃ incorporated/ μ g DNA is greater in the highly repeated sequences than for the other fractions. The Δ pmol CH₃ incorporated/ μ g DNA represents the difference in incorporation obtained between non-differentiating control DNA and the corresponding DNA fraction from differentiating cells. The last column of Table 1 expresses the relative change in hypomethylation of each fraction as a percentage of the incorporation measured for the corresponding fraction of non-differentiating cells. Per cent hypomethylation is highest in the highly repetitive DNA, followed by the single copy sequences. For this last fraction a Δ value of 0.04 pmol/ μ g DNA represents an average of 25 symmetrically methylated CpG sites per gene undergoing activation from zero value (1659 genes for a genome of 70 000 genes). This value seems more plausible provided that all these genes undergo demethylation during differentiation.

Genome-wide demethylation occurs in the absence of DNA synthesis

Genome-wide demethylation may occur by a passive or active mechanism. For example, in differentiating erythroleukemia cells, Adams *et al.* (11) suggested that transient demethylation may not reflect the selective removal of methylcytosine from pre-existing DNA but that delayed methylation of newly synthesised strands could possibly give rise to a transient increase in hemimethylated sites. This could arise due to a failure of endogenous DNA methyltransferase 1 to keep pace with replication. In order to assess the role of DNA replication in genome-wide demethylation, DNA synthesis in myoblasts was blocked with either aphidicolin or L-mimosine. Aphidicolin blocks DNA synthesis (29–31) whereas L-mimosine, a plant amino acid, reversibly inhibits cell cycle progression in late G₁ phase (29,32–34). Aphidicolin and L-mimosine, at the concentrations used, did not inhibit or stimulate DNA methyltransferase or 5-MCDG (data not shown). At a concentration of 3 μ M, aphidicolin inhibited DNA synthesis by >99%, whereas the same level of inhibition of DNA synthesis required 400 μ M L-mimosine (data not shown). Cells were pretreated for 16 h

Table 1. State of DNA methylation of genomic DNA fractionated by Cot values

DNA fractions	Reassociation kinetics Cot values	Per cent of total DNA	pmol CH ³ /μg DNA		Hypomethylation (Δ pmol CH ³ /μg DNA)	Δ pmol CH ³ /μg DNA (% pmol CH ³ /μg DNA)
			Non-differentiating (a)	Differentiating (b)	(c)	Non-differentiating (c/a)
Total DNA	Not fractionated	100	1.19 ± 0.05	1.71 ± 0.04	0.52	43
Highly repeated	0.05	34	1.12 ± 0.05	2.15 ± 0.03	1.03	91
Moderately repeated	0.05–10	24	0.58 ± 0.04	0.71 ± 0.03	0.13	22
Low copy	10–500	19	0.17 ± 0.006	0.22 ± 0.02	0.05	29
Single copy	500–2000	23	0.09 ± 0.004	0.13 ± 0.006	0.04	44

Relative hypomethylation of DNA was calculated from results obtained with the *SssI* methyl-accepting assay carried out on control non-differentiating and differentiating cells. Δ is the difference between the non-differentiated control and the differentiating cells.

