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DNA METHYLATION AND ONCOGENESIS

by

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ABSTRACT

DNA methylation is a postreplicative covalent modification of the DNA which is catalysed by the DNA methyltransferase enzyme. DNA methylation plays an important role in controlling the gene expression profile of mammalian cells. The hypothesis presented in this thesis is that the expression of the DNA methyltransferase gene is upregulated by cellular oncogenic pathways, and that this induction of MeTase activity results in DNA hypermethylation and plays a causal role in cellular transformation. Novel DNA methyltransferase inhibitors may inhibit the excessive activity of DNA methyltransferase in cancer cells and induce the original cellular genetic program. These inhibitors may also be used to turn on alternative gene expression programs. Therefore specific DNA methyltransferase antagonists might provide us with therapeutics directed at a nodal point in the regulation of genetic information.

RESUMÉ

La methylation de l' ADN est une modification covalente effectuée par l'enzyme DNA methyltransferase (DNA MeTase). Cette methylation joue un rôle important dans le control de l'expression génic specific à chaque cellule mammifère. L'hypothèse presentée dans cette thèse est que l'expression génique de l'enzyme DNA Methyltransferase est activitée par les signaux cellulaires (oncogénique) et que cette activation augmente la méthylation general de la cellule ce qui causerait une transformation cellulaire vers une cellule cancéruese. L'inhibition du surplus d'activité de l'enzyme DNA MeTase dans une cellule cancéreuse par de nouveaux inhibiteurs pourraient permettre à cette cellule de retourner a son programme original de différenciation. Ces mémes inhibiteurs pourraient être utilisés pour activer un programme d'expression génique alternatif. Donc, ces inhibiteurs pourraient devenir un outil thérapeutique pour manipuler directement l'information génétique d'une cellule.

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Chapter 1: Regulation of the DNA Methyltransferase by the Ras-AP-1 signaling Pathway. J. Rouleau, A.R.MacLeod and M.Szyf, J. Biol. Chem . **270**, 1595-1601 (1995).

The candidate contributed primarily at the intellectual level with most of the work being done by J. Rouleau, and Dr. M. Szyf, tissue culture by Ms. Johanne Theberge, Northern blot analysis in Fig. 5A was done by Ms. Vera Bozovic.

Chapter 2: Regulation of DNA Methylation by the Ras Signaling Pathway. A.R. MacLeod, J. Rouleau and M. Szyf , J.Biol.Chem. **270**, 11327-11337 (1995).

The candidate is responsible for most of the work but received much appreciated technical help with some of the tissue culture from Ms.Vera Bozovic.

Chapter 3: Expression of Antisense to the DNA Methyltransferase mRNA induces DNA Demethylation and Inhibits Tumorigenesis. A.R. MacLeod and M. Szyf, J. Biol. Chem. **270**, 8037-8043 (1995).

Candidate is responsible for most of the work again receiving technical help with some of the tissue culture from Ms. Vera Bozovic.

Chapter 4: Expression of antisense mRNA to the DNA Methyltransferase inhibits v-Ha-Ras initiated transformation of C2C12 cells. A.R. MacLeod, V. Bozovic, J. Rouleau and M.Szyf. (Submitted manuscript).

The candidate is responsible for part of the work. Tissue culture, soft agar assay, thymidine incorporation and injection of mice was done by Ms. Vera Bozovic.

Chapter 5: Antisense oligonucleotides to the DNA Methyltransferase inhibit tumor growth *in vivo*. A.R. MacLeod, V. Bozovic, E. Van Hoffe and M Szyf. (submitted manuscript).

The candidate is responsible for most of the work, help with tissue culture was again provided by Ms. Vera Bozovic, oligonucleotide design by Dr. M.Szyf and Dr. E. Van Hoffe and the synthesis of oligonucleotides was done by Hybridon Inc. Worcester MA.

Contribution to original Knowledge

1. I have demonstrated that the DNA Methyltransferase (DNA MeTase) is a downstream effector of the Ras signal transduction pathway.

2. I have shown that modulation of the Ras pathway can lead to stable changes in DNA MeTase activity and to stable changes in DNA methylation patterns of the genome.

3. I have shown that modulation of the Ras pathway leads to changes in both the nature and levels of the AP1 transcription complexes in the nucleus of Y1 cells.

4. I have shown that downregulation of the DNA MeTase by expression of antisense mRNA can reverse the transformed phenotype of the Y1 adrenocortical cell line.

5. I have shown that inhibition of the DNA MeTase by antisense expression in Y1 cells results in induction of an active cell death program (apoptosis) when these cells are deprived of serum.

6. I have shown that expression of v-Ha-Ras in C2C12 myoblast leads to an increase in the steady state level of DNA MeTase mRNA and activity.

7. I have shown that expression of v-Ha-Ras in C2C12 myoblasts results in DNA hypermethylation coincident with the transformation of these cells.

8. I have shown that expression of antisense to the DNA MeTase in C2C12 cells transfected with v-Ha -Ras blocks the Ras initiated transformation and hypermethylation.

9. I have demonstrated that the ability of C2C12 cells transfected with v-Ha-Ras to form tumors in syngeneic mice is inhibited by the co-expression of antisense to the DNA MeTase.

10. I have shown that antisense phosphorothioates oligonucleotides to the DNA MeTase can inhibit DNA MeTase mRNA and activity, and reverse the transformed phenotype of Y1 cells *in vitro* .

11. I have shown that antisense phosphorothioates oligonucleotides to the DNA MeTase have therapeutic anticancer potential in that they can inhibit the growth of Y1 adrenocortical cells in syngeneic LAF-1 mice.

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I would like to keep these acknowledgements brief because a few written words can't describe the appreciation I have for all the friends I have been lucky enough to work with and for in the last few years.

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Table of Contents

Introduction and literature review

Pharmacology has been traditionally interested in discovering small molecule drugs that interact with cell surface receptors or enzymes and modulate their activity. However many disease states such as cancer involve alterations in the genetic program of the cells involved. Therefore as modern biology gains insight into how our cells interpret our genetic information, there is hope that important targets for pharmacological intervention will be discovered. Once one understands the genetic program and the ways by which it is maintained, changed and regulated, we might be able to develop new agents that act by modulating the flow of genetic information from DNA to protein. In this thesis I would like to discuss one important function that I believe could serve as a target in cancer therapy or other diseases that involve an altered genetic program. The candidate is the DNA Methyltransferase, the enzyme that controls methylation of DNA. In this thesis entitled "DNA METHYLATION AND ONCOGENESIS" I will discuss the fundamental aspects of DNA methylation that indicate that it is an important regulator of genomic functions. I will present what is known about the mechanisms regulating DNA methylation and what has been recently learned from the regulation of DNA methylation regarding its role in progression of cancer and ultimately discuss how one can utilise this understanding towards the development of pharmacological therapeutics for cancer patients. The hypothesis tested is that: **Deregulation of the DNA Methyltransferase is a causal event in some oncogenic transformations and that selective inhibition of DNA MeTase has antitumorigenic potential.**

In order to test the above hypothesis I performed sets of experiments to address four specific questions:

A) Can the DNA MeTase promoter activity be transactivated by the Ras-AP-1 signal transduction pathway.

B) Can modulation of the Ras-AP-1 pathway lead to stable changes in genome methylation patterns.

C) Can inhibition of DNA MeTase inhibit transformation and dedifferentiation of C2C12

myoblast cells initiated by v-Ha-Ras.

D) What is the therapeutic potential of inhibiting the DNA MeTase by antisense oligonucleotides.

To appreciate the logic behind the design of these experiments an extensive literature search follows and is divided into three sections:

1) DNA Methylation. This section describes formation of DNA methylation patterns and their possible role in gene regulation, replication and cancer.

2) Cellular Signal transduction pathways. This section describes receptor protein tyrosine kinases. The Ras-AP-1 signal transduction pathway and its possible role in gene expression and in oncogenic transformation.

3) Antitumorigenic potential of DNA MeTase inhibition and antisense technology. This section describes proposed mechanisms of action of antisense mRNA and oligonucleotides and discusses the therapeutic potential of such an approach directed to the DNA MeTase.

DNA METHYLATION

Genetic information encoded by DNA is often described in terms of an alphabet, comprised only of the four letters G, A, T and C. Different combinations of these four characters code for proteins that determine the make up and function of a cell. However, what is not often mentioned, is the fact that organisms from bacteria to man contain more letters than just G, A, T and C. The additional letters of their genomes are DNA bases modified by methylation (reviewed by Razin and Szyf, 1984). It is also becoming increasingly evident that these modified bases play important roles in regulating the flow of genetic information. In bacteria and some lower eukaryotes, both 6-methyl-adenine and 5-methylcytosine can be found (Razin and Szyf, 1984), however in higher eukaryotes such as vertebrates, only cytosines modified at the 5 position by methylation are found. In

vertebrates, methylated cytosines are generally found in the dinucleotide sequence CG (Gruenbaum et al, 1981), however some reports have also indicated the presence of methylated cytosines at the dinucleotide sequences CC, CT and CA (Woodcock et al., 1987, 1988; Toth et al., 1990). Most of the work investigating the biological significance of DNA methylation has focused on the modification of cytosine residues in the dinucleotide sequence CG. A recent report suggests that methylated cytosines other than those residing at the CG dinucleotide sequence are concentrated at origins of replication (Tasheva and Roufa, 1994). In this review I will focus primarily on CG methylation since the role of other methylated dinucleotide sequences is poorly understood. The modification of the cytosine moiety occurs shortly after the time of replication and is accomplished by the transfer of a methyl group from the methyl donor S-adenosyl-L-methionine to carbon 5 of the cytosine residue forming a stable covalent bond (Gruenbaum et al., 1982; Wu and Santi 1985) (see Fig. 1). DNA methylation and DNA replication are carried out by two independent sets of enzymatic machinery thus interesting possibilities for pharmacological modulations of one system independent of the other exist (Szyf, 1994).

DNA Methylation patterns

One of the perplexing questions in biology is how cells of multicellular organisms that contain the identical genetic information can be made to adopt such varied phenotypes and perform such diverse functions. The observation that bases modified by DNA methylation are not distributed randomly throughout the genome and that tissue specific patterns of methylation exist (Waalwijk and Flavell, 1978a), suggested that this covalent modification by methylation might encode "epigenetic information" allowing a single genome to express itself in many different ways (Holliday and Pugh, 1975; Holliday, 1990; Razin and Riggs, 1980). Early studies aimed to determine whether different tissue contained different levels of this epigenetic information and whether this correlated with the state of differentiation of the tissue (Ehrlich 1982, Gama-Sosa et al., 1983, Gruenbaum et al., 1981, Razin et al., 1984). These and other studies employing more sophisticated techniques that enabled the

analysis of the level of cytosine methylation at a specific dinucleotide sequence (Gruenbaum et al., 1981), concluded that between 70 and 80% of cytosines residing at the dinucleotide sequence -CG- are methylated in most tissues and cell lines analysed (Razin et al. 1984). Therefore, the total level of genomic DNA methylation was not responsible for determining the different cellular phenotypes. The use of the MspI/HpaII restriction enzyme pair (Waalwijk an Flavell, 1978) combined with Southern blotting and hybridisation with gene specific probes has been the most useful means to analyse methylation patterns at specific loci. HpaII cleaves the sequence CCGG, a subset of the CpG dinucleotide sequences, only when the site is unmethylated while MspI will cleave the same sequence irrespective of its state of methylation. By comparing the pattern of HpaII cleavage of specific genes, one could now entertain questions relating DNA methylation to the expression of genes, and whether or not these methylation patterns are stable or amenable to change under certain conditions. A large number of genes have since been similarly analysed and it was found that in general, genes are methylated at CG sites in nonexpressing tissues whereas they are nonmethylated in tissues actively expressing the gene (for a review see Yisraeli and Szyf, 1984). The fact that methylated genes are generally not expressed suggested the possibility that DNA methylation is functioning as a transcriptional inactivation signal. This is not to say that exceptions to this rule do not exist where the correlation between expression and demethylation of specific sites is not as precise as was originally expected (Yisraeli and Szyf, 1984).

Despite the strong correlation between gene expression and DNA methylation, the possibility exists that methylation patterns merely reflect the state of activity of a gene and are not actively involved in its regulation. To answer this question, an understanding of how DNA methylation patterns are formed is required.

The DNA MeTase enzyme.

