DNA Methyltransferase Is a Downstream Effector of Cellular Transformation Triggered by Simian Virus 40 Large T Antigen*

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This paper tests the hypothesis that DNA methyltransferase plays a causal role in cellular transformation induced by SV40 T antigen. We show that T antigen expression results in elevation of DNA methyltransferase (McTase) mRNA, DNA McTase protein levels, and global genomic DNA methylation. A T antigen mutant that has lost the ability to bind pRb does not induce DNA McTase. This up-regulation of DNA McTase by T antigen occurs mainly at the posttranscriptional level by altering mRNA stability. Inhibition of DNA McTase by antisense oligonucleotide inhibitors results in inhibition of induction of cellular transformation by T antigen as determined by a transient transfection and soft agar assay. These results suggest that elevation of DNA McTase is an essential component of the oncogenic program induced by T antigen.

In mammalian cells 60–80% of CpG dinucleotides are methylated, forming a pattern that correlates with gene expression (1, 2). The DNA 5-cytosine methyltransferase (DNA MeTase) is the enzyme responsible for the establishment of this pattern (3). Several reports have demonstrated that cancer cells bear increased levels of DNA McTase (4), that increased DNA MeTase is an early event in tumor progression in an animal model of lung carcinogenesis (5), and that regional hypermethylation is characteristic of tumor cells (6, 7). However, the extent to which DNA McTase activity is elevated in cancer cells in vivo is still debatable (8).

A basic question is whether an increase in DNA McTase activity is a critical downstream component of oncogenic pathways (9) or whether it is an aberrant consequence of the transformed state? One molecular link between DNA McTase and an oncogenic pathway is the observation that its expression in mouse cells is up-regulated at the transcriptional level by alteration of mRNA stability. Inhibition of DNA McTase by antisense oligonucleotide inhibitors results in inhibition of induction of cellular transformation by T antigen as determined by a transient transfection and soft agar assay. These results suggest that elevation of DNA McTase is an essential component of the oncogenic program induced by T antigen.

If increased DNA MeTase is a necessary constituent of cellular transformation, it should be induced by diverse oncogenic pathways. SV40 T antigen is one of the most studied viral oncoproteins that can induce frequent tumors when expressed as a transgene in mice (16), can immortalize primary cell lines (17) or transform immortalized cells (18), but transforms primary cells only when expressed in conjunction with Ras or other components of its signaling pathway (19). Thus, T antigen induces a very effective transformation pathway that is complementary to, but different from, the one induced by Ras. Extensive studies have established that T antigen transformation is a consequence of its ability to physically interact with the tumor suppressors pRb (20) and p53 (21) as well as yet noncharacterized functions. A recent observation has shown that two human SV40-transformed lines express higher levels of DNA McTase protein than their nontransformed counterparts (22). Is it possible that similar to the Ras signaling pathway, the T antigen-tumor suppressor pathway utilizes DNA McTase as a downstream effector? This paper tests this hypothesis by determining whether expression of T antigen increases the levels of DNA McTase mRNA and protein in the cell, defining the level of gene expression regulation at which T antigen acts, and testing whether DNA McTase plays a causal role in T antigen triggered cellular transformation?

EXPERIMENTAL PROCEDURES

Cell Culture and DNA-mediated Gene Transfer—Balb/c 3T3 cells (ATCC) were maintained as monolayers in Dulbecco's modified Eagle's medium which was supplemented with 10% heat-inactivated fetal calf serum (Immucor, Montreal). All other media and reagents for cell culture were obtained from Life Technologies, Inc. Cells (1 × 10^6) were plated on a 150-mm dish (Nunc) 15 h before transfection. The pZIP U19 (to A55/Neo T antigen expressing vector (20)) (this plasmid expresses high levels of T antigen and is highly transforming in our experience at 37 °C, it was not temperature-sensitive in our experience) was introduced into Balb/c 3T3 cells with 1 μg of pUCSNeo as a selectable marker by DNA mediated gene transfer using the calcium phosphate protocol. Selection was initiated 48 h after transfection by adding 1 ng/ml G418 (Life Technologies, Inc.) to the medium. G418-resistant cells were cloned in selective medium.