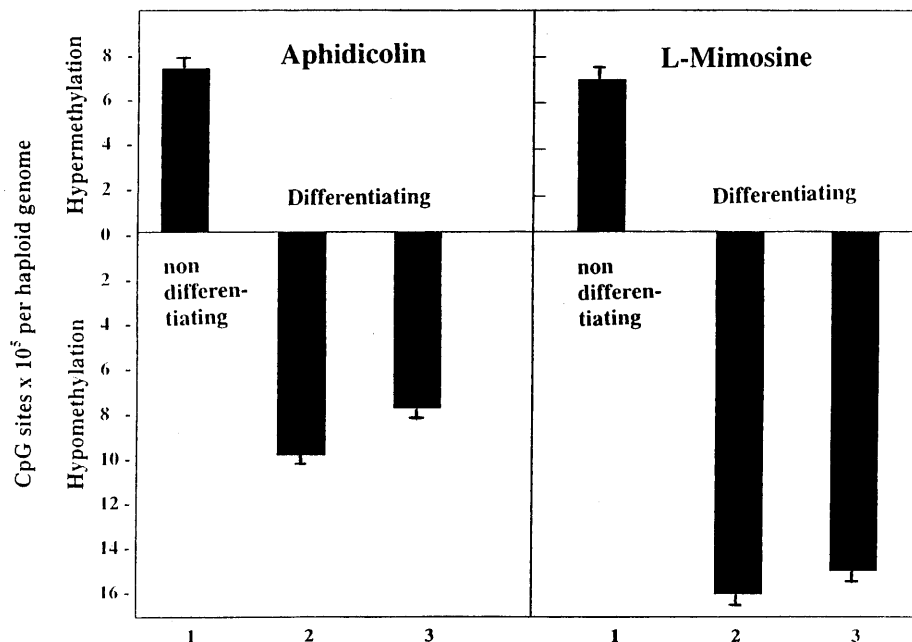


Figure 3. Effects of aphidicolin and L-mimosine (inhibitors of DNA synthesis) on the genome-wide demethylation of differentiating myoblasts. Bars 1 represent the effect of the drugs on growing non-differentiating cells (hypermethylation of DNA). Bars 2 represent differentiating myoblasts grown in the absence of the inhibitors of DNA synthesis. Bars 3 represent differentiating myoblasts grown in the presence of either aphidicolin or L-mimosine. An assay of *SssI* methyltransferase was run in triplicate as described in Materials and Methods.

with either aphidicolin or L-mimosine before they were moved to differentiation medium. The drugs were also added to the low serum differentiation medium. After 24 h of further incubation in the low serum medium, cells were collected and DNA extracted and tested for methylation. Figure 3 shows the results of the *SssI* methyl-accepting assay. It can clearly be seen that cells grown in normal medium in contact for 16 h with either aphidicolin or L-mimosine were hypermethylated (bar 1). Hypermethylation caused by drugs interfering with DNA synthesis has already been documented for other systems (24; and references therein). However, when the cells were further incubated for 24 h in differentiation medium in the presence of

either aphidicolin or L-mimosine, genome-wide demethylation took place, albeit at a somewhat lower level than for differentiating cells incubated in the absence of the two drugs (Fig. 3, compare bars 2 and 3). The somewhat smaller hypomethylation observed for differentiating myoblasts grown in the presence of aphidicolin may be explained by a slight toxic effect of the drug. In the presence of 3 μM aphidicolin some cells with picnotic nuclei detached from the collagen-coated Petri dishes. These results strongly suggest that significant DNA demethylation can occur despite the absence of DNA synthesis. Therefore, genome-wide demethylation is in part an active process due to an enzymatic reaction.

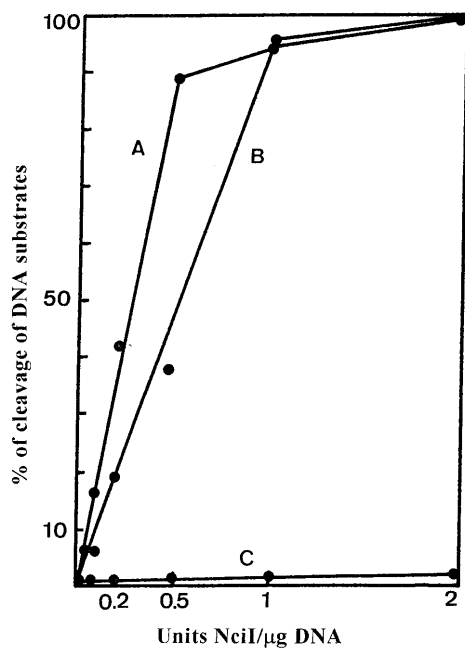


Figure 4. Selective cleavage of model substrate oligonucleotides by *NciI*. Assays were carried out as described in Materials and Methods. (A) Cleavage of the non-methylated oligonucleotide. (B) Cleavage of the hemimethylated oligonucleotide. (C) Cleavage of the symmetrically methylated oligonucleotide (methylated on both strands).