The enzyme that catalyses the DNA methylation reaction is the DNA Methyltransferase (MeTase), a 190 kDa modular protein with several interesting domains: a catalytic domain that is homologous to other cytosine DNA methyltransferases, a Zinc finger DNA binding domain (Bestor et al., 1988) and a nuclear localisation domain that mediates the association of the DNA MeTase with the replication fork (Leonhardt et al., 1992). The catalytic mechanism of action of cytosine DNA methyltransferases as suggested by Wu and Santi (1985) is supported by the resolution of the crystal structure of the bacterial HhaI methyltransferase bound to DNA (Cheng et al., 1993). This model predicts that the first step of DNA methylation involves the generation of a covalent intermediate between the enzyme and the carbon at the 6th position of the cytosine base. The transfer of a methyl group to the carbon at the 5th position disrupts the covalent bond and releases the enzyme. Understanding the mechanism of catalysis of the enzyme is critical for the rational design of inhibitors of DNA MeTase. Based on this mechanism it was suggested that substituting a fluoro group at the 5th position would inhibit transfer of the methyl group to cytosine resulting in a stable covalent complex between the enzyme and cytosine in DNA (Wu and Santi, 1985). This prediction has been verified by a large number of experiments and 5-fluorocytosine modified DNA is used as a mechanism based inhibitor of DNA MeTase and was also used as the substrate in the crystal structure resolving the structure of DNA MeTase (Osterman et al., 1988; Chen et al., 1991; Cheng et al., 1993). A similar mechanism of action has been proposed for the DNA methylation inhibitor cytidine which also forms, following its incorporation into DNA, a stable covalent bond with the DNA MeTase (Santi et al., 1984). While mechanism based suicide substrates are very efficient inhibitors of DNA MeTase, they cause trapping of the enzyme to the modified DNA. Trapping of a large number of DNA MeTase molecule to DNA might disrupt DNA function and result in toxicity (Juttermann et al., 1994). This potential toxicity might limit the use of such inhibitors in therapy. Furthermore, 5-azaCytidine is known to inhibit many cellular processes (see below), thus the biological response to treatment with these inhibitors might

reflect many functions, not solely inhibition of DNA methylation.

To date only one enzyme has been shown to directly catalyse the methylation of mammalian DNA. This enzyme has been shown to mediate the methylation of both DNA molecules that are methylated on one strand (hemimethylated DNA), as well as non methylated DNA (Bestor et al., 1983; Bestor et al., 1988). Earlier reports however demonstrated that the mammalian MeTase is much more efficient at methylation of hemimethylated DNA strands (Gruenbaum et al., 1982). The cDNA of both the mouse (Bestor et al., 1988) and human DNA MeTase (Yen et al., 1992) were cloned but there is no evidence as of yet for the existence of more than one cytosine DNA MeTase. It is clear that even if additional DNA MeTases exist, the one identified to date is responsible for maintaining and generating a large number of methylation patterns, since overexpression of this DNA MeTase cDNA results in hypermethylation, and suggesting that the cDNA cloned by Bestor et al., (1988) also mediates de novo methylation (Wu et al., 1993). Furthermore, knock out of the DNA MeTase by homologous recombination results in DNA demethylation and embryonic lethality in homozygous mice (Li et al, 1992). As the same enzyme is present in different cells bearing different patterns of methylation, the critical question is how can one enzyme generate such diverse tissue specific methylation patterns?

Inheritance of methylation patterns: maintenance versus de novo methylation

Given that cells of a certain tissue maintain their specific DNA methylation pattern for generations, there must be a mechanism responsible for replicating the pattern. One simple model explaining the maintenance of cell specific patterns of methylation was proposed by Razin and Riggs (Razin and Riggs, 1980) (see Fig.2). Replication results in the generation of a hemimethylated double stranded DNA molecule composed of the methylated parental strand and the nonmethylated nascent strand. The hemimethylated DNA will now serve as an efficient substrate for the DNA MeTase. The Razin and Riggs model proposes that

DNA methylation patterns are inherited during replication because the DNA MeTase is very efficient in methylation of CpG moieties in hemimethylated DNA (maintenance methylation) but relatively inefficient in methylation of nonmethylated substrates (*de novo* methylation). Therefore, only sites that were methylated in the parental strand will be methylated in the daughter strand and nonmethylated sites will remain so. Thus the methylation patterns will be faithfully maintained from generation to generation. According to this model the memory of the methylation pattern is carried by the template parental strand methylation pattern solely. Therefore, a simple non-site selective DNA MeTase can impart the faithful inheritance of specific methylation patterns. The hypothesis that the DNA MeTase enzyme has very high affinity for hemimethylated strands over non methylated DNA was supported by in vitro experiments that compared the methylation activity of a partially purified mammalian DNA MeTase using hemimethylated versus nonmethylated substrates (Gruenbaum et al., 1982). Therefore, according to this model if a methylation of a site is lost, it will not be recovered since methylation is dependent on the presence of a methylated template. This model can also explain how in parental imprinting, an allele specific methylation pattern is carried over through multiple generations from fertilisation to adulthood. The allele that was originally methylated in gametogenesis will maintain it's methylation pattern through multiple replication cycles because of the properties of the MeTase that efficiently methylates a hemimethylated site (generated by replication of a methylated site) whereas the nonmethylated allele will not be methylated in development since the DNA MeTase is very inefficient in methylation sites that are nonmethylated on both strands.

Whereas the "semiconservative inheritance" model described above is very appealing because of its simplicity, a long line of evidence has established that the memory of DNA methylation patterns is not carried over only by the state of methylation of the parental strand (reviewed in Szyf, 1991). At the time that the model was proposed evidence was emerging that showed methylation patterns are altered in development by demethylation and de novo methylation events (Monk et al., 1987, Shemer et al., 1991; Kafri et al., 1992, Brandeis et al., 1993b; Kafri et al., 1993, Razin and Kafri, 1994). It is now clear that parentally imprinted genes form their methylation patterns de novo late in development and

these methylation patterns might be dictated by signals other than hemimethylation. Moreover, Sapienza et al., have shown that the pattern of imprinting is dependent on the genetic background of the other parent suggesting that transacting factors determine methylation patterns (Sapienza et al., 1989). As these processes result in specific alterations in methylation patterns, factors other than the original pattern of methylation must determine methylation patterns. Razin and Riggs suggested that these processes are determined by other enzymatic activities, such as a unique de novo methylase and are not carried out by the maintenance methylase. Recently, Laird and Jaenisch also suggested that mammalian cells bear another DNA MeTase activity which is specific for de novo methylation (Laird and Janisch, 1994). Whether an additional de novo methylase activity exists is still an open question. However, it is clear that some methylation events must involve elements in addition to the DNA MeTase and the state of parental strand methylation. One of the strongest pieces of data supporting this hypothesis is the well documented observation of partially methylated sites that are consistently methylated in a fraction of a clonal population (Yisraeli and Szyf, 1984, Turker et al., 1989). Partial methylation in a clonal population implies a loss of methyl groups during replication. If maintenance methylation is solely responsible for the inheritance of methylation, then each loss of a methyl group during a replication event must be irreversible. If de novo methylation is not specifically targeted, it will result in a drift in the methylation pattern. While this well established phenomenon was originally dismissed as representing different populations of cells even in a seemingly single lineage group of cells, it is now clear that clonal populations of cells maintain partially methylated sites for many generations (Turker et al., 1989). Moreover, when a fragment bearing a partially methylated site has been cloned and thus lost its methylation pattern, is reintroduced into the cell, it will acquire the same partially methylated pattern. This strongly suggests that the tendency of this site to be methylated is determined by portable signals in the sequence itself and that each round of methylation involves a specific de novo methylation event. Partial methylation of a site therefore reflects its affinity or probability to become methylated at each round of replication. Hemimethylated sites were also shown to be maintained as such for several generations (Toth et al., 1990). The persistence of hemimethylated sites in a proliferating

cell is inconsistent with the semiconservative model of inheritance of methylation patterns even in somatic cells.

A clear example of site and cell specific de novo methylation in somatic cells is the specific de novo methylation of an exogenous nonmethylated transfected 21-hydroxylase gene in the adrenocortical cell line Y1 (Szyf et al., 1989; Szyf et al., 1990). Other sequences are not methylated in Y1 cells and the 21-hydroxylase gene is not methylated in other cells. Since there is no obvious difference in DNA MeTase between the different cells and since methylation in Y1 cells occurs only at specific sequences, it suggests that sequence and cell specific signals for methylation exist in Y1 cells.

Whereas both Razin and Riggs (Razin and Riggs 1980) and Laird and Jaenisch (Laird and Jaenisch, 1994) models differentiate between de novo and maintenance methylation as two fundamentally different modes of transmission of the methylation pattern, Szyf previously suggested a unifying hypothesis to explain the generation of new methylation patterns by de novo methylation and demethylation, their propagation in specific lineages and their parental inheritance. According to this model DNA methylation patterns are determined by an interaction between signals in the DNA sequence and proteins that interact with these signals and target specific sites for DNA methylation.

The model proposed by Szyf (see Fig.3) suggests that DNA methylation like transcription and other genome functions such as DNA replication is determined by DNA cis-acting signals ("centers of methylation" or "demethylation") and transacting factors or modifying proteins that interact with these signals and target juxtaposed sequences for methylation or demethylation. Whereas modifying proteins have not been yet identified, a number of studies by Turker's group have demonstrated a cis acting "de novo methylation center" in the upstream region of the mouse APRT gene (Turker et al., 1991; Mummaneni et al., 1993) this sequence can also lead to inactivation of the APRT gene supporting the hypothesis that centers of methylation also function as centers of inactivation (Mummaneni et al., 1995). The transfected APRT methylation mimics the pattern of methylation of the endogenous sites even though the transfected gene is originally nonmethylated demonstrating clearly that the memory of the methylation pattern is not carried over by the methylation pattern of the parental strand but by the sequence itself and the repertoire of

factors that are present in the cell and interact with it. In addition to signals that enhance de novo methylation, other cis acting signals that protect juxtaposed sequences from de novo methylation, "hypomethylation signals", were identified in the CpG island of the thy-1 gene (Szyf et al., 1990) and HPRT (Brandeis et al., 1994). The presence of cis acting centers of methylation signals that can interact with ubiquitous or cell specific factors has been demonstrated. However, the identity of the proteins that interact with these signals remains to be determined.

According to this model the pattern of methylation is not determined by the original "hemimethylated" state but by signals encoded in the DNA as well as cell specific or ubiquitous proteins that can interpret this signal. This model can apply for both de novo and maintenance methylation, both might be determined by DNA-protein interactions. The identification of these proteins will most probably unveil important targets for therapeutics aimed at specifically modifying methylation at a site or a subset of sites.

Biological role of DNA Methylation

Despite the strong correlation between methylation and gene inactivation, recent attention has focused on tissue specific DNA binding factors and transcription factors as regulators of tissue specific gene expression and has relegated DNA methylation to a secondary role. However there are two examples where the presence of different transacting factors can not explain differential gene expression, X inactivation and parental imprinting. Both involve allele specific gene expression, where only one of a pair of identical allelic genes is expressed even though they are exposed to the same transacting factors within the cell (X inactivation reviews; Gartler and Riggs, 1983; Migeon, 1994; for parental imprinting reviews; Peterson and Sapienza, 1993; Efstratiadis, 1994). There must be some "epigenetic' mechanism that marks genetically identical alleles for differential expression. In X inactivation in females, almost all of the genes located on one of the pair of X chromosomes are inactivated in the trophoblast cells and later in the primitive ectoderm that will develop into the embryo proper (Lyon, 1992). Intensive genetic dissection of this

phenomenon has shown that inactivation of the X chromosome spreads from an "inactivation center" in cis in one of the chromosomal pair and is controlled by the Xce locus (X-controlling element) which maps in the mouse to this center (Lyon, 1992). Riggs has suggested that methylation of DNA might be the mechanism of spreading of inactivation where differential methylation is initiated at the inactivation center and then travels in cis along the chromosome (Riggs, 1975). In accordance with this model it has been shown that X-linked genes are methylated on the inactive chromosome and nonmethylated in strategic sites on the active X-chromosome (Keith et al., 1986; Pfeifer et al., 1989; Pfeifer et al., 1990a; Pfeifer et al., 1980b; Singer-Sam et al., 1990) and several groups have shown that the DNA methylation inhibitor 5-azaC could activate silent X-inactivated genes (Mohandas, 1981; Hansen and Gartler, 1990).

