Induction of Myogenic Differentiation by an Expression Vector Encoding the DNA Methyltransferase cDNA Sequence in the Antisense Orientation*

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To test the hypothesis that DNA methylation controls the state of differentiation of a mammalian cell, we transfected the stable mesenchymal line 10T1/2 with an expression vector encoding sequences from the DNA methyltransferase (DNA MeTase) cDNA in the antisense orientation. 10T1/2 cells transfected with the antisense construct (pZaM), but not with the vector alone, exhibit morphological changes, convert into multinucleated tubular cells, and express the skeletal myosin heavy chain protein. The conversion to myogenic phenotype is a late event and is dependent on the number of replication events that the cell has undergone, suggesting that induction of myogenesis is a multistep process. Demethylation of sequences that are not involved in the myogenic process is detected at early passages, while demethylation and expression of the MyoD gene is a late event. This report establishes for the first time that demethylation is a very early event in commitment to myogenic differentiation, while demethylation and expression of MyoD is a late event. We suggest that other genes serve as the initial targets for demethylation and commitment of mesenchymal cells to myogenesis. The cell lines described in this report can serve as an important system for identifying these genes.

One of the open questions in molecular biology is whether DNA methylation plays a critical role in regulating gene expression (1), differentiation (2), development, and induction of cellular transformation (3) as has been suggested by numerous experiments. In vertebrates, the cytosine moieties at a fraction of the CpG sequences is methylated (60–80%) at the 5' position, generating a pattern of methylation that is gene- and tissue-specific (4). Several lines of evidence support the hypothesis that DNA methylation plays a role in biological regulation, but none of them have resulted in conclusive evidence. First, the pattern of methylation is correlated with gene expression levels, but some DNA methylation patterns do not correlate with gene expression (5), and gene inactivation precedes DNA methylation in the process of X inactivation (6), globin switching (7), and silencing of genes in cultured cell lines (8). Second, in vitro methylation of certain genes will inhibit their expression when introduced into the cell by DNA-mediated gene transfer (9), but in vitro methyl-

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10T1/2 (24). This model was chosen because 10T1/2 is a stable mesenchymal cell line that exhibits a non-detectable rate of spontaneous reversion to other cellular phenotypes (14), but undergoes a well characterized pathway of differentiation when treated with 5-azaC or transfected with one of the myogenic master regulatory genes (18).

**MATERIALS AND METHODS**

DNA and RNA Analyses—Preparation of genomic DNA and total cellular RNA, purification of DNA fragments for cloning and labeling (using the random-primer labeling kit from Boehringer Mannheim), blotting DNA and RNA onto Hybond-N+ (Amersham), and all standard molecular biology manipulations were performed according to Ausubel et al. (38). MapI and HpaII restriction enzymes (Boehringer Mannheim) were added to DNA at a concentration of 2.5 units/μg for 8 h at 37°C. Radionucleotides were purchased from Amersham, and other DNA modification enzymes were purchased from Boehringer Mannheim or GIBCO-Bethesda Research Laboratories.

Plasmid Construction—pZEM is a eukaryotic expression vector that contains the metallothionein promoter and the human growth hormone 3' region and poly(A) tail (Uhler and McKnight (25)). A 0.6-kb EcoRI-BamHI fragment containing the most 5' sequences of the DNA MeTase cDNA (23) was subcloned, following modification of the EcoRI site into a BamHI recognition sequence, in the BglII site of pZEM in the reverse orientation to the direction of transcription of the metallothionein promoter (Fig. 1).

Cell Culture and DNA-mediated Gene Transfer—10T1/2 cells (24) were maintained as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum. All media and reagents for cell culture were obtained from GIBCO-BRL. 10T1/2 cells (1 × 10⁶) were plated on a 150-mm dish 15 h before transfection. pZaM (encoding antisense DNA MeTase) or pZEM (control vector) (10 μg) were introduced into the cells by DNA-mediated gene transfer using the calcium phosphate protocol and pUCSVneo (2 μg) as a selectable marker (38). Selection was initiated after 48 h by adding 0.2 mg/ml G418 (GIBCO-BRL) to the cells. G418-resistant cells were cloned in selective medium.