Hemimethylated CpG sites are present during the early phase of myoblast differentiation

The presence of hemimethylated CpG sites in the early phase of cell differentiation has already been documented (13). In our system upon the onset of differentiation there is a drop in both DNA synthesis and DNA methyltransferase activities, which could potentially create hemimethylated sites. The presence of hemimethylated CpG sites in total genome DNA was tested with two different procedures. One is based on the ability of *NciI* to cleave, under certain conditions, CCCGG or CCGGG sites in the hemimethylated but not in the symmetrically methylated form (25; Fig 4). The second procedure is based on the capacity of purified DNA methyltransferase 1 to selectively methylate hemimethylated DNA (13). The results in Figure 5, for both methods, clearly show that in non-synchronised dividing mouse myoblasts there is a baseline level of hemimethylated sites already present. Three hours following the onset of myoblast differentiation there is an increase in the number of hemimethylated sites. The increase in hemimethylated sites could account in part for the hypomethylation that we observed during the first 3 h of differentiation (Fig. 2) as *SssI* methyltransferase does not distinguish between hemimethylated and non-methylated CpG sites. As seen in Figure 5, after the first 3 h differentiation there is a steady decline in hemimethylated sites. Concomitant with the decrease in hemimethylated sites there is also an increase in overall hypomethylation of the genome (compare Figs 2 and 5), suggesting that these hemimethylated sites are enzymatically demethylated. Since we have previously shown that hemimethylated DNA is the preferred substrate of 5-MCDG *in vitro*, it was logical to test

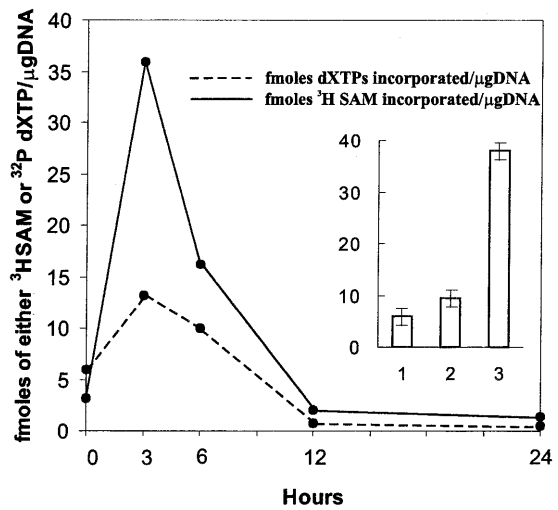


Figure 5. Presence of hemimethylated CpGs in differentiating G8 myoblasts. Hemimethylated DNA was determined as described in Materials and Methods. The curves represent the average values of two independent experiments. The results represented by the dotted line were obtained by a method consisting of sequential cutting and filling-in of *HpaII* (CCGG) and *NciI* (CCCGG and CCGGG) sites. The solid line represents the second procedure, consisting of a very short incubation time of genomic DNA (15 min) with purified HeLa DNA methyltransferase 1. The values obtained are the relative incorporation of [³²P]dXTPs or [³H]SAM per μg DNA. The bar diagrams of the inset represent the presence of hemimethylated CpG sites in differentiating cells (24 h time point). Cells were grown and differentiated: (1) in the absence of morpholino oligonucleotides; (2) in the presence of control morpholino oligonucleotides (random sequence); (3) in the presence of the morpholino antisense oligonucleotides (5-MCDG). Hemimethylated DNA was determined by incubating the DNA for 15 min in the presence of purified Dnmt1 and [³H]SAM.

whether this enzyme is responsible for the genome-wide demethylation occurring in differentiating mouse myoblasts.

Transfection of myoblasts with G/T mismatch DNA glycosylase antisense morpholino oligonucleotides decreases the level of 5-MCDG activity and inhibits genome-wide demethylation

It has previously been shown that *in vitro* the preferred substrate of 5-MCDG is hemimethylated DNA (21,35). From Figure 5 it can be seen that following the onset of differentiation there is an initial increase followed by a rapid loss of hemimethylated CpG sites. It is possible that these hemimethylated sites are acted on by 5-MCDG, thereby generating genome-wide demethylation. In order to test this hypothesis, the level of endogenous 5-MCDG present in myoblasts was depleted with morpholino antisense oligonucleotides as described in Materials and Methods. The results in Figure 6 show that upon differentiation there is an ~3-fold increase in glycosylase activity. Transfection with control morpholino oligonucleotides does not alter the level of glycosylase activity (compare bars 2 and 3). An additional control carried out with the morpholino oligonucleotide of the unrelated sequence of p68 helicase had no effect on 5-MCDG activity (results not shown). However, differentiating myoblasts transfected with the G/T mismatch DNA glycosylase antisense morpholino