Parental imprinting is the selective expression of one member of a pair of identical genes based on its parental origin. It has been observed for a while that the maternally and paternally inherited genomes despite their almost identical genetic information do not function equally (Efstratiadis, 1994). A minority of genes are expressed only when inherited from one parent whereas the other copy inherited from the other parent is silent, these are called imprinted genes (Peterson and Sapienza, 1993). In the recent few years a number of such genes were identified to be expressed monoallelically based on the parent of origin of the allele (H19, IgFII, IgFIIr). Mouse embryos that inherit both sets of chromosomes from either maternal or paternal origin are nonviable (Mcgrath and Solter, 1984), suggesting that the proper development of a mouse requires both the maternal and paternal genomes, and implies that an epigenetic "tag" mediates the reciprocal expression from both sets of chromosomes. It is clear that these genes are imprinted at gametogenesis and the imprint is maintained through development whether the gene is expressed in specific tissues (mouse IgFII) or whether it is ubiquitously expressed (human IgfII). As the imprinting signal has to be maintained through rounds of replication during the developmental process, a highly stable epigenetic "tag" must be involved. DNA methylation is the only known covalent modification of mammalian DNA and is therefore a good candidate for the imprinting signal. DNA methylation is an attractive hypothesis because it was postulated that once a pattern of methylation is established by de novo

methylation during gametogenesis it will be maintained during successive replications by semiconservative maintenance methylation (Razin and Riggs 1980) as discussed above. Thus the methylation pattern can carry through fertilisation and successive rounds of replication the memory of the parental origin of the specific allele. DNA methylation is the only known specific modification of DNA that could be faithfully inherited. Since methylation is generally thought to inactivate transcription, it was suggested that the imprinted allele is methylated whereas the nonimprinted allele is hypomethylated. It should be noted that because of the requirement for the "epigenetic" information to cross the generation barrier, parental imprinting provides perhaps the strongest argument for DNA methylation as the carrier of epigenetic information. Whereas early experiments with parentally imprinted transgenes showed remarkable correlation between "imprinting" and hypermethylation, later experiments have shown that imprinted genes do not escape the rounds of demethylation and remethylation that occur during preimplantation and postimplantation stages (Kafri et al., 1992; Razin and Kafri, 1994) and that the final pattern of methylation of imprinted genes is established later in development. Although the correlation of site specific methylation and imprinting has been confusing, general inhibition of DNA methylation by a knockout mutation of the DNA methyltransferase gene provides insights into its involvement. In a MeTase null/null background the transcription activity of two imprinted gene IgfII and IgfIIr is extinct whereas the expression of H19 is biallelic (Li et al., 1993). These experiments, measuring the impact of a general inhibition of DNA methylation on parentally imprinted gene expression, establish a causal relationship between DNA methylation and expression of imprinted genes.

To go beyond the level of correlatory evidence one has to actively intervene and modulate the biological system in question. To date, it is technically impossible to specifically change the methylation state of an endogenous gene in the cell without altering many other cellular processes. However, one can methylate a cloned gene in vitro using bacterial enzymes such as HpaII (which methylates the sequence CCGG (Singer et al., 1979) or SssI (which methylates all CG dinucleotide sequences (Nur et al., 1985) or by in vitro synthesising a hemimethylated substrate on a nonmethylated single stranded template replacing dCTP with 5methyl-dCTP (Gruenbaum et al., 1981a, Stein et al., 1982a),

introduce it into a cell line by transfection and compare its activity with a mock methylated exogenously introduced gene (Vardimon et al., 1982; Stein et al., 1982b). A long list of experiments utilising a similar strategy have demonstrated that genes methylated *in vitro* are repressed in mammalian cells. These experiments provided some insight regarding the possible mechanisms involved in repression of gene expression by methylation. The first question to be addressed by these experiments was whether methylation of a specific sequence(s) is required for gene repression or whether a regional nonsite specific methylation of sequences in and around the gene can silence transcription? The second question to be asked is whether methylation inhibits directly the transcription process or whether other proteins that recognise and bind methylated DNA inhibit gene expression? In some cases, such as viral promoters (the E2A adenovirus promoter) (Langner et al., 1984), or the proenkephalin gene (Comb and Goodman, 1990), methylation of specific sites, mainly transcription factor recognition sequences (such as AP-2 in the proenkephalin gene) was sufficient to repress transcription. In other examples, no site specificity was observed and methylation of different regions in the coding region or in associated sequences even juxtaposed plasmid sequences could mediate repression of different genes (Yisraeli et al., 1988; Bryans et al., 1992; Komura et al., 1995). These observations support the conclusion that DNA methylation could interfere with gene expression by at least two different mechanisms. One mechanism involves inhibition of binding of essential transcription factors to DNA (Becker et al., 1988; Comb and Goodman, 1990; Prendergast et al., 1991) while the other mechanism which is nonsite-specific most probably involves the formation of an inactive chromatin structure around the gene (Kass et al., 1993).

Whereas in vitro methylation and transfection experiments support the hypothesis that methylation can repress gene expression, one major criticism of this line of experiments is that the exogenously transfected genes might behave differently than endogenous cellular genes. To demonstrate that DNA methylation plays a role in controlling endogenous gene expression, one has to be able to inhibit methylation of endogenous genes and measure its effects on gene expression. One of the early tools used in inhibiting DNA methylation was the cytidine analogue 5-azaCytidine (5-azaC) which is incorporated into the DNA during replication and covalently binds and inhibits the activity of DNA methyltransferase. 5-azaC

treatment usually results in hypomethylation of DNA. A series of experiments pioneered by Jones's group and repeated by many others showed that many silent genes could be induced by 5-azaC (Constantinides, 1977; reviewed by Jones, 1985). These experiments also suggested that DNA methylation can control the differentiated state of mammalian cells since a number of cell lines could be induced to differentiate with 5-azaC such as Friend erythroleukemia cells (Creusot et al., 1982) and 10T1/2 fibroblasts which differentiated into muscle, fat and osteoclast cells (Taylor and Jones, 1979). Experiments have also suggested that demethylation of a single locus induced by 5azaC was sufficient to convert 10T1/2 cells into determined myoblasts leading to the identification of a gene that was proposed to function as a master regulator of myogenic differentiation, MyoD (Lassar et al., 1986; Davis et al., 1987). The main criticism of 5-azaC experiments is the fact that 5-azaC is a nucleoside analog that can inhibit other DNA metabolising enzymes and might interfere with other DNA binding functions that are critical for maintaining the state of gene expression. Indeed, 5azaC has been shown to induce new developmental phenotypes in Aspergillus whose DNA does not contain 5-methylcytosine (Tamame et al., 1983). The toxicity of 5-azaC is consistent with classic nucleoside analogue effects and does not seem to be mediated by inhibition of DNA methylation (Bouchard et al., 1990). Whereas many investigators have tried to avoid some of these problems by using concentrations that were below the toxic doses, it is stil very hard to determine whether the effects on differentiation are exclusively a consequence of hypomethylation. To circumvent the limitations in 5-azaC, recent experiments utilised an antisense approach to partially inhibit DNA MeTase expression in 10T1/2 cells. These experiments demonstrated that inhibition of DNA methylation results in conversion of the cells to a myogenic phenotype (Szyf et al., 1992) supporting the hypothesis that DNA methylation is involved in controlling the state of differentiation of somatic cells. The results with the antisense 10T1/2 transfectants suggest however that demethylation is a very early event in commitment to myogenic differentiation while expression of MyoD is a later event (Szyf 1992). If this model is true, other genes that are methylated in 10T1/2 cells are induced by demethylation and then activate MyoD to complete the conversion to the muscle phenotype. Whereas most studies focus on the involvement of DNA methylation in regulating gene expression directly, an alternative

hypothesis to be considered is that DNA methylation controls the differentiated state of the cell by mechanisms other than gene expression such as the regulation of DNA replication. In summary, whereas the specific mechanism by which methylation controls the differentiated state of a cell is unclear, the 5-azaC and antisense experiments provide us with evidence that DNA methylation is involved in epigenetic control of genome functions.

DNA methylation and replication

DNA methylation has been traditionally looked at as a mechanism for regulation of gene expression (Razin and Rigs,1980). Most of the functions I have touched on in this review pertain to different facets of regulation of gene expression. However it is obvious that the genome plays other roles such as replication, repair and recombination. DNA methylation as the only covalent modification of the genome is a candidate to influence these functions as well. The role of DNA methylation in controlling replication of E. coli genomes is very well established. In E. coli, the state of methylation of the origin defines its state in terms of replication (Messer et al., 1985; Smith et al., 1985; Boye and Lobner-Olesen, 1990). Once replication of E. coli is initiated, there is a very short lag between initiation of synthesis of the nascent strand of DNA and its methylation by the Dam DNA methyltransferase (Szyf et al., 1984). The hemimethylated origin of replication is then sequestered by the membrane and remains hemimethylated because it is inaccessible to the Dam DNA methyltransferase (Campbell and Kleckner, 1990). The replication dependent state of methylation (hemimethylation) is maintained because the level of the Dam methylase in E. coli cells is limiting (Szyf et al., 1984). The hemimethylated state of the origin signals that replication is not completed and new initiations are inhibited (Russell and Zinder, 1987). Once replication is complete, the origin is methylated and a new round of replication is initiated (Bakker and Smith, 1989). The hypothesis that DNA methylation may also regulate DNA replication in eukaryotes is supported by recent experiments showing that in Neurospora crassa, methylation deficiency mutants lead to postreplicative chromosomal anomalies (Foss, H.M. et al., 1993) and that densely methylated sequence

islands, designated DMIs, have been observed in two Chinese hamster replication origins (S14 and DHFR) (Tasheva and Roufa, 1994). All dCs are methylated in these sequences when the cells are cycling but are not methylated when the cells are arrested (Tasheva and Roufa, 1994). The fact that CA, CC and CT sites are specifically methylated in the origin is surprising since it was generally accepted that DNA methylation in vertebrates occurs mainly at the CG dinucleotide sequences as discussed above. This raises interesting questions as to the biochemical machinery responsible for this methylation. Is there a specific enzyme methylation CC, CT, and CA sequences? Is this enzyme cell cycle regulated? The paper by Tasheva and Roufa proposes a biological role for methylation occurring in sequences other than CG. Roufa et al., suggested three possible functions for the DMIs in mammalian chromosomal origins of replication: association with the nuclear matrix, licensing of activation of particular replication origins during specific developmental programs or molecular signals to mark previously replicated origins (Tasheva and Roufa, 1994).

DNA methylation and cancer

In the early days of molecular biology, scientist were discovering altered cellular genes that when overexpressed or activated by genetic mutation could deregulate cell growth and lead to cancer, these cellular genes were named protooncogenes, and their altered forms oncogenes (Weinberg, 1985). Genes involved in many different cellular roles have since shown the potential to become oncogenes, examples include; tyrosine kinase cell surface receptors of growth hormones (Schlessinger and Ulrich, 1992), intracellular tyrosine kinases (Schlessinger and Ulrich, 1992), intracellular signaling molecules such as Ras (Lowy and Willumsen, 1993), early immediate transcription factors such as Fos, Myc and Jun (Herschman et al., 1991), and translation factors such as the mRNA cap binding factor eIF-4E (Lazaris-Karatzas et al., 1990). Because of these early findings, cancer biologists focused on the aberrant activation of genes in cancer cells. Due to the potential role of DNA methylation in gene regulation it was proposed that

hypomethylation of DNA would induce aberrant gene activation and ultimately lead to cancer. In accordance with this hypothesis, a series of papers showed that the genomes of several cancer cells are widely hypomethylated when either the general level of methylated cytosines is assayed (Gama-Sosa, 1983b; Feinberg et al., 1988) or when specific genes were looked at with methylation sensitive restriction enzymes and Southern blot analysis (Feinberg and Vogelstein, 1983; Goelzet al., 1985; Wahlfors et al., 1992). It is now clear however, that hypomethylation is not directed specifically at oncogenes, since genes that are not involved in cancer progression are also hypomethylated (such as β globin). It is also worth noting that most protooncogenes are usually constitutively expressed proteins and not regulated by methylation. Studies opposing the role of DNA hypomethylation in oncogenic transformation include those presented in this thesis and more recently that of Laird et al., 1995, who induced DNA hypomethylation in mice bearing a germ line mutation leading to adenomatosis polyposis coli neoplasia (APC) by systemic treatment with the DNA MeTase inhibitor 5-azaC, although the authors themselves expected increased polyp formation what they found was a dramatic suppression of intestinal neoplasias. 5-azaC treatment has also been shown to suppress the growth of a highly tumorigenic murine cell line, T984-15 when transplanted in BALB/c nude mice (Walker et al., 1987). Therefore to date, the causal relationship between DNA hypomethylation and cellular transformation is unclear.