Tumorigenicity Assay—For analysis of growth in soft agar, 3 × 10^3 cells were seeded in triplicate onto a six well dish (Falcon) in 4 ml of complete medium containing 0.33% agar solution at 37 °C (24). Cells were fed with 2 ml of medium every 2 days. Growth was scored as colonies containing >10 cells, 21 days after plating.

Oligodeoxynucleotide Treatment and Transient Transfection—Cells were plated on tissue culture grade 100-mm dishes at a density of 1 × 10^6/plate 24 h before treatment. Each treatment was performed in triplicate. The phosphorothioate oligodeoxynucleotides used in this study are HYB101584, which is antisense to a sequence encoding the second putative translation initiation site of DNA McTase (5'-TTT ATT TGA GTC TGC CAT TT-3') and the reverse sequence HYB101585 (5'-TTT ACC GTC TGA GTC CTT-3') as described in Ref. 14. To determine whether DNA McTase antisense oligonucleotides inhibit transformation initiated by transient expression of T antigen, Balb/c 3T3 cells were plated at a density of 2.5 × 10^4/well in a six-well plate 24 h before initiation of oligonucleotide treatment. Oligonucleotides were mixed with 31 μl of Lipofectin (Life Technologies, Inc.) and 4 ml of
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Opti-MEM (Life Technologies, Inc.) were added to the mix. The cells were incubated in the Opti-MEM/oligonucleotide mix for 4 h, following which the mix was replaced with regular growth medium. The cells were treated with 100 nM amounts of either HYB101584, HYB101585, or with Lipofectin carrier alone. 48 h after plating, 2 μg of either pZip U19 (ts A58)Tneo (T ant) or pZip RH8s corresponding to 18S RNA were denatured in DNA MeTase transfection mix with oligonucleotides. The oligonucleotide treatment was then repeated a third time 72 h after plating with oligonucleotides but without plasmids. The cells were then harvested following the third treatment and counted. The viability was determined by trypan blue dye exclusion, and the cells were plated onto soft agar as described above. T antigen RNase protection and methyltransferase assay and nearest neighbor analysis, RNA, nuclear extracts, and DNA were prepared from treated cells as described below. To verify equal efficiency of transfection and expression of T antigen under the different conditions, a sample of the transfected cells was plated on glass coverslips, fixed for immunostaining with methanol, and incubated with an SV40 T antigen monoclonal antibody (Santa Cruz number sc-147) for 1 h in phosphate-buffered saline, 0.1% bovine serum albumin. The signal was detected using Texas Red-conjugated secondary anti-mouse monoclonal antibody (Vector number H0724) using standard techniques as recommended by the manufacturer. No difference in transfection efficiency and expression of T antigen was observed in oligonucleotide-treated transfectants. Nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (data not shown). For the quantification of the number of transfected cells, cells were stained with 4,6-diamidino-2-phenylindole dihydrochloride (data not shown). Cells were harvested for soft agar assay 48 h posttransfection.