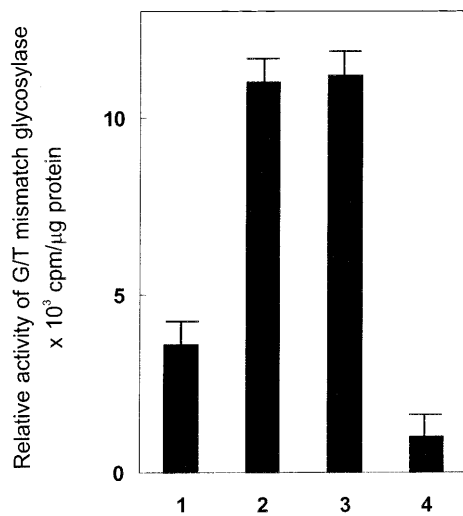


Figure 6. Activity of 5-MCDG in nuclear extracts of cells transfected with morpholino antisense oligonucleotides to 5-MCDG and control morpholino oligonucleotides. Nuclear extracts were prepared either from growing non-differentiating cells or from differentiating cells (post 24 h). Bar 1 represents the control growing, non-differentiating, untransfected cells and bar 2 the corresponding differentiating untransfected cells. Bar 3 represents cells transfected with morpholino control oligos and grown in differentiating medium. Bar 4 represents cells transfected with morpholino antisense oligonucleotides to 5-MCDG and grown in differentiating medium for 24 h.

oligonucleotide have a lower level of glycosylase activity (Fig. 6, bar 4) than cells transfected with control morpholino oligonucleotide (bar 3) and non-differentiating cells (bar 1). At the same time, the results in Figure 7 show that transfection of differentiating cells with antisense morpholino oligonucleotides produces a decrease in genome-wide demethylation (compare bar 3 with bars 1 and 2). The extent of inhibition of hypomethylation by antisense morpholino oligonucleotides varies between experiments and on average (six independent experiments) there was an ~80% decrease in hypomethylation. The random morpholino oligonucleotide sequence used as a control did not give any significant inhibition of hypomethylation when compared with a non-transfected control (Fig. 7, compare bars 1 and 2).

Since at the onset of myoblast differentiation there is an increase in hemimethylated CpG sites, one would expect that cells treated with G/T mismatch DNA glycosylase antisense oligonucleotides should present higher levels of hemimethylated DNA than the controls treated with random sequences. The inset of Figure 5 (bar 3) shows that at 24 h differentiation cells treated with the morpholino antisense oligonucleotides have more hemimethylated DNA than the corresponding non-treated controls (bar 1) or the controls treated with a random oligonucleotide sequence (bar 2). A value of 40 fmol [³H]SAM incorporated/μg DNA represents ~50 000 CpG sites per haploid genome. This is only ~10% of the total demethylated sites observed 24 h post-differentiation. The presence of these hemimethylated sites in cells treated with antisense oligonucleotides could possibly explain the results obtained in Figure 7 (bar 3) with the *SssI* methyl-accepting assay. In these

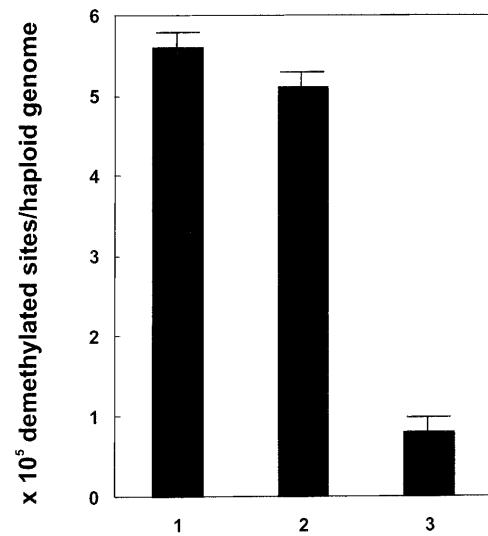


Figure 7. Effect of morpholino antisense oligonucleotides to 5-MCDG on genome-wide demethylation in differentiating myoblasts. Cells were grown and transfected with morpholino oligonucleotides as indicated in Materials and Methods. DNA was extracted 24 h following the onset of differentiation and assayed for demethylation by means of the *SssI* [³H]methyl-accepting assay. Bar 1, differentiating cells exposed to the transforming solution, no DNA; bar 2, differentiating cells transfected with morpholino control oligonucleotide; bar 3, differentiating cells transfected with morpholino antisense oligonucleotides. The state of methylation of DNA was tested with the *SssI* methyl-accepting assay as described in Materials and Methods. All tests were run in triplicate and the vertical bars represent the standard deviation of the mean.

