Communication

Growth Regulation of Mouse DNA Methyltransferase Gene Expression*

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The steady state level of DNA methyltransferase mRNA is markedly increased as growth-arrested Balb/c 3T3 cells progress into the S phase of the cell cycle. mRNA abundance is reduced to the basal level before termination of DNA synthesis activity. Maintenance DNA methylation activity in nuclear extracts follows a similar pattern with two exceptions. (a) A small peak of DNA methylation activity is detected in early G1 phase. (b) The extinction of DNA methylation activity lags behind the termination of DNA synthesis. Nuclear runon experiments demonstrate that the gene is transcribed in growth-arrested cells, and expression of the gene is post-transcriptionally regulated. We suggest that this mode of regulation of the DNA methyltransferase gene might play an important role in determining and maintaining DNA methylation patterns.

Although most CpG dinucleotide sequences in vertebrate genomes are methylated at the 5-position of cytosine, a minor fraction remains hypomethylated (1). These nonmethylated sequences are nonrandomly distributed and constitute a pattern of methylation which is gene- and tissue-specific (2). While numerous experiments have demonstrated that specific DNA methylation patterns could be correlated with patterns of gene expression (2) and more recent experiments have shown that DNA methylation might interfere with binding of transacting factors to promoter elements (3), the mechanisms responsible for generating specific methylation patterns are still obscure.

DNA methylation results from interaction between the DNA methyltransferase and its DNA substrate (4); however, about 20% of the CpG sites escape DNA methylation (1). The cDNA encoding for the mouse DNA methyltransferase has been cloned by Bestor et al. (4), but nothing is yet known about the regulation of its expression.

Based on results obtained from the Escherichia coli dam methylase system, we have suggested that maintaining a limiting methylation capacity in the cell coupled with differential affinities of the DNA methyltransferase protein for different sites might play an important role in determining the final methylation pattern (1, 5). Recent evidence indeed suggests that the level of dam methylase in E. coli plays an important role in maintaining the origin of replication in a hemimethylated state and regulating DNA replication (6, 7). A similar mechanism may play a role in shaping methylation patterns in vertebrate cells. If the level of DNA methyltransferase activity plays such a role, it should be regulated with respect to DNA synthesis activity. In accordance with this hypothesis, we and others have previously shown that DNA methylation activity correlates with the proliferative activity of cells (8-10). In this report, we test the hypothesis that regulation of DNA methyltransferase gene expression is associated with the proliferative state of the cell and determine the level at which it is regulated using the mouse fibroblast Balb/c 3T3 cell as a growth-induced cell system.

RESULTS

Balb/c 3T3 cells are a nontransformed murine embryonal mesenchymal line, isolated by Todaro and Green (11), which can be maintained in a state in which they exhibit stringent density-dependent growth. The ability to obtain large numbers of Balb/c 3T3 cells synchronized at specific portions of the cell cycle have made these cells valuable models for analysis of cellular and molecular events that regulate the progression of the cell cycle (12). The following protocol was used for growth-arresting the cells. Balb/c 3T3 cells were grown to confluency in DMEM containing 10% fetal calf serum, and the medium was then replaced with DMEM containing 0.5% serum for an additional 2 days. To induce the cells into the cell cycle, we replaced the medium with 10% serum containing DMEM.

The steady state levels of the DNA methyltransferase mRNA were determined using Northern blot assays of RNA purified from growth-induced cells (13, 14) at different time intervals following growth stimulation and hybridized to a methyltransferase cDNA probe (4) (Fig. 1A). To determine the amount of total RNA in the different samples, the filter was stripped of radioactivity by boiling in 0.2 × SSC, 1% SDS buffer and hybridized to an 18 S RNA-specific 32P-labeled oligonucleotide (15) using standard hybridization conditions (13) (Fig. 1B). As seen in Fig. 1A, the ~5-kb DNA methyltransferase mRNA (4) is absent in growth-arrested cells and is induced at 8 h, just before the initiation of the DNA synthetic phase of the cycle. An additional band hybridized to our methyltransferase probe and was increased in its intensity as the cells progressed through the cycle. To determine the relative level of DNA methyltransferase mRNA at different phases of the cycle, serial dilutions of total RNA (10–500 ng) were dot-blotted onto nylon filters, and the filter-bound RNA was hybridized sequentially to a methyltransferase and an 18 S probe. The autoradiograms were scanned, and the signal obtained at each point was normalized to the amount of 18 S RNA in each sample (Fig. 1C). DNA synthesis activity

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The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; SDS, sodium dodecyl sulfate; kb, kilobase pair(s); PMSF, phenylmethylsulfonyl fluoride; HEPEs, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenbis(oxyethylene)tetraacetate].