DNA and RNA Analyses—Genomic DNA was prepared from pelleted nuclei, and total cellular RNA was prepared from cytosolic fractions. To quantify the relative abundance of DNA MeTase and T antigen mRNA, total RNA (5 μg) was blotted onto Hybond N+ using the Bio-Rad slot blot apparatus. The filter was hybridized to a 32P-labeled 0.6-kilobase pair cDNA probe encoding the 5′-terminal 1,600 nucleotides of DNA MeTase (c10161) under the direction of a cytomegalovirus promoter (pcDNA 3.1 His DNA MeTase), 10 μg of either pcDNA 3.1 His DNA MeTase or pcDNA His control were mixed with 4 μl of Lipofectin, and transfection was performed as described above. Expression of transfected constructs was verified by immunocytochemistry essentially as described above using the Xpress monoclonal antibody (Invitrogen number 46-0528) for detection of His-tagged proteins and Texas Red secondary monoclonal antibody. Nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (data not shown). Cells were harvested in triplicate for Hybrid-N+ filters bearing 20 μg of immobilized pSKMet5′ (containing a genomic fragment bearing exons 2–4 of the murine DNA MeTase gene) (31) (indicated as Met in Fig. 3), pZip U19 (ts A58)Tneo (T ant), and γ-antigen (γ-actin) plasmids and subjected to autoradiography as described previously (26). The intensity of the autoradiographic bands corresponding to 18S RNA, DNA MeTase transcribed in T antigen expressing transfecants and control cells was determined by scanning densitometry and normalized to the signal obtained for γ-actin. The ratio of DNA MeTase/actin signal in T antigen transfectants was compared with the value obtained for neo transfectants.

RESULTS

To determine whether ectopic expression of T antigen alters the levels of DNA MeTase in the cell, we cotransfected immunolabeled but nontransformed Balb/c 3T3 cells with a T antigen expression vector pZipneoSV40U19tsA58 (20), and G418-resistant colonies were isolated. Quantification of the level of DNA MeTase mRNA as a function of T antigen expression was done with RT-PCR and Northern blots. As shown in Fig. 1A, a strong stimulation of DNA MeTase mRNA by increasing levels of T antigen expression, as assessed by quantifying the hybridization signals obtained with a DNA MeTase probe or T antigen probe and normalizing these signals to the signal observed following hybridization to an 18 S ribosomal RNA probe. Our data show a good correlation between T antigen expression and DNA MeTase levels, with highest levels observed in the cells transfected with the highest expression plasmid.
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T antigen expression elevates DNA MeTase levels. A, T antigen and DNA MeTase mRNA levels in 24 independent T antigen transfectants. RNA was prepared from 24 different T antigen transfectants and subjected to a slot blot analysis of DNA MeTase and T antigen expression as described under “Experimental Procedures.” The results were plotted as DNA MeTase/18 S. Two clones were selected for further studies. T ant 10, a high expresser, and T ant 22, a medium expresser. Four representative neo clones expressing the bacterial neo resistance gene, but not T antigen, were processed similarly. B, Western blot analysis of DNA MeTase and T antigen levels. Nuclear extracts (50 μg) were isolated from 3T3, T ant 10, and T ant 22 cells, electrophoresed on 5% SDS-polyacrylamide gel electrophoresis, and electrotransferred to a Hybond nitrocellulose (Amersham Pharmacia Biotech) membrane. T antigen was detected using antibody (Pab 101, Santa Cruz), and DNA MeTase was detected by a polyclonal antibody raised to a peptide in the catalytic domain (14) using the ECL kit (Amersham Pharmacia Biotech).

determined by a nearest neighbor analysis (10). As shown in Fig. 2A there is a 1.5 (T ant 22)- to 2-fold (T ant 10) reduction in the population of unmethylated CpG dinucleotides in T antigen-transfected cells, suggesting that increased DNA MeTase activity results in a detectable increase in genomic DNA methylation.