experiments we always observed a residual [³H]methyl-accepting activity. Taken together, these results suggest that hemimethylated DNA is indeed a substrate for 5-MCDG. In addition, 5-MCDG seems to be capable of using symmetrically methylated DNA as a substrate.

DISCUSSION

A comparison of the present data with our previous work (9) shows a significant difference in the kinetics of DNA demethylation. This difference could possibly be explained by the use, in the present case, of one selected clone of G8 mouse myoblasts. This clone has a higher basal level of 5-MCDG than the original pool of cells from which it was derived. In addition, the *SssI* methyl-accepting assay presently used is far more sensitive and reliable than the previous assay based on the analysis of CCGG sites. A comparison of the results obtained by hybridisation with DNA microarrays and Figure 2 shows that genome-wide demethylation precedes the turning on of a large number of genes. Results obtained with myoblasts treated for 4 days with 5 μM 5-aza-C also suggest that many muscle-specific genes may be directly or indirectly regulated by DNA methylation (Fig. 1). Furthermore, for the most important genes involved in myogenesis, like myogenin, Scarpa *et al.* have clearly shown that demethylation is required for its activation (10,36). Demethylation of the myogenin gene is rapid and precedes myogenic differentiation. Using a different approach, Szyf *et al.* (37) showed that overexpression of DNA methyltransferase 1 cDNA in the antisense orientation in 10T½

cells triggered DNA hypomethylation and myogenesis. These cells were converted into multinucleated tubular cells and expressed skeletal myosin heavy chain protein. These results indicate that demethylation of specific genes is crucial for myogenic differentiation. Interestingly, the 5-MCDG (G/T mismatch glycosylase) mRNA expression level is reduced during differentiation and after 5-aza-C treatment. This contrasts with the results obtained by measuring the activity of 5-MCDG in nuclear extracts. In this case there is, at 24 h differentiation, a 2–3-fold increase in the specific activity of the enzyme (Fig. 6). An explanation for this could be covalent modification of the enzyme or of its complex with other proteins. For example, we found that nuclear extract incubated in the presence of the phosphatase inhibitor β -glycerophosphate had three times more 5-MCDG activity than controls incubated in the absence of the inhibitor (unpublished results). This result is consistent with possible phosphorylation of one of the components of the demethylation complex.

One of the first steps in genome-wide demethylation may be the formation of hemimethylated CpG sites. Based on their experiments with differentiating mouse erythroleukemia cells, Adams *et al.* (11) suggested that a delay in the methylation of newly synthesised DNA during the early phase of differentiation could generate hemimethylated sites and transient hypomethylation. A similar observation has been made for the early phase of differentiation of F9 embryonal carcinoma and Friend erythroleukemia cells; hemimethylated sites were also detected (13). Formation of hemimethylated DNA has also been documented for specific genes, such as α -actin transfected into L8 myoblasts (24) and for the avian vitellogenin gene *in vivo* (38). In this last example, upon estradiol treatment the promoter of the avian vitellogenin II gene becomes demethylated first on one strand, followed 24 h later by the second strand (38). A similar mechanism is possibly responsible for glucocorticoid-induced demethylation of the hepatic tyrosine aminotransferase gene (39). The results in Figure 5 are in agreement with the above observations since during the early phase of myoblast differentiation there is also an increase in hemimethylated DNA. The quick disappearance of these hemimethylated sites could constitute in part the overall demethylation shown in Figure 2. It is conceivable that 5-MCDG may be implicated in the removal of asymmetrical methylcytosine, since we have shown in *in vitro* experiments that this enzyme prefers hemimethylated DNA as substrate (21,33). The results in Figures 6 and 7 clearly show that antisense morpholino oligonucleotides to 5-MCDG (G/T mismatch DNA glycosylase), which lower the level of glycosylase activity, also partially inhibited genome-wide demethylation. A partial rather than total inhibition of hypomethylation is to be expected, since during the first few hours following incubation in serum-poor medium there is still formation of hemimethylated sites. As shown in Figure 5 (inset, bar 3), 24 h after the onset of differentiation cells treated with antisense oligonucleotides still had high levels of hemimethylated CpG sites when compared with the controls (bars 1 and 2). These hemimethylated sites could possibly serve as a substrate for the SssI [³H]methyl-accepting assay and explain the residual hypomethylation observed in cells treated with antisense 5-MCDG oligonucleotides (Fig. 7, bar 3). As already mentioned in Results, these hemimethylated sites represent only a fraction of all demethylated sites, implying that