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Regulation of DNA Methyltransferase Gene Expression

Fig. 1. Induction of DNA methyltransferase mRNA levels following growth stimulation by serum. A, RNA from 3 x 10^6 serum-starved Balb/c 3T3 cells and from cells induced with 10% fetal calf serum at different time points postinduction were subjected to Northern blot hybridization analysis using a 32P-labeled 0.6-kb BamHI fragment encoding the most 5' sequences of the DNA methyltransferase cDNA (4). The second arrow indicates the position of the ~5-kb DNA methyltransferase mRNA. B, the filter-bound RNA was rehybridized with an 18 S RNA-specific 32P-labeled oligonucleotide (15). The arrow indicates the position of 18 S RNA. C, relative level of DNA methyltransferase mRNA at different time points following serum induction determined by slot blot hybridization analysis using a 32P-labeled methyltransferase cDNA probe. The signal obtained at each point was normalized to the amount of total RNA at each sample determined by hybridization to a 18 S RNA-specific probe. Each value is presented as a mean of three determinations ± S.D. D, DNA synthesis in serum-starved and serum-induced Balb/c 3T3 cells at different time points following induction was assayed by measuring the incorporation of 1 μCi of [3H]thymidine into aliquots of cells which were withdrawn from the bulk cultures at the indicated time points and were incubated for an additional 2 h at 37 °C. Each value is presented as means of six determinations ± S.D.

at each time point was determined by measuring incorporation of 1 μCi of [3H]thymidine into DNA, measured as trichloroacetic acid-precipitable counts, in aliquots (0.5 ml) of cells withdrawn from the bulk culture and incubated for an additional 2 h at 37 °C (Fig. 1D). As observed in Fig. 1 the level of DNA methyltransferase mRNA is positively induced 3 h prior to initiation of DNA synthesis and returns to almost basal levels before the cessation of DNA synthesis. This demonstrates that the steady state level of methylase mRNA is tightly regulated with the proliferative state of the cell.

Inasmuch as we have previously demonstrated that DNA methyltransferase activity follows a time course which is concordant with DNA synthesis in rat-regenerating liver and in concanavalin A-induced splenocytes (8), we wondered whether the reduction or methylation mRNA at a time when DNA synthesis was still at its peak reflected a different mechanism operating in these cells or whether DNA methylation activity exhibited a different time course than that observed with its mRNA. We determined maintenance DNA methyltransferase activity in nuclear extracts of growth-starved Balb/c 3T3 cells at different time points following growth induction by serum. Nuclei were isolated from 1-3 x 10^6 cells (8), and crude nuclear extracts were prepared by 0.3 m NaCl extraction (8). One μg of nuclear extract was assayed for DNA methyltransferase activity immediately in a 50-μl reaction using 0.1 μg of a synthetic 33-base pair hemimethylated oligonucleotide (Fig. 2B) as substrate and 1 μCi of S-[methyl-3H]adenosyl-l-methionine (78.9 Ci/mmol) as the ethyl donor in a 20 mM Tris-HCl (pH 7.4), 10 mM EDTA, 25% glycerol, 0.2 mM PMSF, and 20 mM β-mercaptoethanol buffer. The results of a representative experiment illustrated in Fig. 2 demonstrate that maintenance DNA methylation activity in Balb/c 3T3 cells is regulated with DNA synthesis activity with two exceptions. (a) A small peak of DNA methylation activity is detected at early G1 phase. This implies that other mechanisms in addition to message abundance regulate DNA methylation activity in the cell. (b) DNA methylation activity remains elevated following completion of DNA synthesis. While DNA methylation activity is present throughout the DNA synthesis phase as has been shown before (8), the ratio of methylation activity to DNA synthesis activity changes in the late phases of DNA synthesis.