It is hard to determine whether this hypermethylation indicates complete hypermethylation of a limited set of specific sites or whether it reflects a limited increase in the frequency of methylation of all sites. Comparing the state of methylation of specific sites in different transfectants is an arduous task because of the tendency of cells in culture to exhibit fluctuations in the state of methylation of CpG sites. A previous report has shown, however, that increased ectopic expression of DNA MeTase leads to hypermethylation of CpG islands (28). We therefore studied by bisulfite mapping the state of methylation of the CG-rich region in the first exon of the MyoD gene (GenBank accession number M84918) in the higher expressing T antigen clone, T ant 10. CpG islands in general and MyoD in particular have been shown to undergo hypermethylation upon cellular transformation (29). The bisulfite method enables one to look at a large number of copies of the studied gene, thus enabling a representative picture of the state of methylation of each specific site in the population of cells (27). As observed in Fig. 2, B and D, the MyoD region analyzed in our study is hypermethylated in T antigen transfectants relative to neo controls. Of the 18 CpG sites analyzed, a total of 9 sites were found to be hypermethylated in the T ant 10 stable line relative to neo controls (Fig. 2B). When the results for all of the CpG sites in this region in all the sequenced clones (18 for neo, 16 for T ant 10) were pooled and averaged for comparative analysis, the average methylation of CpG sites was found to be 39% for neo transfectants and 71% for T ant 10 transfectants. In order to determine whether this genomic hypermethylation extends to CG sites not contained in CG-rich areas, we chose to map the region upstream to the second exon of the DNA Methyltransferase (dnmt 1) locus. As shown in Fig. 2C and D, the methylation status of the dnmt 1 locus does not change substantially with T antigen overexpression. Of the seven CpG sites analyzed in this region, only one site (CpG 101) was shown to be partially hypermethylated in the T ant 10 stable line relative to the neo control line. Interestingly, another site (CpG 157) was actually hypomethylated in T ant 10 relative to the neo control.

The methylation status of other sites was not substantially different between the clones examined. When the results for all of the CpG sites for all sequenced clones were pooled and averaged, there was no significant difference in the average methylation of CpG sites, contrary to the results for the MyoD locus. One possible interpretation of this data could be that CpG sites contained in CG-rich areas of the genome are preferentially hypermethylated as a result of DNA MeTase induction. However, confirmation of this hypothesis would require substantially more study of many additional regions. Whereas it is unclear whether hypermethylation of specific sites plays a causal role in T antigen-mediated transformation, our results support the hypothesis that T antigen expression stimulates DNA methylation activity in mammalian cells. In accordance with previous observations (30), we show here that an increase in DNA MeTase activity is followed by an increase in overall genomic DNA methylation and that there is localized hypermethylation of at least one CG-rich site.

One of the established mechanisms of transformation by T antigen is its ability to bind and inactivate the tumor suppressor Rb (20). To determine whether induction of DNA MeTase is dependent on Rb inactivation, we took advantage of a previously characterized mutant of T antigen, T ant Rb– (bearing a
FIG. 2. **State of DNA methylation in T antigen transfectants.** A, nearest neighbor analysis of methylation at CpG dinucleotides. DNA from 3T3 cells and the T antigen transfectant clones T ant 10 and 22 was subjected to nearest neighbor analysis as described under “Experimental Procedures.” The relative abundance of nonmethylated and methylated cytosines was quantified (Scanalytics), and the results are plotted as the percentage of nonmethylated cytosines from the total population of cytosines residing in the dinucleotide sequence CpG.

B, bisulfite mapping of the MyoD locus in T antigen and neo transfectants. DNA was extracted from T ant 10 transfectants (T ant 10) as well as 3T3 neo controls (NEO). The first line is a physical map of the MyoD genomic region (exons are indicated by filled boxes, intronic sequence is indicated by a line). A blowup of the region amplified is shown under the physical map; the different CpG sites in the fragment are presented as ovals. The percentage of methylated cytosines per site in the 16–18 clones analyzed per treatment were determined and are presented as different shadings of the circles representing each of the sites as indicated. Fully methylated sites (>75%) are represented as filled ovals, mainly methylated sites (50–75%) are indicated as ovals with a checkerboard pattern, partially methylated sites are indicated as shaded ovals, and nonmethylated sites (0–24%) are indicated as open ovals. Sites that were hypermethylated in T antigen transfectants are indicated by arrows. The numbering is according to GenBank™ accession number M84918. The average methylation of all CpG sites in all sequenced clones for T antigen (T ant 10) and neo (NEO) transfectants is presented in the lower panel as the percentage of unmethylated cytosines. The difference between methylation status of the MyoD locus for T ant 10 and neo transfectants was determined to be statistically significant (t test, p < 0.05).