symmetrically methylated DNA could also possibly serve as a substrate for the glycosylase *in vivo*. Preliminary experiments clearly indicate that in this system 5-MCDG is part of a complex of different proteins (B.Zhu, unpublished results). Transfection of the cells with control morpholino oligonucleotides had only a limited effect on glycosylase activity and genome-wide demethylation when compared with mock-transfected cells.

We have previously shown that 20-fold overexpression of 5-MCDG in human kidney 293 cells was sufficient to trigger demethylation of the promoter of a reporter gene without causing genome-wide demethylation (40). This is in apparent contradiction to the results obtained with differentiating myoblasts. In myoblasts genome-wide demethylation is initiated by placing the cells in serum-poor medium, leading to a lowering of DNA methyltransferase 1 activity (9) and the formation of hemimethylated DNA. As this presumably occurs at random sites throughout the genome, it generates hemimethylated sites that are acted upon by 5-MCDG to contribute to genome-wide hypomethylation.

On the other hand, in the former system the kidney cells were actively dividing in continuous culture in serum-rich medium. The absence of a trigger to generate hemimethylated DNA may explain the lack of extensive genome-wide demethylation despite the high levels of 5-MCDG in the kidney cells. In addition, in 293 kidney cells demethylation was specifically targeted to the reporter gene by formation of a complex between 5-MCDG and the retinoid X receptor (the promoter contained several tandem repeats of a retinoid X receptor-binding site). Thus demethylation of a specific site in this case could be achieved in the absence of overall genome-wide hypomethylation. What is the triggering mechanism(s) of DNA demethylation? Based on our past (9) and present results, it is conceivable that the presence of hemimethylated DNA in replicating cells where DNA methyltransferase 1 activity decreases very rapidly (onset of differentiation) could be one of the signals for the glycosylase to remove 5-methylcytosines from hemimethylated DNA. The DNA repair machinery could then replace it with cytosine. Whether genome-wide demethylation is a random or gene-specific process is unknown. *A priori* one cannot exclude the possibility that some other factors present in the demethylation complex may provide specificity of demethylation of a set of genes.

The mechanism of genome-wide demethylation described above is probably different but related to that responsible for the paternal genome demethylation observed in fertilised mouse oocytes (1,2). In oocytes very low levels of G/T mismatch DNA glycosylase (5-MCDG) mRNA were observed (41). In sharp contrast, in mixed spermatogenic germ cell nuclear extracts (42,43) and in mature human sperm cells (J.-P.Jost, unpublished results) highly efficient base excision repair activities are present. It is also noteworthy that demethylation of the paternal genome is rapid and takes place in the absence of replication. This suggests that in this particular case the demethylation of DNA occurs exclusively on symmetrically methylated CpG sites.

As we have seen in Figure 2, ~48 h following the onset of genome-wide demethylation there is remethylation of the genome. This *de novo* methylation of DNA could possibly be due to an isoform of Dnmt1 recently discovered in differentiated myotubes (44), or alternatively to Dnmt3a and 3b (45).

The *de novo* methylation occurring 2–4 days after the onset of differentiation could possibly be responsible, in part, for the silencing of genes that are down-regulated.

ACKNOWLEDGEMENTS

We would like to thank Mr P. Müller for synthesis of the oligonucleotides and Mrs I. Oberfoell for the photographic work. This work was sponsored by the Novartis Research Foundation, Basel, Switzerland.

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