It has become obvious in recent years that cancer cannot be understood simply in terms of aberrant gene activation. In fact, it is the loss of function or inactivation of certain critical genes that is gaining support as a primary underlying mechanism in oncogenic transformation. The loss of function of these tumor suppressing genes can lead to cancer. Spectacular progress has been made in the identification of tumor suppressor genes, whereas some were identified by positional cloning from cancers caused by loss of function of specific genes, such as Wilms Tumor (Hastie, 1994), and Neurofibromatosis I (NF-1) Cawthon, et al., 1990), many other proteins were identified by an elaborate dissection of the biochemical events controlling the cell cycle such as the inhibitors of cyclin dependent kinases (cdks), p16 and p21 (Elledge and Harper, 1995). It appears that

most tumor suppressors play different roles in controlling the progression of the cell cycle. Only when the bias towards oncogene activation in cancer was lifted was it possible to entertain the idea that overexpression of the DNA MeTase and DNA hypermethylation might induce transformation by the inactivation of such critical loci. In accordance with this hypothesis, hypermethylation was documented in specific loci in a number of cancer cell types by S. Baylin's group (de Bustros et al., 1988). For example, abnormal methylation of the calcitonin gene marks progression of chronic myelogenous leukemia (Nelkin et al., 1991). Makos et al have shown that distinct regional hypermethylation patterns occur on chromosome 17p in human lung and colon cancer DNA. Interestingly, hypermethylation occurs in a region that is frequently reduced to homozygosity in both tumor types, suggesting that this region bears genes whose inactivation is critical for progression of cancer (Makos et al., 1992). It has since been shown that inhibition of tumor suppressor activity in a large number of tumors that bear no apparent genetic alterations is associated with hypermethylation such as Rb tumors (Ohtani-Fujita et al., 1993) WT (Royer-Pokora, et al., 1992) and renal carcinoma (Herman et al., 1994). It has also been suggested that inactivation by hypermethylation of parentally imprinted genes such as H19 is involved in WT (Taniguchi, et al., 1995; Ogawa et al., 1993) and possibly in the somatic overgrowth syndrome Beckwith-Wieldemann (Ogawa et al., 1993; Weksberg, et al., 1993).

The hypermethylation in cancer cells appears to demonstrate some site specificity by being targeted to specific chromosomal loci and specific group of sequences. One class of sequences that appear to be targeted for hypermethylation in cancer cell lines is the CpG rich island sequences (Antequera et al., 1990) which are usually found in the 5' region of housekeeping genes and are hypomethylated in most normal somatic tissues (Bird et al., 1985). Jones et al. have shown that hypermethylation of the MyoD CpG island occurs in transformed cell lines ex vivo (Jones et al., 1990) and proceeds progressively during cellular transformation (Rideout et al., 1994). Abnormal methylation of CpG island sequences on chromosomes 11p and 3p was also previously described by Baylin et al., (1986) and de Bustros et al., (1988) in human lung tumors. Hypermethylation of CpG islands also accompanies oncogene-induced transformation of human bronchial epithelial

cells (Vertino et al., 1993). Another clinically and pharmacologically relevant example is the methylation of CpG islands of the estrogen receptor in human breast cancer cells which is associated with hormone resistance (Ottaviano et al., 1994). One additional group of genes that has been shown to undergo specific hypermethylation is tissue specific genes that characterize the differentiated state of the normal parental cell and are inactivated in the dedifferentiated cancer cell type. The adrenal specific 21-hydroxylase gene (C21) is an example of this phenomenon. This gene is hypomethylated and expressed in the adrenal cortex and is hypermethylated and inactivated in the adrenocortical carcinoma cell line Y1 (Szyf et al., 1989, 1990). When an exogenous C21 is introduced into Y1 cells it is specifically de novo methylated suggesting that the cancer cell maintains the capacity to specifically recognise this gene and target it for de novo methylation and inactivation (Szyf et al., 1989).

What are the mechanism responsible for regional hypermethylation in cancer cells and what is its role in carcinogenesis? A possible mechanism for hypermethylation of DNA in cancer cell is the hyperactivation of the DNA MeTase observed in many cancer cells (Kautien and Jones 1986: El Deiry et al., 1991). What is the mechanism responsible for the increased DNA MeTase activity in cancer cells? Evidence to be presented in this thesis demonstrates that oncogenic signal transduction pathways can lead to deregulation of the DNA MeTase and to DNA hypermethylation of critical loci (see Fig. 4).

Cellular Signal transduction Pathways

Cells modulate their energy expenditure in response to external cues. Single cell organisms will vary their activities in response mainly to nutrient levels or toxic signals from the local environment. Multicellular organisms however have evolved complex mechanisms to coordinate the behaviour of one cell with that of its neighbours. Many of these cell to cell communication lines are controlled by extracellular signalling molecules. The desired signal is often transduced from the exterior of the cell across the plasma membrane by transmembrane receptor molecules. Perhaps the most widely characterised

of these molecules are the receptor protein-tyrosine kinases (RPTKs). The interaction of growth factors, cytokines, and hormones with specific RPTK molecules activates the intrinsic tyrosine kinase activity of the receptor molecule which then triggers an intracellular cascade of biochemical signals ultimately resulting in the activation or repression of various subsets of genes. Included among RPTKs are the epidermal growth factor (EGF) receptor family, platelet-derived growth factor (PDGF) receptor family , insulin (INS) receptor family , fibroblast growth factor (FGF) receptor family, hepatocyte growth factor (HGF) receptor family and the nerve growth factor (NGF) receptor family, all of which contain several family members (reviewed in Van der geer, Hunter and lindberg 1994). The signals activated by RPTK-ligand interaction are involved in many cellular programs, including cellular proliferation and differentiation. Because of these properties it is not surprising that genetic aberrations of the proteins involved in these pathways are associated with developmental abnormalities and cancer.

In this section I will discuss activation of RPTK molecules in general and then will focus on the Ras signal transduction pathway, signalling to the nucleus and the significant advances in understanding that have recently come to light.

Receptor Protein Tyrosine Kinase (RPTK) Activation

The signal transduction pathway through a receptor protein tyrosine kinase is activated when the ligand binds its receptor. The series of events that follow are outlined below (see Fig.5).

1. After ligand binding, the receptor undergoes dimerization. This is thought to occur via a ligand induced conformational change in the receptor making dimerization energetically favourable.

2. Receptor dimerization is followed by intermolecular autophosphorylation. The

dimerization of the receptors places their cytoplasmic tails in close proximity to one another. This contact between the two cytoplasmic tails is thought to induce conformational changes inducing protein tyrosine-kinase activity, leading to transphosphorylation of each receptor by its dimer partner. This auto transphosphorylation occurs at specific sites, most of which lie outside the catalytic domain itself.

3. Transphosphorylation activates the intrinsic protein-tyrosine kinase activity of the dimeric RPTK enabling phosphorylation of cytoplasmic targets thus initiating the intracellular cascade of biochemical events (Ullrich and Schlessinger, 1990; vav der Geer et al., 1994).

4. The autophosphorylated dimer has increased affinity for cellular substrates. Experiments using kinase-inactive RPTK mutants demonstrate the absolute requirement for phosphorylation of cytoplasmic substrates for signalling (Schlessinger and Ullrich 1992).

SH2 containing adaptor proteins

As mentioned above the autophosphorylation of RPTKs occurs at defined sites. These phosphorylated tyrosines serve as ports to which adaptor proteins or enzymes dock via their SH2 domains.

SH2 domains or Src like homology domains are approximately 100 residues in length and are not required for catalytic activity (Sadowski et al 1986; Pawson 1988). Subsequently it was shown that SH2 domains bind to phosphorylated tyrosine (P.tyr) residues in sequence specific manners, that is the amino acid sequences surrounding the P.tyr residue in the RPTK determine the affinity of SH2 containing proteins for binding (Songyang et al 1993). The affinity of proteins containing SH2 domains for the P.tyr of RPTKs provides a mechanism for recruitment and activation of cytoplasmic substrates

upon stimulation of the receptor. There are many SH2 containing proteins that are known substrates for RPTKs. These include enzymes such as the phospholipases, PLCγ1 and PLCγ2, the GTPase activating protein RasGAP, VAV, phosholipase 3' kinase (PI3 kinase), protein tyrosine phosphatases and others. The activity of these enzymes can be modulated when bound to RPTK P.tyr residues through their SH2 domains. Interaction of these enzymes with a RPTK may make them attractive substrates for phosphorylation themselves, an event that may be either activating or inhibitory (Carpenter et al., 1993). Other SH2 containing proteins such as GRB2, SHC and NCK serve primarily as adaptors between RPTKs and the next element in the pathway (Van der geer, Hunter and lindberg 1994). Binding of these SH2 adaptor molecules to the RPTK may serve to increase the local concentration of associated molecules. This seems to be the mechanism by which GRB2 (the adaptor) brings the associated protein SOS (a guanine nucleotide exchange factor) to the inner side of the plasma membrane thus allowing it to activate its target Ras (Chardin et al 1993) (see Ras pathway below). Another feature common to many of these adaptor molecules is the presence of Src like homology 3 (SH3) domains (Pawson, 1988). SH3 domains also participate in protein-protein interactions and are thought to provide the other binding site of SH2-SH3 adaptor proteins (Cicchetti et al., 1992). In fact the adaptor protein GRB2 or its *C. Elegans* homolog SEM-5 is a 217 amino acid protein that contains nothing more than an SH2 domain located between two SH3 domains. Another class of SH2 containing proteins are the so called "docking" proteins such as the insulin receptor substrate IRS-1. These docking proteins are phosphorylated at multiple tyrosine sites upon receptor activation and then themselves bind a collection of SH2 containing proteins (Van der geer, Hunter and lindberg 1994). It seems that these docking proteins are ideal to generate diversity in a pathway because of the number of combinations of phosphorylation-SH2 protein interactions possible from one receptor activation event.

Ras Activation

The 21 kD membrane bound GTP-binding protein Ras functions as a crucial mediator of many biological responses triggered by ligand bound RPTKs and receptors associated with tyrosine kinases (Feig, 1993) (see Fig.5). Microinjection experiments with neutralising anti-Ras antibodies and experiments using dominant-negative mutants of Ras indicate that Ras lies downstream of receptor and nonreceptor protein tyrosine kinases and upstream of a kinase cascade including the proteins Raf, MEK (MAP kinase kinase) and MAPK (MAP kinase) (Satoh, et al., 1992). Stimulation of this kinase cascade plays a pivotal role in the transmission of extracellular signals to the nucleus. The three cellular Ras proteins, H-Ras, N-Ras, and K-Ras act as a molecular switch for transmission of these signals because they can exist in one of two states, an active one where Ras contains a bound guanosine triphosphate (GTP) molecule, or an inactive state where Ras contains a bound guanosine diphosphate (GDP). Activation of Ras can be measured directly by an increase in the ratio of GTP/GDP guanine nucleotides bound to RAS. The levels of Ras-GTP are regulated by guanosine nucleotide releasing factors (GNRFs) and GTPase activating proteins (GAPs) (Polakis and McCormick, 1993). Activation occurs by the replacement of bound GDP with GTP. The rate limiting step in this exchange is the release of bound GDP, this is catalysed by GNRP proteins such as SOS and VAV. Deactivation occurs by the hydrolysis of bound GTP to GDP. Ras has intrinsic GTPase activity that is dramatically enhanced by the interaction with GTPase activating proteins such as GAP and NF1. Genetic studies in C. Elegans, Drosophila, and Saccharomyces cerevisae combined with biochemical studies in animal cells have revealed the proteins involved and the mechanisms by which information flows from tyrosine kinases to Ras (Broek, et al., 1987; Clark, et al. 1992; Lowenstein, et al., 1992). These studies identified an adaptor protein containing one SH2 domain flanked by two SH3 domains (SEM5 in *C.elegans* or GRB2 its mammalian homolog), a guanine nucleotide releasing factor (Sos in Drosophila or mSOS1,2 its mammalian homolog). Recent studies have shown that GRB2 is bound to mammalian SOS1 through its two SH3 domains (Egan, et al., 1993) and that the GRB2-SOS complex

can bind directly to phosphorylated tyrosine residues of RPTK molecules. Thus the ligand mediated autophosphorylation of a RPTK enables the GRB2-SOS complex to bind to phosphorylated tyrosine through its SH2 domain bringing the SOS part of the complex in close enough proximity to the membrane bound Ras to exert its function as a GNRF, thus activating Ras.