Immunostaining—Cells from each clone (1 × 10⁴) were plated in six well dishes in triplicates at five passages (early) or 11 passages (late) posttransfection and maintained in the appropriate medium for 14 days. The cells were fixed in methanol/acetic acid (v/v) and immunostained for myosin heavy chain with the anti-myosin monoclonal IgG (27), and the cells were counterstained with a hematoxylin solution (Sigma).

RESULTS

A 0.6-kb BamHI-EcoRI fragment from the 5' region of DNA MeTase cDNA (R6), which contains the ATG translation initiation codon (25), was subcloned in the mammalian expression vector pZEM (26) in the reverse orientation relative to the metallothionein promoter contained in this vector (see physical map in Fig. 1). The metallothionein promoter is a ubiquitous promoter (25). The DNA MeTase antisense expression construct (pZaM) was introduced into 10T1/2 cells by DNA-mediated gene transfer together with pUCSVneo as a selectable marker. As a control, we transfected 10T1/2 cells with the expression vector pZEM. G418-resistant colonies were isolated and propagated for both constructs. The pZaM constructs expressed the expected mRNA product as determined by Northern blot analysis and hybridization to the MT region probe. Positive clones harbored a 1.3-kb fragment hybridizing to the MT promoter region. These clones also expressed the expected ~1.3-kb chimeric mRNA as determined by a Northern blot analysis (B). Total RNA (5 μg) prepared from the two transfectant lines (4 and 5), nontransfected cells (no) and cells transfected with the vector alone (pZEM) was subjected to Northern blot analysis and hybridization with the 0.6-kb DNA MeTase cDNA probe (described in the physical map). Under these conditions the sense 5.2-kb mRNA, which is relatively nonabundant in 10T1/2 cells, is not detected.
of differentiation. The latter explanation is highly improbable because repeated transfections gave similar results and because spontaneous induction to myogenic cell types has not been previously reported, nor has it been observed in our laboratory, with other plasmid constructs. Thus, if the myogenic cells are a contaminant they should be diluted out, as myogenesis usually involves exit from the proliferative cycle (28). However, if myogenic differentiation is a multistep process, then the fraction of myogenic cells should increase in late passages of the transfectant cell line. To test this hypothesis, we passaged pZaM4 and pZaM5 for an additional nine passages and monitored the rate of myogenic transformation following nine passages of the pZaM transfectant. While few, mostly mononuclear, cells were stained with MF20 (Fig. 2, bottom left panel). The controls showed no trace of MHC reactivity. The late cultures also exhibited a remarkable qualitative change from the early cultures. As observed in Fig. 3, the DNA MeTase antisense transfectants were morphologically transformed into multinucleated syncitia exhibiting tubular as well as round morphologies. The multinuclear cells contain a vast number of nuclei and cover an appreciable amount of the surface of the tissue culture dish. Many of the tubular, as well as the round syncitia, express high levels of MHC as demonstrated by the intense staining with MF20 (Fig. 2, bottom left panel). One of the unusual characteristics of the DNA MeTase antisense transfectants is the fact that the myogenic transformation of the cells occurred in the presence of 10% fetal calf serum which is different from what has been known for 5-azaC-induced myoblasts, C2 myogenic cell lines, and MyoD transformed 10T1/2 (14, 28). One of the characteristics of the antisense transfectants is their very high level of proliferative activity which is probably essential for the generation of the huge syncitia with a remarkable high density of nuclei (Fig. 3, bottom panel, compare with a similar size area in the control panel and Fig. 2, bottom left panel).

To further exclude the possibility that the myogenic cells are a rare contaminant, we subcloned pZaM4 and -5 cells from an early passage. The myogenic properties of the resulting clones were evaluated by morphological examination and staining with the monoclonal antibody MF20. 50% of the subclones from each of our transfectants contained cells that stained positive with MF20 (Table I). The frequency of myogenic transformation was variable in the different subclones ranging between a few cells per well up to 20% of the population. The distribution of frequencies of myogenic conversion in different subclones (Table I) suggests that myogenic differentiation of 10T1/2 cells is a multistep process. This experiment demonstrates that expression of a DNA MeTase antisense construct commits mesenchymal cells to myogenic differentiation in a multistep process.