C, bisulfite mapping of the *dnmt1* locus in T antigen and neo transfectants. DNA was extracted from T ant 10 transfectants (T ant 10) as well as neo controls (NEO). The first line is a physical map of the *dnmt1* genomic region residing upstream to the second exon (exons are indicated by filled boxes, intronic sequence is indicated by a line). Similarly to the MyoD region in B, a blowup of the region amplified is shown under the physical map, with the 7 CpG sites in the fragment presented as ovals. The percentage of methylated cytosines per site in the 9–17 clones analyzed per treatment were determined and are presented as different shadings of the circles representing each of the sites as indicated as described above. The numbering is according to GenBank™ accession number M84387. The average methylation of all CpG sites in all sequenced clones for T antigen (T ant 10) and neo (NEO) transfectants is presented in the lower panel as the percentage of unmethylated cytosines. The difference between methylation status of the *dnmt1* locus for T ant 10 and neo transfectants was not determined to be statistically significant (t test, p = 0.14).

D, representative bisulfite mapping sequencing films of T antigen (T Ant 10) and neo (NEO) clones at the MyoD and *dnmt1* loci. One representative sequencing film of bisulfite-treated DNA from each genotype is presented.
E107K point mutation in the Rb pocket), which does not bind Rb (31). This mutation has been shown to selectively impair interactions between Rb and T antigen while leaving p53 and p300 interactions with T antigen unimpaired (31). Balb/c transfectants expressing high levels of T ant Rb - as determined by Northern analysis (Fig. 3, A and B) and Western blot analysis (Fig. 3C) do not express induced levels of DNA MeTase mRNA (Fig. 3, A and B) and protein (Fig. 3C). The variability of the DNA MeTase mRNA levels in T ant Rb - clones is in the order of the variability in neo clones (compare Fig. 1A and 3A). These results suggest that induction of DNA MeTase is dependent on tumor suppressor inactivation by T antigen. It is yet unclear whether Rb is directly involved in regulating DNA MeTase mRNA levels or whether the changes in DNA MeTase mRNA are a consequence of the multiple downstream effectors of Rb. It is also possible that other proteins that are inactivated by T antigen are involved in regulating DNA MeTase.

Two modes of regulation of DNA MeTase activity have been described previously: first, transcriptional regulation by the Ras signaling pathway (10, 11) and second, posttranscriptional regulation at the level of message stability is involved in cell cycle regulation of DNA MeTase (26). We found no evidence for transcriptional regulation of DNA MeTase by T antigen. First, expression of a previously described DNA MeTase-CAT expression vector bearing a transcriptional regulatory region of the murine DNA MeTase (11) is not induced by coexpression of T antigen but is induced by coexpression of c-Jun, suggesting that T antigen activity is not mediated by induction of AP-1 (data not shown). Second, a nuclear runon assay (Fig. 4A) shows that there is no significant change in the transcriptional rate of the DNA MeTase gene in the presence of T antigen. Quantification of this experiment is presented in the bottom panel of Fig. 4A.

To determine whether the stability of DNA MeTase mRNA is altered as a consequence of T antigen expression, the T antigen transfectants as well as 3T3 controls were treated with actinomycin D (an inhibitor of mRNA transcription) for 0, 1, 4, or 7 h and the level of DNA MeTase mRNA was assessed using a Northern blot analysis (the signal was normalized to the level of 18S rRNA in each lane). While the levels of mRNA in Balb/c 3T3 cells decline to undetectable levels by 7 h (Fig. 4B) (this result has been repeated in three independent experiments), the profile of decline in DNA MeTase mRNA obtained for the T antigen transfectants is markedly different. The mRNA in T ant 22 remains at 50% of control (0 h) levels after 7 h (Fig. 4B, left panels) and even up to 24 h treatment (data not shown). In T ant 10, the level of DNA MeTase mRNA remains at, or close to, 100% for the duration of the actinomycin D treatment (Fig. 4B, middle panels). The rate of decay of c-fos mRNA (which expression increased 2–3-fold more in T antigen transfectants versus Balb/c 3T3), a gene known to be regulated by stabilization of its message (32), is unchanged (Fig. 4B, bottom).