From Ras to the nucleus

Activation of Ras triggers a cascade of kinase activities (see Fig. 6). Some of these cascade pathway result in translocation of a signal to the nucleus and to changes in gene expression profiles of the cell. However, what are the immediate downstream targets of activated Ras? Recent work indicates that one of the primary targets of Ras is Raf-1. The *raf* protooncogene product, p74 ^{raf}, is a serine-threonine kinase which can be activated by p21 Ras alone or in cooperation with protein kinase C (PKC) in response to several growth factors (Pronk, et al., 1993). Ras-GTP has also been shown to interact directly with the N-terminal regulatory region of Raf-1 (Barnard, et al., 1995; Brtva et al., 1995). However in vitro, this association is not sufficient to activate Raf-1 suggesting a third component is necessary for ras-GTP activation of Raf-1. Raf-1 can phosphorylate and activate MAP kinase kinase (also known as MEK), a dual specificity protein kinase, which in turn phosphorylates and activates MAP kinases at their Thr and Tyr activating sites (Dent et al., 1992). MAP kinase is a family of enzymes, and at least one form has been shown to translocate to the nucleus upon serum stimulation, once in the nucleus it can phosphorylate and activate transcription factors such as Elk-1 and serum response factor (SRF) (Treisman, 1994; Rivera, et al., 1993). Also included in the Map Kinase family are the ERKs and JNKs (Jun N-terminal kinases). Raf-1 activated MEK can in turn activate Erk1 and 2 by phosphorylation of conserved threonine and tyrosine residues (Cobb et al., 1991; Ahn et al., 1991; Crews and Erikson, 1992). Although different cell types use different pathways to activate Erks, the Raf-1 protein is a common element in all the various signals leading to these activations. Further downstream to Erk2 is the serine/threonine kinase

$p90^{rsk}$ (ribosomal S6 kinase) (Strugill et al.,1988). Many of the putative targets of $p90^{rsk}$ kinase are proteins localised to the nucleus and involved in transcriptional regulation (Blenis, 1993; Davis, 1993).

Recent studies demonstrate that activated Ras protein induces transcription of genes that are regulated by promoters containing AP-1 binding sites (Imler et al., 1988). Activation of c-Jun, one component of the AP-1 complex is achieved by phosphorylation at Ser63 and Ser73 (Binetruy et al., 1991; Pulverer et al., 1991) by a Jun Kinase (JNK). JNK exists in several isoforms all of which are members of the mitogen- activated protein kinase (MAPK) family (Hibi et al., 1993) and like Erk1 and Erk2 are activated by phosphorylation of conserved tyrosine and threonine residues (Derijard et al., 1994). The kinase responsible for JNK activation is termed MEK kinase (MEKK) which preferentially activates JNK rather than Erk2 (Lange-Carter et al., 1993). It is important to note that Raf does not participate in MEKK activation. Therefore, Ras activation triggers two divergent signalling cascades, one initiated by Raf-1 and leading to Erk1 and 2 activation while the other is initiated by MEKK resulting in JNK activation (Minden et al., 1994). It is interesting that although these pathways diverge after the point of Ras activation they seem to reunite in the nucleus, independently activating both partners of the AP-1 transcription complex, c-fos and c-jun.

Both Erks and JNKs phosphorylate different transcription factors (Karin, 1994). Activation of Erk1 and 2 results in the phosphorylation and activation of TCF/Elk1. Elk1 (ternary complex factor, $p62^{TCF}$) is part of a ternary complex along with the transcription factor, SRF (serum response factor, $p67^{SRF}$). Both Elk1 and SRF transcription factors form a ternary complex at the c-fos serum response element (SRE) upon phosphorylation of Elk1 C-terminal region through Erks (Gille et al., 1992; Marais et al., 1993). This complex formation at the SRE stimulates transcription activation of the protooncogene c-fos upon stimulation by serum growth factors and mitogens. On the other hand JNKs phosphorylate and activate the c-Jun protein directly (Su et al., 1994). The increased transcription of the c-fos gene and the increased activity of c-jun protein results in greatly enhanced transactivation of AP-1 responsive genes.

While it stands to reason that the AP-1 complex is not the only transcription factor activated by the tyrosine kinase receptor signal transduction pathways, it is most probably one of the nodal points where many of the different signals converge.

Recent studies probing signalling pathways activated in response to interferons (IFNs) and cytokines expose a much more direct means of communication to the nucleus than the complex cascade of kinases discussed above. Receptors for IFNs lack intrinsic tyrosine kinase activity but their activation has been shown to induce the tyrosine phosphorylation and activation of several IFN-stimulated gene factors (ISGFs). ISGFs are transcription factor complexes that when phosphorylated, rapidly translocate to the nucleus and induce transcription of target genes (Montminy, M., 1993; Larner et al., 1993; Shuai et al, 1993). Components of ISGF complexes include the Stat proteins (signal transducers and activators of transcription) stat 84, 91 and 113. The kinases responsible for the phosphorylations belong to the family of the non-receptor protein tyrosine kinases and are called JAKs (just another kinase). Family members include JAK1, 2 and Tyk2 (Muller, M. et al., 1993). Thus activation of JAKs by interferon binding to its receptor leads to phosphorylation of Stat proteins capable of direct translocation to the nucleus and transcriptional activation of target genes. An important note highlighting the potential cross talk of various signalling pathways is the finding that Stat proteins are themselves SH2 containing proteins that can interact with phosphotyrosine residues of receptor protein tyrosine kinases (RPTKs) (Fu, X-Y. and Zhang, J-J., 1993).

Therapeutic potential of DNA MeTase inhibition and selective inhibition using antisense technology

DNA methyltransferase as a therapeutic target

There are several findings that suggest that not only is DNA MeTase involved in oncogenic transformation but that it could serve as an ideal therapeutic antitumor target. First, cancer cells often exhibit greatly increased levels of DNA MeTase activity (Kautiainen and Jones, 1986) and DNA hypermethylation as discussed above. Second, because the level of MeTase activity is the key determinant of the pattern of DNA methylation, partial inhibition of DNA methyltransferase activity should result in a change in the methylation pattern. If aberrant hypermethylation in cancer cells is caused by overexpression of the MeTase, then partial inhibition of methylation should restore the original pattern. Third, partial inhibition of DNA MeTase activity is potentially non toxic since mice heterozygous for the DNA MeTase null mutation and indistinguishable from wild type litter mates (Li et al., 1992) even though DNA Methylation is reduced by 50%. Fourth, inhibition of DNA MeTase is not merely cytostatic or cytotoxic but can alter the genetic program of a cell and take it to a more differentiated state (LEUKEMIA, vol 7 supplemental monograph 1, 1993).

The most commonly used DNA methyltransferase inhibitor is the cytosine analog 5-azaCytidine (5-azaC) or its deoxy analog, 5-aza-2'-deoxycytidine (5azaCdR, Decitabine) which contains a nitrogen substitution of carbon 5. 5-azaC inhibits methylation only after its nucleotide derivative is incorporated into the DNA. Wu and Santi suggested that an irreversible covalent complex is formed between the DNA methyltransferase enzyme and carbon 6 in the cytosine moiety. Methylase activity in the nucleus is depleted and replication proceeds in the absence of methylation (Jones, 1984). As mentioned previously a problem with interpreting the results obtained with 5-azaC and a major limitation of its therapeutic potential is the fact that 5-azaC is a nucleoside analog which is toxic at high concentrations in vivo and in vitro (Jones, 1984). 5-azaC inhibits t- RNA methylation,

protein synthesis and DNA replication and might inhibit other enzymes that use pyrimidines as substrates such as cytidine deaminase, cytidine kinase and ribonucleotide reductase. 5-azaC is a weak mutagen in bacterial and eukaryotic systems and can cause chromosome damage at high concentrations (Jones, 1984). The gene-induction property of 5-azaC have been used to treat sickle cell anemia (Charache et al., 1982) or thalassemia (Ley et al., 1982). The normal developmental program involves silencing and methylation of the fetal g globin. 5-azaC can cause demethylation and activation of the fetal g globin gene and compensate for the defect in the adult b-globin. These clinical trials however were terminated because of the toxic side effects of 5-azaC.

New specific inhibitors of DNA methyltransferase are required to realise the full therapeutic potential of anti MeTase therapy. The advent of antisense technology enables one to design antisense oligonucleotides that are specific to DNA methyltransferase mRNA sequences. Results presented in this thesis indicate that they might possess anticancer activity. The recent resolving of the crystal structure of the HhaI methylase (Koomar et al., 1994) presented a detailed atomic structure of the DNA methyltransferase and will enable the rational design of highly specific antagonists. These novel antagonist will be potential candidates for anticancer and gene induction therapy. The potential advantage of anti MeTase therapy over alternative chemotherapy approaches is that it targets a potential regulator of the cancer state rather than nonspecifically targeting replicating cells.

Antisense Technology

Prior to the evolution of modern molecular biological techniques, DNA and RNA were used as potential therapeutic agents. DNA from various sources was shown to posses antitumorigenic potential that was dependent on size, base composition and secondary structure (Glick, 1967; Glick and Goldberg, 1965). Much of the work with RNA as therapeutics focused on the ability of these polynucleotide to induce interferon (Isaacs et al, 1963). In stark contrast to the use of polynucleotides as general inducers of cellular functions is their more recent use as rationally designed inhibitors of genetic information.

The rational design of such inhibitors derives from an understanding of nucleic acid structure and function and dependent on Watson-Crick hybridisation (Watson and Crick, 1953). In addition, the demonstration that nucleic acid hybridisation is feasible (Gillespie and Spiegelman, 1965) along with improvements in diagnostic probe technology (Thomson and Gillespie, 1990) both paved the way for the development of the antisense concept. An antisense RNA or DNA molecules is so named because it is complementary in sequence to the RNA coding for a specific protein. Because of this complementarity, hybridisation of the antisense molecule to the protein coding RNA is possible through Watson and Crick base pairings. This hybridisation is thought to interfere with or modulate the flow of genetic information from gene to protein, in effect, altering the intermediary metabolism of RNA.

The precise means by which antisense oligonucleotides exert their biological effects are complex and currently little is known about the mechanisms invoked following oligonucleotide binding to its mRNA target. However there are reports of antisense activities functioning at many different levels along the path to mature protein production. Oligonucleotides may interact with complementary DNA sequences in the genome and inhibit either initiation or elongation of transcription, by blocking interactions with critical factors involved in in this process (Gasparro et al., 1990; Gasparro et al. 1989). The above mechanism of transcriptional arrest necessitates the denaturation of the double stranded DNA duplex in order for the complementary antisense molecule to reach its target. This denaturation is a high energy process, thus attempts have been made to inhibit transcription by interacting with non denatured double stranded DNA. Such interactions would lead to the formation of triple stranded DNA (triple helix), by non Watson and Crick hydrogen bonding. However because the interaction is through weak hydrogen bonds other than Watson and Crick (Felsenfeld et al, 1957) both the strength of the interaction and the specificity to which the molecules can recognise the target are dramatically reduced. In fact, oligonucleotides that have been shown to form triple helix structures generally are composed of long stretches of purines that base pair with purines in the target DNA duplex (Cooney et al. 1988). Another key step in the intermediary metabolism of most mRNAs is the removal of intronic sequences by splicing. Proper

splicing of the message requires sequence and structure specific interactions with the proteins of the splicing machinery. Therefore, oligonucleotides directed at splice-acceptor sites may prevent proper interaction with splicing proteins and inhibit the production of mature mRNA. Recently an oligonucleotide was reported that could actually induce alternative splicing, suggesting that oligonucleotide may be effective in cases where splicing defects occur (Dominski and Kole,1993). Most antisense oligonucleotides that have been designed and tested have attempted to induce translational arrest by interactions at the translation initiation codon. The region of complementarity of the oligonucleotide is designed to flank the translation initiation codon on both sides however the exact position of the initiation codon within the oligonucleotide can vary greatly. Hybridisation in the region of the initiation codon is thought to inhibit the proper interaction of the mRNA with components of the translation machinery thus stalling translation. The stalling of translation may also allow factors involved in the degradation of mRNAs greater access to their substrates. Target RNA molecules that have been reported to be inhibited by translational arrest include HIV (Agrawal et al, 1988), n-myc (Rosolen et al., 1990) as well as many cellular genes (Vasanthakumar and Ahmed, 19989; Sburlati et al., 1991; Zheng et al., 1989; Maier et al., 1990). RNA molecules can adopt a variety of energetically favourable three-dimentional structures, which are thought to influence processes such stability, poly adenylation and transport (Baskerville and Ellington, 1995). Disruption of these secondary structures by oligonucleotides could potentially modify one or more of these steps in RNA metabolism. An example of such a strategy is the disruption by complementary oligonucleotides to the stem-loop structure present in all RNA species of HIV, the TAR element. Oligonucleotides were shown to bind TAR and disrupt the structure and inhibit TAR-mediated production of a reporter gene (Vickers et al.1991).