MyoD has been shown to be one of the primary loci induced in 5-azaC-treated 10T1/2 cells and is suggested to be a nodal point during specification of the muscle cell lineage (18). To determine whether the induction of myogenesis in the pZaM-transfected cells involved similar genetic switches, we hybridized RNA that was purified from the different transfectants with a cDNA probe for MyoD (14). MyoD expression is detected only in late passage pZaM transfecteds (Fig. 4B, b and c), where an appreciable percentage of the cells exhibit a myogenic phenotype (Figs. 2 and 3). This suggests that the events involved in induction of myogenesis in cells that express antisense DNA MeTase message are similar to those involved in pharmacological inhibition of DNA MeTase using 5-azaC. This experiment also suggests that expression of MyoD is a late event in myogenic differentiation.

While it is not clear whether demethylation of MyoD is the prime target of 5-azaC induction of myogenesis (14), it has been demonstrated that the CpG island contained in the first exon of the MyoD gene is methylated in nonmyogenic cell lines and is partially demethylated in 5-azaC-treated cells (29). DNA purified from control pZEM transfecteds and pZaM transfecteds from early (fifth passage) and late subcultures (ninth and eleventh passages), was digested with HpaII or MspI and analyzed by Southern blot analysis for methylation of MyoD using the complete cDNA (29) as a probe (Fig. 4A). The MspI digest of 10T1/2 DNA shows three major bands: 1.06 kb resulting from cleavage of MspI sites 9 and downstream in the 3' region of the gene; 0.56 kb resulting from MspI sites 8 and 9 in the second and third exons.
are Dercent of tissue culture dish surface covered with Dositivelv staining cells.

grew per 10-cm culture dish. Clones were isolated, propagated in six-well dishes for 14 days, and immunostained for myosin heavy chain with probe encoding mouse MyoD (29). The arrow indicates the position was subjected to digestion with posttransfection and 7, flanking the CG island in the MyoD gene (29).

of the 0.76-kb band resulting from demethylation of CCGG sites 6 and 7 are methylated in 10T1/2 cells and many other nonmyogenic cell lines but not in the myogenic C2 and azamyoblast cell lines (29). Similarly, 10T1/2 pZEM2 and pZEM3 DNA are methylated at these sites as implied by the absence of the 0.79-kb fragment indicating cleavage of site 6 and 7 flanking the CpG island contained within the first exon of the gene (for physical map of sites see Ref. 29). Sites 6 and 7 are methylated in 10T1/2 cells and many other nonmyogenic cell lines but not in the myogenic C2 and azamyoblast cell lines (29). Similarly, 10T1/2 pZEM2 and pZEM3 DNA are methylated at these sites as implied by the absence of the 0.79-kb fragment (indicated by the arrow in Fig. 4A) in HpaII digests of the DNAs and its replacement by a 0.88-kb fragment. The presence of the 1.0- and 0.56-kb fragments indicates that sites in the 3' of the gene are not methylated (29). Transfectants expressing antisense to the DNA MeTase are also methylated at the CCGG sites flanking the CpG island at early passages following transfection (Fig. 4Aa), correlating with the low frequency of myogenic transformation at this stage. Late passage pZaM transfectants show partial demethylation of the CCGG sites flanking the first exon of the MyoD gene (Fig. 4Ac), as indicated by the appearance of the 0.79-kb fragment, correlating with the partial transformation of these cells to myoblasts (Fig. 2). This analysis demonstrates that the demethylation events induced by the antisense DNA MeTase message are qualitatively similar to those induced by 5-azaC and that demethylation of MyoD is a late event in myogenic induction in our system that correlates with the onset of expression of the MyoD gene.