DNA is presented per clone for each locus. Lollipops indicate the specific CpG sites by their position, shaded as described above. The numbering is according to GenBank™ accession number M84918 for the MyoD locus and M84387 for the dnmt1 locus. (Not shown in the films are the sixth and seventh CpG sites (sites number 290 and 303), which are demethylated in both T ant 10 and neo clones as indicated in the map). The DNA was subjected to bisulfite treatment as described in the methods. The genomic region bearing 18 CpG sites in the first exon of the MyoD gene and the genomic region bearing 7 CpG sites upstream to the second exon of the dnmt1 gene was amplified by PCR using the primers indicated under “Experimental Procedures” and sequenced. Unmethylated cytosines are converted to thymidines by this protocol, whereas methylated cytosines are protected and are visualized as cytosines.
fore, T antigen expression affects the stability of DNA MeTase mRNA without affecting the general ability of the cell to degrade mRNA. Further experiments will be required to determine what are the downstream effectors of Rb that modulate the stability of DNA MeTase mRNA and whether the change in stability of the mRNA can account for all of the increased expression of DNA MeTase. Our data suggest, however, that the level of DNA MeTase can be regulated by viral antigens acting on tumor suppressors at a different level than the transcriptional up-regulation induced by protooncogenes such as Ras (10, 11).

We have utilized DNA MeTase-specific antisense oligonucleotides that we have developed recently (14) to test the hypothesis that DNA MeTase is a critical downstream effector of Rb that modulate cellular transformation. This oligonucleotide has been shown previously to reduce DNA MeTase mRNA in Y1 carcinoma cells and inhibit tumorigenesis ex vivo and in vivo (14). We first determined that the antisense oligonucleotides can effectively inhibit DNA MeTase in our system. Balb/c cells were treated with 100 nM DNA MeTase antisense oligonucleotide (HYB101584) (14) as well as a reverse control phosphorothioate oligonucleotide (HYB101585) (14) in the presence of a lipid carrier (Lipofectin) and transiently transfected with pZipneoSV40U19tsA58. We first determined whether DNA MeTase antisense oligonucleotide treatment (for 72 h) reduces the level of DNA MeTase mRNA, relative to treatment with the nonspecific oligonucleotide control as described previously (14). Cellular RNA prepared from the treated cells was subjected to an RNase protection assay for DNA MeTase mRNA, using a previously described DNA MeTase-specific riboprobe (11). The results shown in Fig. 5A show that T antigen-transfected Balb/c cells express lower levels of DNA MeTase mRNA following antisense treatment than those treated with equal concentrations of control oligonucleotides. Scanning densitometry values show nearly 90% inhibition of message expression for antisense-treated cells relative to reverse-treated control cells when normalized to the signal obtained for 18 S ribosomal RNA in the same hybridization reaction.

To determine whether this message level inhibition translated into a decrease in DNA methyltransferase activity, an activity assay was performed using nuclear extracts prepared from oligonucleotide-treated cells. As described previously (26), the assay tests the ability of cellular extracts to incorporate a methyl group from tritiated S-adenosylmethionine into a hemi-