Different classes of genes are induced at different stages of progression through the cell cycle (16), and both transcriptional and post-transcriptional levels of regulation of these genes have been documented (17-21). The mechanisms responsible for the coordinated regulation of S phase-specific genes are still unknown. As the DNA methyltransferase gene might be a good model for studying these mechanisms, it was important to determine at the level at which the expression of this gene is regulated. To determine whether the expression of the methyltransferase gene is determined at the transcriptional level, we resorted to nuclear runon assays. Nuclei were prepared from growth-arrested and serum-induced Balb/c 3T3 cells at different time points after induction. Nuclei were prepared from 3 x 10^6 cells at each time point by resuspension of the cell pellet in a 0.1% Nonidet P-40, 0.3 m sucrose, 60
The nuclei were further purified by washing in 1 ml of buffer containing 50% glycerol, 20 mM Tris-HCl (pH 7.9), 50 mM KCl, 15 mM NaCl, 4 mM MgCl₂, 4 mM MnCl₂, 50 mM NaCl, 0.4 mM EDTA, 0.5 mM PMSF, 10 mM creatine phosphate, and 20 units of RNasin buffer and centrifugation at 14,000 × g for 2 min. The nuclei were resuspended in 1 ml of the same buffer and stored at -70 °C. For a nuclear transcription assay, 35 μl of nuclear suspension for each time point was incubated with 150 μCi of [α-32P]UTP (800 Ci/mM) in a buffer containing 1 mM each of GTP, CTP, and ATP, 0.3 M (NH₄)₂SO₄, 100 mM Tris-HCl (pH 7.9), 4 mM MgCl₂, 4 mM MnCl₂, 50 mM NaCl, 0.4 mM EDTA, 0.5 mM PMSF, 10 mM creatine phosphate, and 20 units of RNasin for 30 min at 28 °C. The labeled RNA was purified with a NAP-5 column (Pharmacia LKB Biotechnology Inc.) following DNase I (100 µg/ml) and proteinase K (100 mg/ml) digestion. For hybridization of RNA to the various probes, 10 μg of pMET (a pGEM-3 plasmid containing a 2.7-kb genomic fragment encoding the 5' region of the DNA methyltransferase gene), pGEM-3, or pSP6-γ-actin (a plasmid containing the cDNA for the human γ-actin gene (23)) were immobilized onto a Hybond-N+ nylon filter on a slot blot apparatus using alkaline conditions. The filters were prehybridized by incubation at 42 °C for 16 h in a solution containing 50 mM HEPES (pH 7), 0.75 M NaCl, 50% formamide, 0.5% SDS, 2 mM EDTA, 10 × Denhardt's solution (22), 200 µg/ml herring sperm DNA, and 10 µg/ml poly(rA) oligoribonucleotide. One × 10⁶ dpm of 32P-labeled RNA transcribed at each time point was added to the prehybridization buffer and hybridized with the immobilized plasmids for 72 h at 42 °C. The filters were washed with 2 × SSC, 1% SDS twice at room temperature and then three times with 0.2 × SSC, 1% SDS at 55 °C. Autoradiography (Fig. 3A) was followed by densitometric analysis of the relative intensity ± S.D. (n = 3) (Fig. 3C). The abundance of the methyltransferase mRNA in the cytosol at each time point following serum induction was determined as described above (Fig. 3, B and C). This experiment demonstrates that while the abundance of the methyltransferase message is markedly induced before the onset of the S phase of the cycle (at 8–13 h postinduction, Fig. 3, B and C), the methyltransferase gene is transcribed in resting cell nuclei and does not follow a similar change in transcription activity. This suggests that post-transcriptional regulation is the major determinant of methyltransferase mRNA abundance in the cytosol.