The demethylation of MyoD might be an effect of its expression. To test whether expression of antisense DNA MeTase message results in changes in the methylation pattern of other areas in the genome that are not involved in myogenesis, we tested the state of methylation of the 21-hydroxylase gene (C21) using a similar MspI/HpaII analysis. DNA was digested with BamHI and then with MspI or HpaII, Southern blotted, and hybridized with a 3.8-kb genomic fragment containing most of the coding sequences of the gene as a probe (Fig. 5). This gene is specifically expressed in the adrenal and is heavily methylated in nonadrenal tissues and cell lines (8). The gene is heavily methylated in 10T1/2 cells as indicated by the absence of the 1.0- and 0.8-kb fragments which are present in the MspI digests in the HpaII cleavage. This gene undergoes partial demethylation in the pZaM transfectants as indicated by the diminution of the intensity of the 3.8-kb fragment (indicating the fully methylated state) and the appearance of new HpaII fragments resulting from additional hypomethylation events as indicated by the dark arrows. This assay demonstrates that expression of an antisense DNA MeTase message results in partial inhibition of methylation in other areas of the genome irrespective of their transcription status.

Two alternative models might explain the late onset of myogenic induction in mesenchymal cells transfected with the DNA MeTase antisense construct. (a) as the proposed mechanism of hypomethylation by antisense DNA MeTase message is passive inhibition of methylation during replication, this mechanism of hypomethylation will result in a gradual increase in the fraction of hypomethylated DNA at each round of replication. Thus, if the extent of myogenic differentiation is a function of the fraction of cells that are

| Clone | 0 cells | <100 cells | 1-5% | 5-15% | 15-25% | Total |
|-------|---------|------------|------|-------|--------|-------|
| pZaM 4 | 22      | 13         | 4    | 6     | 1      | 40    |
| pZaM 5 | 19      | 15         | 1    | 4     | 3      | 48    |

Fig. 4. Demethylation and expression of MyoD in 10T1/2 cells harboring the DNA MeTase antisense plasmid. Transfected 10T1/2 clones (as indicated) were cultured for different times posttransfection (6 passages (a), 9 passages (b), and 11 passages (c)). A, DNA (10 μg) extracted from the cell at the indicated time intervals was subjected to digestion with MspI (M) (MspI digestion is identical for all clones tested, therefore, only one MspI digestion lane is shown) or HpaII (H), Southern blot transfer and hybridization with a cDNA probe encoding mouse MyoD (29). The arrow indicates the position of the 0.76-kb band resulting from demethylation of CCGG sites 6 and 7, flanking the CG island in the MyoD gene (29).

B, cellular RNA (10 μg) was extracted from the cells at the indicated time intervals and subjected to Northern blot analysis using the MyoD cDNA probe. The arrow indicates the expected position of MyoD.

Fig. 5. Demethylation of the C21 gene in 10T1/2 pZaM transfectants. A, DNA (10 μg) was extracted from 10T1/2 pZEM or pZaM transfectants and subjected to digestion with MspI (M) or HpaII (H), and then with BamHI, Southern blot analysis, and hybridization with a mouse probe for the C21 gene (3.8-kb BamHI fragment (8)). The 3.8-kb fragment represents genes that were methylated at all HpaII sites contained in the 3.8-kb BamHI fragment (shaded arrow). Fragments resulting from hypomethylation at the pZaM transfectants are indicated by dark arrows. The level of demethylation of the C21 gene at different passages (8, 13, and 14) was determined by subjecting DNA extracted from pZaM 4 and 5 clones to BamHI and HpaII digestion (B).

Expression of DNA MeTase Antisense
hypothesized that the fraction of myogenic cells should increase with every passage of the transfectant cell line. If this model is correct, then a gradual increase in MHC-expressing cells should be observed with every passage of the transfectant cell line. (b) alternatively, the commitment of 10T1/2 cells to myogenic differentiation is triggered immediately after the initial demethylation, but expression of the full myogenic phenotype requires an ordered sequence of events which is dependent on a series of replication cycles. According to this model the kinetics of transformation to a myogenic phenotype will exhibit an abrupt transition from nondifferentiated to differentiated phenotype at a defined time after transfection. To address this question we have monitored the rate of myogenic conversion of two independent pZaM-transfected clones at different passages posttransfection by immunostaining with the MF20 aMHC antibody. The results of such an analysis demonstrate that the rate of myogenic conversion of the transfectant cell lines is negligible for almost nine passages posttransfection, while a dramatic increase occurs between 10 and 11 passages posttransfection (Fig. 6A). This supports the second model that demethylation triggers a replication-dependent sequence of steps resulting in full myogenic differentiation. The kinetics of demethylation of the C21 gene is also consistent with this model because the pattern of hypomethylation of the C21 gene is similar for early and late passages (Fig. 5B). This suggests that most of the general demethylation occurred at early stages after transfection and that the pattern of methylation was then stabilized.