![Fig. 3. A T antigen mutant that does not bind Rb cannot trigger an increase in DNA MeTase levels. A, quantification of a Northern blot analysis of six transfectants with a T antigen bearing a E107K mutation in the Rb binding pocket (31) (T ant Rb−), a neo clone, and a Balb/c control. The results were plotted as described in Fig. 1A. B, Northern blot analysis of T antigen and DNA MeTase mRNA expression in T antigen and T antigen Rb− transfectants. Balb/c 3T3 cells were transfected with a T antigen construct bearing a E107K mutation in the Rb pocket (31). RNA (20 μg) extracted from 3T3 and T ant 10, a neo control, and T antigen Rb− stable transfectants was subjected to a Northern blot analysis as described under “Experimental Procedures” and hybridized to either T antigen, 18 S, or DNA MeTase probes as indicated. C, Western blot analysis of T antigen and DNA MeTase expression of nuclear extracts (50 μg) prepared from 3T3 cells T ant 10, T ant Rb−, and neo transfectants. The antibodies were the same as in Fig. 1B.](image101x245.jpg)

![Fig. 4. T antigen regulates DNA MeTase at the posttranscriptional level. A, run-on assay. Nuclei from 3T3 cells and the T ant 10 transfectants were isolated, and transcription was allowed to resume in the presence of [32P]UTP as described previously under “Experimental Procedures” (26). Equal counts of each reaction were hybridized (representative duplicate experiments are presented in two horizontal panels) to Hybond N+ membranes (Amersham Pharmacia Biotech) on which γ-actin, DNA MeTase (Met), and U 19/ts A 58 T antigen (T ant.) plasmids were immobilized. The average hybridization intensities of three separate experiments were obtained by scanning densitometry (Scanalytics) and presented in the bottom panels with S.E. values as indicated. No significant difference in MeTase hybridization intensities was detected for 3T3 and T ant 10 transfectants. B, Northern blot analysis of actinomycin D-treated 3T3, T ant 10, and T ant 22 cells. Equal numbers of cells were plated on three 10-cm plates per clone and allowed to grow for 4 days without serum replenishment. After 4 days serum was added with 5 μg/ml actinomycin D, and the cells were allowed to grow for 0, 1, 4, or 7 h. RNA was collected, and 10 μg was subjected to Northern blot analysis and hybridization with MeTase, 18 S RNA probes as described under “Experimental Procedures.” The intensity of the signal hybridizing to DNA MeTase relative to 18 S RNA was determined (by densitometry) for each time point and plotted in the bottom panel. The Northern blots were hybridized to c-fos, and the relative signal hybridizing to c-fos was determined.](image346x516.jpg)
methylated substrate. Quantification of this assay in Fig. 5B revealed, similarly to the results of the RNase protection assay, a dramatic (9-fold) and highly significant (p < 0.001) inhibition of DNA methyltransferase activity in antisense relative to reverse oligonucleotide-treated cells.

We then determined whether the inhibition of DNA methyltransferase message levels and activity results in generalized genomic hypomethylation. Nearest neighbor analysis was used to test this hypothesis, as shown in Fig. 5C. The level of methylation of CpG dinucleotides in Balb/c controls is approximately 65% (data not shown), a result similar to that obtained for Balb/c neo control stable transfectants (Fig. 2A). As expected, reverse oligonucleotide treatment showed no significant change in methylation levels from control Balb/c cells. However, antisense-treated cells showed a remarkable reduction in the level of DNA methylation. Only 20% of the CpG sites in the genome were methylated following antisense treatment (Fig. 5C).