Another component of antisense oligonucleotide mediated gene regulation is the enzyme RNase H. RNase H is a ubiquitous enzyme that degrades the RNA strand of an RNA-DNA duplex. It is present in diverse organisms from viruses to humans (for review see Crouch and Dirksen, 1985). Although RNase H activity is greatest in the nucleus of cells other cellular functions such as inactivation of viral sequences have been suggested (Crum, et al., 1985). RNase H activation by oligonucleotides is thought to be the "terminating"

mechanism by which many of these function, for this reason the kinetics of activation and the precise recognition elements for RNase H are being investigated (Walder and Walder, 1988). Recently, oligonucleotides comprised of methylphosphonate backbones have been shown to bind to their target and increase RNase H activation over non modified olgonucleotides (Quartin,1989). Conversely, oligonucleotides that contain 2' methoxy modifications bind equally well to their targets yet do not support RNase H activity (Schatz, et al., 1990; Hogrefe et al., 1990; Inoue et al.,1988). This later class of molecule has potential to induce alternate splicing because it does not degrade the RNA template.

It became apparent at an early stage that advances in the chemistry of olignucleotides would be required if they were to be eventually used as therapeutics because of their rapid degradation by nucleases (Wickstrom, 1986). The most effective modified oligos in terms of stability were the phosphate analogs, the phosphorothioates. These molecules showed great resistance to nuclease mediated degradation (De Clerquet al.,1969). Recent efforts to modify the chemistry of oligonucleotides aim to increase stability to a greater extent, increase delivery by modifying the lipophillicity of the compounds, and to generate chimeric molecules that take advantage a receptor mediated cellular uptake systems (for review see Crooke and Lebleu, 1993).

The long term therapeutic potential of antisense technology remains unclear, however encouraging results from several laboratories in tissue culture experiments and in animal models warrant continued efforts.

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Regulation of the DNA Methyltransferase by the Ras-AP-1 Signaling Pathway*

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Using deletion analysis and site-specific mutagenesis to map the 5′ regulatory region of the DNA methyltransferase (MeTase) gene, we show that a 106-bp sequence (at −1744 to −1650) bearing three AP-1 sites is responsible for induction of DNA *MeTase* promoter activity. Using transient cotransfection chloramphenicol acetyltransferase assays in P19 cells, we show that the DNA *MeTase* promoter is induced by c-Jun or Ha-Ras but not by a dominant negative mutant of Jun, ∂9. The activation of the DNA MeTase promoter by Jun is inhibited in a ligand dependent manner by the glucocorticoid receptor. Stable expression of Ha-Ras in P19 cells results in induction of transcription of the DNA *MeTase* mRNA as determined by nuclear run-on assays and the steady state levels of DNA MeTase mRNA as determined by an RNase protection assay. These experiments establish a potential molecular link between nodal cellular signaling pathways and the control of expression of the DNA *MeTase* gene. This provides us with a possible molecular explanation for the hyperactivation of DNA MeTase in many cancer cells and suggests that DNA *MeTase* is one possible downstream effector of Ras.

The establishment of a pattern of DNA methylation of CpG dinucleotide sequences is critical for normal development of vertebrates (1). It is well established that regulated changes in the pattern of DNA methylation occur during development (2, 3) and cellular differentiation (4), and that aberrant changes in the pattern of methylation occur during cellular transformation (5–7). We have previously suggested that one mode of control of the pattern of methylation is at the level of regulation of the DNA *methyltransferase* (*MeTase*)[1] gene which encodes the activity that catalyzes the transfer of methyl groups to DNA (8, 9). If the level of DNA MeTase activity in the cell is an important determinant of DNA methylation patterns, then it should be coordinated with cell growth and development. As the 5′ region of the DNA *MeTase* was recently cloned (10), we can now address the question whether it contains such regulatory elements.

Many cancer cell lines express high levels of DNA MeTase activity (11) and human colon carcinoma cell lines have been shown to express dramatically higher levels of DNA *MeTase* mRNA than normal cells in a manner that correlates with the state of malignancy of the cell (12). While some sites have been shown to be hypomethylated in cancer cells (5), critical regions of the genome are hypermethylated, such as CpG islands (13), candidate tumor suppressor genes (14), cell type-specific genes (15), genes responsible for terminal differentiation such as *MyoD* (16), and areas of the genome associated with tumor suppression (17). Several lines of evidence suggest that this methylation plays an important role in the inactivation of genes in tumor cells and the process of cellular transformation: (*a*) *in vitro* methylation of CpG islands results in inactivation of these genes following transfection (18) and (*b*) treatment of cells with the methylase inhibitor 5-azacytidine (14, 19) or expression of an antisense to the DNA *MeTase* mRNA results in reactivation of this class of genes (20). Recent experiments have shown that forced expression of an endogenous DNA *MeTase* cDNA in NIH 3T3 cells leads to their transformation (21) and expression of an antisense to the DNA *MeTase* in the adrenocortical carcinoma cell line Y1, or treatment of Y1 cells with 5-azacytidine leads to reversal of transformation.[2] The molecular mechanisms responsible for the increased DNA methylation activity in the process of cellular transformation are unknown. One possible explanation is that the regulatory elements of the gene are responsive to some oncogenic signaling pathways (23) in addition to the cell cycle regulation of DNA *MeTase* gene expression which occurs mainly at the post-transcriptional level (24).

The DNA *MeTase* 2-kb 5′ upstream region bears one consensus AP-1 site located at −1744 and six AP-1 sites with one or two mismatches (10). This study tests the hypothesis that overexpression of signaling pathways leading to activation of Jun (25–27) can result in hyperinduction of the DNA *MeTase* promoter.

MATERIALS AND METHODS

Cell Culture and CAT Assays—P19 cells (from Dr. McBurney) were plated at a density of 8×10^4/well in a six-well tissue culture dish (Nunc) and transiently transfected with the appropriate amounts of plasmid DNA using the calcium phosphate precipitation method as described previously (10, 28). CAT assays were performed in triplicate as described previously (20, 29). For stable transfections, (1×10^6) P19 cells were plated on a 150-mm dish (Nunc) 15 h before transfection. The pZEM^{ras} expression vector bearing a 2.3 *Bam*HI-*Eco*RI fragment encoding v-Ha-*ras* from the MMTV-v-Ha-*ras* plasmid or the pZEM control vector (10 μg) (30) were cointroduced into P19 cells with pUCSVneo (1 μg) as a selectable marker by DNA mediated gene transfer using the calcium phosphate protocol (28). G418-resistant cells were cloned and propagated in selective medium (0.5 mg/ml G418, Life Technologies, Inc.).

Plasmid Construction—The physical maps of the deletion constructs are presented in Fig. 3 and the sequences deleted in the different constructs are listed in Table I. The construction of pMet CAT+(−2.3) and pMet CAT+ ∂1 was described previously (10). To generate pMet

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[1] The abbreviations used are: MeTase, methyltransferase; CAT, chloramphenicol acetyltransferase; bp, base pair(s); kb, kilobase(s).

[2] R. A. MacLeod and M. Szyf, submitted for publication.

TABLE I
Deletion constructs of the DNA MeTase promoter

The sequences of the 5' region of the DNA *MeTase* promoter that were removed in each of the deletion constructs described in Fig. 3. The first number indicates the 5' boundary of the deleted sequence (relative to the transcription initiation site (10)) and the second number indicates the 3' boundary.

Deletion construct (∂) no.	Bases deleted			
	From	To	From	To
1.	−2.0	−0.18		
2.	−2.0	−0.39		
3. 4. 5	−1.35	−0.39		
6. 7. 8	−2.0	−1.75	−1.65	−0.39

CAT+ ∂6, pMet CAT+ ∂7 M/Wt and the pMet CAT+ ∂8 Wt/M, the sequence encoding −1.75 to −1.65 of the DNA *MeTase* 5' (10) was amplified using the 5' oligo containing the AP-1 site at −1744 (5'-AATGCAGCATGACTCATGCT-3') and a 3' oligo encoding the AP-1 site at −1650 (5'-AGGAGCTGTCAGTCAGGGTC-3'). To generate the M/Wt version, the 5' oligo was the same except that the sequence encoding the AP-1 site (underlined in the sequence presented above) included in the oligo was mutated to TGAGGTCA. To generate Wt/M the 3' oligo was mutated so that the antisense 3' AP-1 site was changed to TGCCTCA. The fragment was amplified from 10 ng of SKmet+ (10) using Hot tub (Amersham Corp.) and its recommended buffer using the following cycle: 1) 95 °C 5 min, 2) 45 °C 1 min, 3) 95 °C 1 min, 4) 55 °C 1 min, 5) 72 °C 0.5 min, followed by 20 cycles of steps 3–5. The 0.1-kb polymerase chain reaction fragment was subcloned into PCRII (InVitrogen), verified by sequencing, excised by *Xba*I-*Hin*dIII, and inserted upstream to −0.39 in pMetCAT as indicated in Fig. 3 and Table I. The expression vectors encoding the human *glucocorticoid receptor* (HGCRC) (31) c-*jun* (RSV-cJun) (26), ∂9 v-*jun* (32) were previously described. The *jun* constructs were kindly provided by Dr. M. Karin. To construct pZEM^{ras}, we subcloned a 2.3 *Eco*RI-*Bam*HI fragment encoding v-Ha-*ras* from the MMTV-v-Ha-*ras* construct (33) into the *Bgl*II site of pZEM (30) after transforming the *Eco*RI site into a *Bam*HI site using a *Bam*HI linker (Life Technologies, Inc.).

Gel Retardation Assays—Pairs of complementary 21-mer oligodeoxynucleotides were annealed and 0.4 μg of the double-stranded oligodeoxynucleotides were end-labeled with [γ-^{32}P]ATP (Amersham Corp.) using T4 polynucleotide kinase (Boehringer Mannheim) and purified by excision from an 8% polyacrylamide gel (28). Binding assays were performed by incubating 1 ng (10^3 cpm) of an end-labeled oligomer with 3 μg of crude nuclear extract from P19 cells (24) as described by Ausubel *et al.* (28).

DNase Footprinting Assays—DNase footprinting reactions were performed as recommended by Promega and as described in Ausubel *et al.* (28). A 648-bp *Bam*HI-*Sau*3AI fragment containing four putative AP-1 binding sites (at −1744, −1650, −1547, and −1514) (10) was cloned at the *Bam*HI site of the pGEM3 vector (Promega), digested with *Sma*I (Boehringer Mannheim), treated with calf intestinal phosphatase (Boehringer Mannheim), end-labeled with [γ-^{32}P]ATP (Amersham Corp.) using T4 polynucleotide kinase (Boehringer Mannheim), and then subjected to *Eco*RI digestion. The radiolabeled fragment (2×10^4 dpm) was incubated with 1 footprint unit of the cloned and bacterially expressed c-Jun (Promega), and the binding reaction was subjected to DNase I digestion (0.15 unit) for 3 min at room temperature.

RNase Protection Assays—RNA was prepared from exponentially growing cells using standard protocols. RNase protection assays were performed as described in reference 10 using a 0.7-kb *Hin*dIII-*Bam*HI fragment (−0.39 to +0.318) as a riboprobe (probe A in Rouleau *et al.* (10)).

Nuclear Run-on Assays—Nuclei were prepared from 3×10^6 exponentially growing pZEM and pZEM^{ras} transfected P19 cells and were incubated with [α-^{32}P]UTP (800 Ci/mmol) as described previously (24). The transcribed ^{32}P-labeled RNA (1×10^6 dpm/sample) was hybridized with pSKMet5' plasmid (10) and SK plasmid as a control that were immobilized onto a Hybond-N+ filter as described previously (24).

RESULTS

c-Jun and Ha-Ras Transactivate the DNA MeTase Promoter—To test the hypothesis that the DNA *MeTase* promoter is activated by high levels of AP-1 activity we cotransfected a fusion construct expressing the CAT reporter gene under the direction of 2 kb of the DNA *MeTase* 5' upstream region (pMetCAT+) containing the minimal promoter and the AP-1 recognition sequences (see physical map in Fig. 3) (10), with expression vectors encoding the protooncogene c-*jun* (RSV-c-*jun*) (26) into P19 cells. Recent results have demonstrated that activated Ras indirectly induces transcription of genes that are regulated by promoters that contain AP-1 recognition sequences and that this induction is mediated by activation of c-Jun by phosphorylation (26). To test the hypothesis that activation of the Ras signaling pathway can induce DNA *MeTase* promoter activity, we cotransfected an expression vector encoding the Ha-Ras protein (pZEM^{ras}) and pMetCAT+. To rule out variability in culture conditions and transfection efficiencies as an explanation for the induction of pMetCAT+ by Jun and Ras, we performed multiple experiments (each different experiment was performed in triplicate) at different times using different cultures of P19 cells. In each experiment an increasing dose of either pZEM^{ras} or RSV-c-*jun* (2–10 μg) was used, the maximal fold induction was determined and the average fold induction for all experiments was calculated. The results shown in Fig. 1*A* demonstrate that both Jun and Ras can induce the DNA MeTase promoter (10–30-fold for Jun and 8–15-fold for Ras) from very low basal levels (the results are of statistical significance as determined by a t test, $p < 0.001$). This induction of pMetCAT+ is not a nonspecific enhancement of transfection efficiency or transcription from plasmid DNA because all transfections had equal amounts of total plasmid DNA.