DISCUSSION

In this report, we demonstrate that partial inhibition of DNA methylation by DNA-mediated gene transfer of a DNA MeTase antisense vector results in destabilization of the phenotypic identity of a stable mesenchymal cell line. We would like to suggest that general demethylation occurred early after transfection as demonstrated by the demethylation of the C21 gene, which is not involved in myogenesis (Fig. 5). The methylation pattern was then most probably stabilized by cellular regulatory mechanisms of DNA MeTase gene expression (31), as suggested by the stable pattern of methylation of the C21 gene in pZaM transfectants (Fig. 5B). This initial demethylation triggered a sequence of events leading to differentiation of the cells into other cell types, the most identifiable form being myogenic transformation. The transfectants described in this report provide us with a model to study the biological implications of deregulation of DNA MeTase gene expression. We have previously suggested that regulation of DNA methylation activity with the rate of proliferation is critical for maintaining the phenotypic identity of a cell (30-32). The experiments described in this report support this hypothesis. These cells also provide us with a model to study the molecular events involved in cellular differentiation.

How does general inhibition of DNA methylation cause cells to embark on a well defined pathway to differentiation? One model is that demethylation of MyoD and the resulting induction of its expression is the primary event that triggers myogenic differentiation of mesenchymal cells (14). This model is supported by the fact that transfection of 10T1/2 cells with MyoD induces rapid myogenic conversion of 10T1/2 cells (14), that specific demethylation of MyoD is observed in myogenic cells (29), and that methylation of the MyoD promoter inhibits its expression in 10T1/2 cells (33). Our results suggest that commitment to myogenic differentiation is induced by demethylation events upstream of demethylation and expression of MyoD (Figs. 4 and 6). Our results will also exclude myogenin (34), as well as other previously characterized inducers of myogenesis as the prime target for demethylation-induced myogenesis (35), because these genes are immediate inducers of myogenesis and do not exhibit the cell-passage-dependent pattern of induction observed with our transfectants. We would like to suggest that the assay utilized for identifying these genes which was based on direct transformation of mesenchymal cells into myogenic cells inevitably selected for downstream regulators of myogenesis. Our approach clearly suggests that upstream regulators must exist, moreover the DNA MeTase antisense transfectants provide us with an excellent system to identify these upstream regulator(s) utilizing the cell-passage-dependent myogenesis assay described in this paper. It is clear that myogenic differentiation requires the removal of inhibitory signals which are regulated by serum factors such as the MyoD inhibitor Id in addition to expression of MyoD (18). Some of these additional events might also be controlled by DNA methylation. The fact that the pZaM transfectants exhibit myogenic differentiation independent of the absence of serum factors suggests that deregulation of methylation activity results in a more comprehensive induction of the differentiation program than the one observed in azamycoblasts or MyoD-transfected 10T1/2 cells (14, 18).

This paper provides us with the first direct evidence that:

![Fig. 6. Kinetics of induction of MHC expression in 10T1/2 cells transfected with DNA MeTase antisense constructs.](image-url)
(a) DNA methylation regulates the state of differentiation of a mammalian cell and (b) limited deregulation of the general DNA methylation machinery is sufficient to alter the differentiation profile of a cell in a specific manner. One interesting question is whether mammalian cells utilize a similar mechanism in vivo to induce differentiation. We have previously shown that differentiation of cells involves a genome-wide hypomethylation (36, 37). Our results provide evidence that specific induction of differentiation might indeed result in a general inhibition of methylation.

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