Having established that the antisense oligonucleotides effectively inhibited DNA methyltransferase message and activity levels and DNA methylation levels in Balb/c cells, we devised a cell culture assay to test the role of DNA methyltransferase as a downstream effector of T antigen. To show that DNA methyltransferase is directly involved in initiation of the transformed phenotype, we transiently transfected Balb/c 3T3 cells with the pZipneoSV40U19tsA58 T antigen expression vector following pretreatment with either 100 nM antisense or reverse oligonucleotides. As expected, transient transfection of normal Balb/c 3T3 cells by a T antigen expression vector for 48 h results in their transformation as indicated by their ability to grow in an anchorage independent manner on soft agar (Fig. 6A). Transfection with a neo expression vector or a vector directing the expression of a T antigen that is defective in its ability to interact with Rb does not result in cellular transformation similar to the results observed following stable transfection. Thus, since this assay does not require drug selection it allows us to study the initial stages of transformation induced by T antigen. To determine whether DNA MeTase is required for initiation of cell transformation, the cells were pretreated with 100 nM amounts of either DNA MeTase antisense or reverse oligonucleotides 24 h prior to introduction of T antigen into the cells. Treatment was terminated following an additional 48 h exposure to the oligonucleotides, and the cells were plated onto soft agar in the absence of any further treatment. As observed in Fig. 6A, exposure of cells at the initial stages of transformation by T antigen to DNA MeTase antisense results in significant inhibition of cellular transformation as determined by a soft agar assay. Since the antisense oligonucleotide is not present during growth on soft agar, it supports the
transient expression of DNA MeTase in Balb/c cells induces amino acids (33) into Balb/c cells and plated the transiently transfected cells after 48 h in soft agar. As observed in Fig. 6B, transient expression of DNA MeTase in Balb/c cells induces anchorage-independent growth on soft agar, an indicator of cellular transformation.

**DISCUSSION**

Our manuscript shows that similar to the Ras oncogenic pathway, SV40 large T antigen elevates cellular DNA MeTase mRNA and protein levels (10, 11). This activity of T antigen is dependent on its interaction with the tumor suppressor pRb, suggesting that overexpression of DNA MeTase lies downstream to inactivation of the tumor suppressor Rb. It is unclear yet whether Rb has a direct effect on DNA MeTase or whether these effects are mediated through interactions with other cell cycle regulators. It is also unclear whether other proteins that interact with T antigen are also involved in regulating DNA MeTase. Whereas up-regulation by the Ras oncogene involves transcriptional activation, T antigen up-regulates DNA MeTase at the posttranscriptional level.

This paper also tests the hypothesis that an increase in DNA MeTase levels is critical for cellular transformation triggered by T antigen. The fact that DNA MeTase has been shown to be critical for two different pathways of oncogenesis is consistent with a central role for DNA MeTase in cellular transformation (9, 12). DNA MeTase is critical for the initial stages of transformation by T antigen, since inhibition of DNA MeTase at the time of introduction of T antigen into the cells by transient transfection prevents cellular transformation.

What might be the possible role of DNA MeTase in cellular transformation? One hypothesis that has been supported by a growing body of evidence is that an increase in DNA MeTase might lead to an aberrant increase in methylation and inactivation of tumor suppressors (6), suggesting that increased levels of DNA MeTase cause cellular transformation by ectopic methylation of tumor suppressor genes. In contrast, our data surprisingly shows that DNA MeTase expression is controlled by tumor suppressors. Therefore activation of DNA MeTase might be the downstream target of a transformation program triggered by tumor suppressors. An alternative hypothesis is that the DNA MeTase protein is directly involved in controlling cellular growth (9, 12). Such a model is consistent with the requirement for increased DNA MeTase protein in both Ras and T antigen pathways. An interesting observation is that methyl deficient diets, which cause hypomethylation of DNA, also result in induction of DNA MeTase levels up to 20–23 population doublings. Whereas it is generally accepted that many tumor suppressors are inactivated by methylation, the data presented here are consistent with the hypothesis that DNA MeTase levels are controlled by tumor suppressors.

While additional experiments are required to support or nullify the hypothesis that an increase in DNA MeTase is directly involved in cellular transformation, the fact that a viral oncogene can affect the cellular DNA methylation machinery raises new directions and possibilities for understanding cellular transformation processes.

**Acknowledgments**—We thank Dr. Almazan for providing us with the T antigen expression vector and Dr. Howard for his kind gift of T antigen expression vector. We thank Johann Theberge for superb technical assistance. We thank Shyam Ramachandran for his critical reading of the manuscript.

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