To demonstrate that this transactivation of the DNA *MeTase* promoter by Ras and Jun is dependent on functional Jun activity rather than a nonspecific effect, we cotransfected pMetCAT+ (4 μg/ml) and increasing concentrations of a transdominant negative mutant of Jun, ∂9 which lacks the transactivation domain of Jun but expresses the DNA binding domain (32). The total concentration of transfected plasmid DNA was maintained constant by adding SK plasmid DNA. As demonstrated in Fig. 1*B*, ∂9 cannot transactivate the DNA *MeTase* promoter. Coexpression of ∂9 with Jun (4 μg/ml) results in a dose-dependent inhibition of transactivation by Jun as expected. When ∂9 is coexpressed with Ras (4 μg/ml), the induction of the DNA *MeTase* promoter by Ras is repressed even at low concentrations of the ∂9 plasmid. This demonstrates that both Ras and Jun induce the DNA *MeTase* by a common pathway.

The Glucocorticoid Receptor Inhibits the Transactivation of the DNA MeTase Promoter by Jun—One important characteristic of AP-1 induced genes is the modulation of this induction by the glucocorticoid receptor (35, 36). Nuclear receptors such as the glucocorticoid receptor serve as modulators of differentiation and cellular growth in many systems, for example, the well characterized antimitotic and cytotoxic effect of glucocorticoid on T cells (37). Several groups have recently established that nuclear receptors can modulate the activity of AP-1 complexes by binding overlapping AP-1 recognition sequences (38) or by protein-protein interactions with Jun (35, 36). These interactions have been postulated to explain the antimitotic effects of glucocorticoids. We tested the hypothesis that the glucocorticoid receptor can modulate the transactivation of DNA *MeTase* by AP-1 in a ligand dependent manner. We transiently transfected pMetCAT+ (10 μg/ml) with the glucocorticoid receptor expression vector HGCRC (10 μg/ml) in the presence or absence of inducing concentrations of Jun (4 μg/ml) and in the presence or absence of 0.1 μM dexamethasone. Fig. 2 is an average of three independent experiments that were performed at different times using different cultures of P19 cells. As observed in Fig. 2, dexamethasone *per se* does not affect either the basal level of activity of the DNA *MeTase* promoter or its induction by Jun, most probably because of lack of ex-

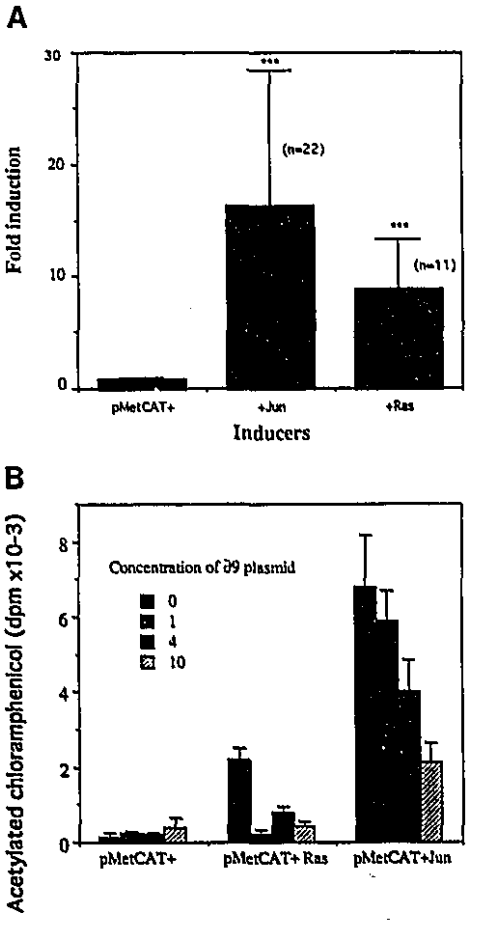

FIG. 1. **Fos and Ras induce the mouse DNA *MeTase* promoter.** *A*, P19 cells, an embryonic carcinoma cell line (22), were transfected with 10 μg of pMetCAT+ (see Fig. 3 for physical map) and increasing concentrations (1–10 μg) of expression vectors expressing c-*jun* (RSV-c-*jun*) (29) or Ha-*ras* (pZEM^{ras}). Cells were harvested after 48 h and CAT activity was determined as described under "Materials and Methods." Each experiment was performed in triplicate. Twenty-two independent experiments using independent cultures of P19 cells at different times were performed for the Jun expression vector and eleven experiments with the Ha-*ras*. The maximal fold induction above the values obtained with pMetCAT+ was determined per construct for each experiment. Each value is presented as mean of the values obtained in all experiments ±S.D. The difference between Jun- and Ras-induced pMetCAT + expression and the noninduced control is highly significant $p < 0.001$. *B*, P19 cells were transfected with either pMetCAT+ (4 μg) and SK−, pMetCAT+ (4 μg) and RSV-cJun (4 μg), or pMetCAT+ (4 μg) and pZEM^{ras} (4 μg) and an increasing concentrations of a vector encoding the transdominant negative mutant of Jun ∂9-v-Jun which was kindly provided by Dr. B. Wasylik (32). The total concentration of plasmid DNA was kept constant by adding SK plasmid to the transfection mix. The values are presented as an average of three determinations ±S.D.

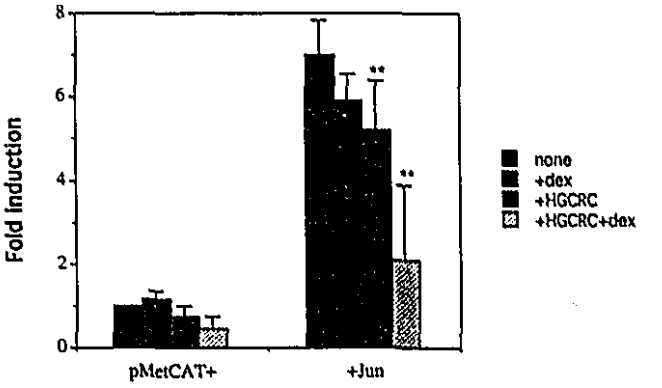

FIG. 2. **Inhibition of Jun-dependent induction of the DNA *MeTase* promoter by the glucocorticoid receptor.** *A*, Ten μg of pMetCAT+ were cotransfected in triplicate into P19 cells with either SK plasmid (pMetCAT+), SK plasmid and 10 μg of the *human glucocorticoid receptor* expression vector (*HGCRC*) (27), RSV-c-*jun* (+*Jun*), or RSV-c-*jun* and the HGCRC (as indicated in the figure). One set of transfectants was treated with 0.1 μM of dexamethasone (Sigma) for 24 h before harvesting (+*dex*) and CAT activity was determined in extracts prepared from the different transfected cells 48-h posttransfection as described under "Materials and Methods." The fold induction over pMetCAT+ (10 μg with SK−) was determined. The experiment was repeated three different times using three different cultures of P19 cells. The values are presented as an average of three independent experiments ±S.D. The statistical significance of the difference between Jun and Jun + HGCRC transfectants and Jun as well as the statistical difference between dexamethasone treated HGCRC + Jun and Jun transfectants was determined using a *t* test, $p < 0.05$.

pression of the glucocorticoid receptor in P19 cells. Cotransfection of the glucocorticoid receptor has a limited inhibitory effect on the expression of the basal and Jun-induced promoter activity resulting most probably from low concentrations of dexamethasone in the the growth medium. However, a significant ($p < 0.05$) inhibition of DNA *MeTase* promoter activity is observed in the presence of both the glucocorticoid receptor and its ligand demonstrating that the glucocorticoid receptor inhibits the expression of the DNA *MeTase* promoter in a ligand dependent manner. This demonstrates that the inhibition is not a nonspecific effect of the glucocorticoid plasmid or dexamethasone. This experiment therefore suggests that transactivation of DNA *MeTase* by Jun can be modulated by the glucocorticoid receptor and that the DNA *MeTase* can be responsive to both the Ras signaling pathway as well as to nuclear receptors.

Deletion Mapping of AP-1 Responsive Elements—To delineate the sequences responsible for regulation of the DNA *MeTase* by AP-1 and to determine whether induction of DNA *MeTase* promoter by AP-1 is mediated by direct interaction of AP-1 with the DNA *MeTase* 5′ region, we carried out a deletion analysis of the 2-kb 5′ upstream region of the DNA *MeTase* (Fig. 3). The different deletion constructs (10 μg) were cotransfected with either SK plasmid or RSV-cJun (4 μg). AP-1 inducibility is lost when the 5′ region including all the potential AP-1 sites upstream to the minimal promoter at −0.18 is deleted. Very limited activity is observed when only one nonconsensus AP-1 site at −290 is present in the construct (−0.39), however, a deletion of most of the 1-kb sequence between −1.3 and −0.39 is still inducible with Jun suggesting that AP-1 inducibility is encoded between −2 and −1.35. The region contained between −2 and −1.35 does not confer inducibility in our assay when it is inserted in the antisense orientation. When both this region and the promoter are inserted in the antisense orientation to CAT, no CAT activity is detected supporting the conclusion that the activity detected in our assays is directed by the DNA *MeTase* promoter. Four potential AP-1 sites are located in the sequence contained between −2 and −1.35. The AP-1 site at −1744 (TGACTCA) is a consensus site, the site at −1650 TGACTGA and 1547 (TGACTCT) bears one mismatch and the sites at −1514 bear two mismatches with the consensus site. To test the hypothesis that AP-1 inducibility is encoded by the 5′ consensus AP-1 site at −1744 and −1650 we amplified the sequence contained between −1744 and −1650 using the primers described under "Materials and Methods" and inserted it upstream to −0.39 (Fig. 3). The 118-bp fragment encoding the two 5′ AP-1 sites confers full inducibility with Jun upon the DNA *MeTase* promoter. The 5′ AP-1 site plays a more significant role in Jun inducibility since introduction of two mismatches (TGAGGCA) into the 5′ AP-1 site (M/Wt) inhibits the inducibility of the construct while introduction of the same mutation to the 3′ site does not have the same effect (Wt/M). It