**DISCUSSION**

Our paper defines some basic properties of the regulation of DNA methyltransferase gene expression. First, the cell responds to a mitogenic stimulus by adjusting the level of mRNA encoding the DNA methyltransferase. The message levels are induced about 3 h before the onset of DNA synthesis. Second, the induction of mRNA levels is followed by elevation of maintenance methylase activity. This strongly suggests that DNA methyltransferase activity at the onset of the DNA synthesis is determined by the abundance of its mRNA. Third, DNA methyltransferase mRNA is significantly reduced as the methyltransferase activity reaches its peak, suggesting that the methyltransferase is down-regulated by factors which are present in the later phases of DNA synthesis. One possible mode of regulation might involve feedback regulation by the level of methyltransferase activity. This mode of regulation suggests that it is important to prevent excess synthesis of methyltransferase as is the case in resting cells. Fourth, other modes of regulation of DNA methyltransferase activity must exist as the short peak of methyltransferase activity observed early after serum induction (Fig. 2A) is not preceded by an induction of mRNA (Figs. 1 and 3). Fifth, the level of DNA methyltransferase mRNA is post-transcriptionally determined as the gene is transcribed in resting cells and does not exhibit a significant change in transcription following stimulation of DNA synthesis. Post-transcriptional regulation has been shown to be an important mode of regulating other genes involved in the DNA synthesis phase such as thymidine kinase and histone genes (17–19, 24), as well as in controlling the expression of early G₁ genes such as fos and myc (25, 26). However, the DNA methyltransferase gene seems to differ from these other cell cycle-regulated genes inasmuch as it does not seem to be transcriptionally regulated. Our observations stress the general significance and role of post-transcriptional regulation. As implied by our
results, a factor must exist that regulates either the natura-
lation or stability of the DNA methyltransferase mRNA and is
regulated with the proliferative state of the cell. This factor
might play a more general role in regulation of gene expression
during the cell cycle. The DNA methyltransferase gene pro-
vides a good model for analyzing the mechanisms involved in
regulating message stability with the proliferative state of the
cell. One interesting question that remains to be answered is
whether cycling cells regulate DNA methyltransferase gene
expression with the S phase of the cell cycle as well. The
changes in expression of the methyltransferase gene following
growth stimulation of quiescent cells may not be a cell cycle
effect but rather be related to other signals that determine
the proliferative state of the cell.

What is the biological role of cell cycle regulation of the
methyltransferase gene? DNA methylation patterns have
been shown to be of importance in regulating the transcription
of genes and maintaining the status of gene expression of
somatic cells while differentiation has been associated with
changes in the DNA methylation pattern (27-30). Adjusting
the abundance of enzyme and the availability of the substrate
might play an important role in the maintenance of patterns
of methylation. A controlled or aberrant switch in the regu-
lation of DNA methyltransferase activity could also play a
role in altering the pattern of methylation. Although many
observations stress the limited and site-specific nature of
DNA methylation, genome wide changes in methylation occur
during differentiation (27-29). Moreover, some changes in DNA
methylation patterns involve wide areas of the genome:
methylation of CpG islands in tumor cell lines (31) or the
hypermethylation of late replicating regions of the genome
(32). Cancer cells have been shown to contain higher levels of
DNA methyltransferase activity than nontumor cells (33),
and senescent cells in culture have been shown to undergo
genome wide hypomethylation (34). We have previously sug-
gested that general changes in DNA methyltransferase activ-
ity might also result in site-specific changes because of differ-
ences in the affinity of different sites to methylation activity
reflecting higher organization of the DNA substrate (5, 35,
36). The higher ratio of DNA methyltransferase to DNA
synthesis activity late in the S phase might play a role in the
hypermethylation of late replicating DNA sequences. The
abundance of DNA methyltransferase activity in tumor cell
lines might reflect loss of cell cycle regulation in cancer cells
as has been observed with other DNA synthesis genes (37).

Regulation of enzymatic activity with the cell cycle suggests
in many cases involvement in controlling some aspects of cell
cycle progression (16). One exciting possibility is that regu-
lation of DNA methyltransferase activity can play a role in
controlling DNA replication. Recent evidence suggests that
initiation of DNA replication in E. coli is controlled by the
state of methylation of dam sites at the origin of replication
(6, 7). We have previously shown that the level of dam
methyltransferase in E. coli is limiting and suggested that this
might play an important role in maintaining a specific status
of methylation (5). Recent evidence has demonstrated that
altering the level of methylase in E. coli will deregulate DNA
replication (6). The regulation of DNA methyltransferase in mamma-
lian cells is consistent with this attractive model.

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