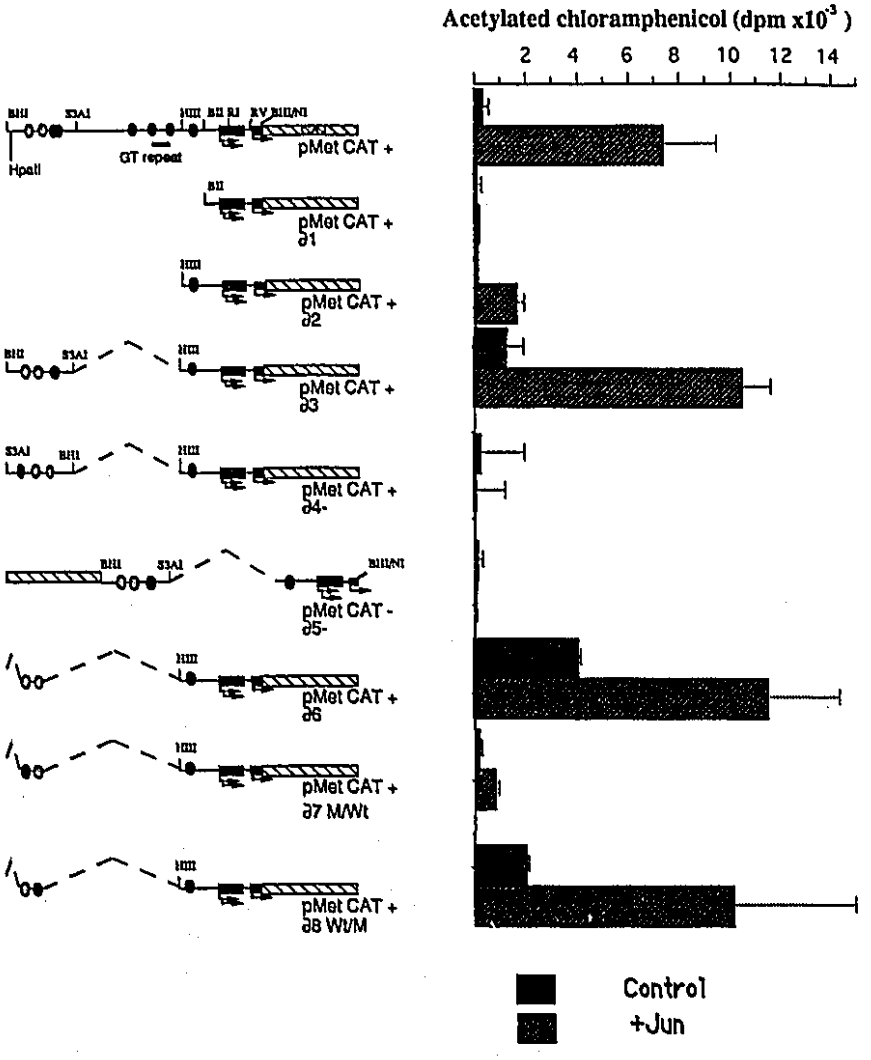

FIG. 3. **Deletion analysis of the DNA MeTase 5′ region.** The physical maps of the different deleted pMetCAT+ constructs are shown in the *right panel* relative to the original pMetCAT+ construct. Regions deleted are indicated by a *broken line*. The boundaries of the deleted sequence are indicated relative to the transcription initiation site and listed in Table I. The first number indicates the 5′ of the deleted sequence and the second number indicates the 3′ boundary. Exons are indicated by *filled boxes*, CAT sequence is *hatched*, transcription initiation sites are indicated by *horizontal arrows*, *shaded ovals* indicate AP-1 sites (forming DNA protein interactions, Fig. 4), *filled ovals* are mismatched AP-1 sites. *HIII*, *Hin*dIII recognition site; *BII*, *Bgl*II; *RI*, *Eco*RI; *RV*, *Eco*RV; *BHI/NI* is an original *Nae*I site that was modified by linkers to a *Bam*HI site, *BHI*, *Bam*HI; *S3AI*, *Sau*IIIAI. *M/Wt*, the AP-1 site at −1750, is mutated to TGAGGCA but the one at −1644 is wild type. *Wt/M*, the AP-1 site at −1750 is wild type but the 3′ site is mutated as above. The different constructs (10 μg) were transfected in triplicate into P19 cells with either 4 μg of SK plasmid (*Control*) or 4 μg of RSV-c-*jun* (*+Jun*), harvested after 48 h, and CAT activity was determined as described above. Each experiment was repeated twice using different plasmid preparations. The results are presented as averages of three determinations ±S.D.

is interesting to note that constructs bearing deletions of the sequence between −1.3 and −0.39 express a higher basal DNA *MeTase* promoter activity than the nondeleted control. This might suggest the presence of a *cis*-repressor recognition sequence encoded in this region. This repression of transactivation of DNA *MeTase* promoter by endogenous AP-1 might be an additional mechanism through which DNA *MeTase* gene expression is controlled and maintained at a basal level under normal conditions. In summary, the deletion experiments demonstrate that the control of the DNA *MeTase* by AP-1 is mediated by an interaction between Jun (AP-1) and the AP-1 site(s) located at −1744 to −1650 upstream to the transcription initiation site.

Physical Interaction between AP-1 Sites in the DNA MeTase Promoter and AP-1 Transactivation Complex—To determine whether the transactivation of the DNA MeTase by AP-1 involves direct interaction of AP-1 with the AP-1 recognition sequences in the DNA MeTase 5′ upstream region, we performed gel retardation and DNase footprinting assays. Nuclear extracts from P19 cells contain a measurable level of DNA-binding activity interacting with a ^{32}P-labeled oligomer from the 5′ MeTase region (−1753, −1734) containing an AP-1 recognition sequence from −1744, −1738 (Met5′ AP-1) (Fig. 4*A*). This DNA-protein complex can be competed out with a 10-fold excess of a nonlabeled oligomer encoding a previously characterized consensus AP-1 recognition sequence (*AP1*) (Fig. 4*A*), with an excess of nonlabeled Met5′ AP-1 oligomer but not with an oligonucleotide sequence containing an AP-1 recognition sequence with two mismatches located at −505–499 (*Met 3′ AP1*) or an oligomer encoding the recognition sequence of the transcription factor Sp1 (Fig. 4*A*). This demonstrates that the AP-1 site at −1744–1738 can specifically interact with the AP-1 complex. This DNA-protein complex could be competed with an excess of nonlabeled AP-1 but not with Sp1 or Met3′ AP-1.

To visualize the specific molecular interactions between Jun and the AP-1 binding region of DNA MeTase 5′ upstream sequences, we incubated a 648 bp fragment containing these sequences with c-Jun protein and subjected the bound products to a DNase I footprint analysis as described in materials and methods (Fig. 4*B*). Two potential AP-1 sites (TGACTCA (−1744, −1738, and TGACTGA(−1650, −1644)) were protected from DNase cleavage as indicated in Fig. 4*B*. These experiments have therefore established that the AP-1 sites in the 5′ of the DNA *MeTase* gene are functional AP-1 sites *in vitro*.

Exogenous Expression of Ras Induces Transcription of the Endogenous DNA MeTase—To determine whether induction of the Ras signaling pathway can induce the expression of the endogenous DNA *MeTase* gene, we introduced either pZEM^{ras} or pZEM into P19 cells by DNA mediated gene transfer. G418-resistant colonies were propagated and Ras expressing and pZEM colonies were identified. Three colonies bearing exogenous pZEM^{ras} or pZEM were randomly selected. The pZEM^{ras}

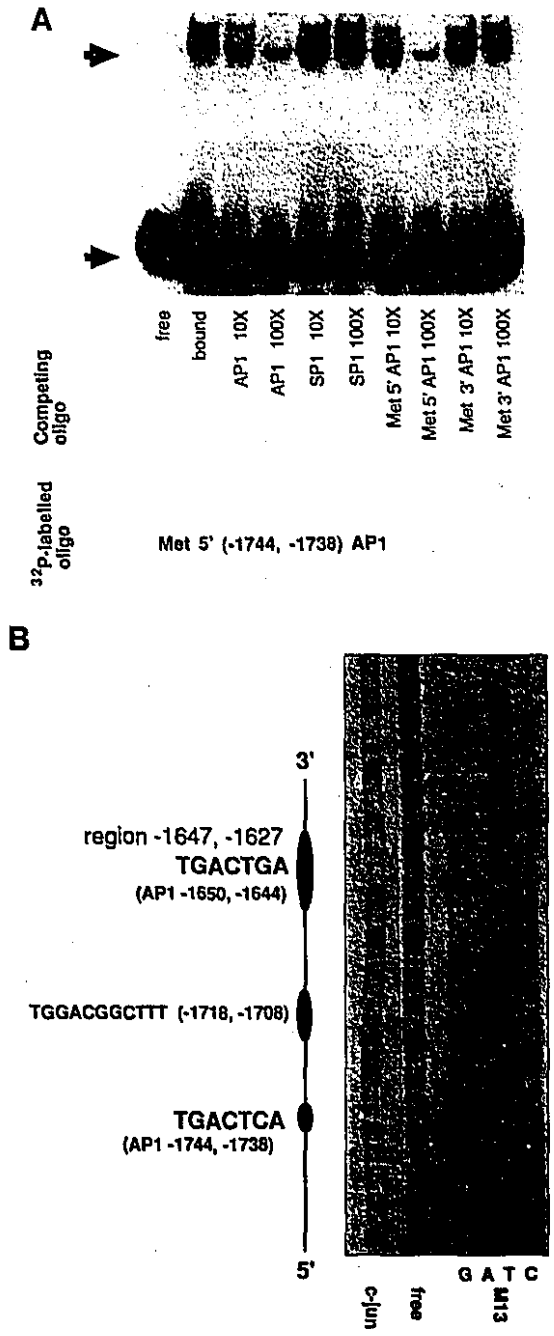

FIG. 4. **Binding of the AP-1 transcription complex to sequences in the DNA MeTase 5′ upstream region.** *A*, A 21-mer double-stranded oligomer, Met 5′ AP-1, encoding the sequence: 5′-AATGCAGCATGACTCATGCT-3′ located at (−1753, −1734) in the DNA MeTase 5′ region (the putative AP-1 recognition sequence is *underlined*) was end-labeled with [γ-^{32}P]ATP (Amersham Corp.) and incubated with 10 μg of P19 nuclear extract. For competition experiments, an excess of nonlabeled double-stranded oligomers was used. The free oligomer (*gray arrow*) and the bound AP-1 complex (*black arrow*) were separated on a native polyacrylamide gel. An excess of labeled substrate was used for all assays. The following competitors were used: (*a*), Met 3′ AP-1 is located at −299 to −275 in the DNA MeTase 5′ region and encodes the sequence 5′-GTTTTGAGGCAGGATTTTGA-3′ (the *underlined* sequence contains one mismatch with the consensus AP-1 recognition sequence); (*b*) AP-1 encodes a consensus AP-1 sequence (5′-CGCTTGATGAGTCAGCCGGAA-3′) (Promega); (*c*) Sp1 encodes the binding recognition sequence of the transcription factor Sp-1 (5′-GATCGATCGGGGCGGGGCGATC-3′). *B*, an *in vitro* DNase footprinting assay was performed on an end labeled 0.6-kb *Bam*HI-*Sau*3AI fragment containing the 5′ AP-1 region of the DNA *MeTase* gene (see Fig. 3) which was incubated with 1 footprint unit (fpu) of a bacterially expressed c-Jun protein (Promega). The AP-1-reacted fragment and a naked (nonreacted) control were subjected to cleavage by DNase I (Boehringer Mannheim) which was followed by electrophoresis and autoradiography. An M13 sequencing reaction (Pharmacia) was used as as a size marker ladder. The regions that are protected from DNase I cleavage by AP-1 are indicated. The footprints at −1744 to −1738 (TGACTCA) and −1650–1644 (TGACTGA) match the AP-1 consensus recognition sequence. The footprint at −1718 to −1708 (TGGACGGCTTT) does not correspond to the consensus AP-1 recognition sequence.

transfectants express exogenous Ras as indicated by the Northern blot presented in Fig. 5*A*. To determine whether Ras induces transcription of DNA MeTase, we prepared nuclei from exponentially growing P19 pZEM control transfectants and P19 lines transfected with Ha-*ras* and subjected them to a run-on transcription assay using [α-^{32}P]UTP. Equal amounts of labeled RNA were hybridized with either immobilized SK (as a control) or SKmet5′. The hybridized filters were exposed to autoradiography after stringent washing. The results presented in Fig. 5*B* show that the two pZEM^{ras} transfectants tested express higher levels of DNA *MeTase* transcript than the pZEM control. To determine the levels of steady state DNA MeTase mRNA, we resorted to RNase protection assays using a 720-bp riboprobe encoding the first two exons of the DNA MeTase and the transcription initiation site (10). This probe displays a group of fragments at 89–99 bp representing the two exons and two initiation sites. Equal amounts of RNA (as determined by hybridization with an 18 S oligonucleotide probe) prepared from three independent transfectants with pZEM and three independent pZEM^{ras} transfectants were subjected to a RNase protection assay as described in Rouleau *et al.* (10) (Fig. 5*C*). The results were quantified by densitometry and the quantification is presented in Fig. 5*D*. The results presented in Fig. 5*D* demonstrate that the *ras* transfectants express higher levels of DNA MeTase mRNA than the pZEM controls (10–20-fold). Two transfectants express high levels of mRNA that is initiated at multiple sites in addition to the standard sites observed with other cell lines and the controls (10). To quantify this difference in transcription rate, three independent pZEM transfectants and three independent pZEM^{ras} transfectants were subjected to a runon analysis, the level of DNA *MeTase* transcript was quantified by densitometry (Masterscan, Scanalytics) and the results were presented as an average of the three independent transfectants (Fig. 5*D*). These results demonstrate that the pZEM^{ras} transfectants express 3-fold higher levels of DNA *MeTase* transcript than the controls. The higher levels of induction of steady state levels of mRNA relative to the change in the transcription rate might either suggest that Ras affects the stability of the DNA *MeTase* mRNA in addition to its rate of transcription, or alternatively, it might reflect differences in the experimental procedures used to measure these two parameters. In summary, the results presented in this section demonstrate that transcription of the endogenous P19 DNA *MeTase* mRNA is induced by forced expression of Ras. The effects that this induction might have on DNA methylation and DNA MeTase activity is, however, complex since Ras induces an increase in demethylase activity[3] in parallel to its induction of transcription of DNA MeTase.

DISCUSSION

This report addresses the hypothesis that the activity of DNA MeTase is regulated by nodal cellular signaling pathways. While this fact by itself is not sufficient to demonstrate that regulation of DNA *MeTase* is an important cellular control point, it is consistent with that hypothesis (23, 39). We demon-

[3] M. Szyf, unpublished results.

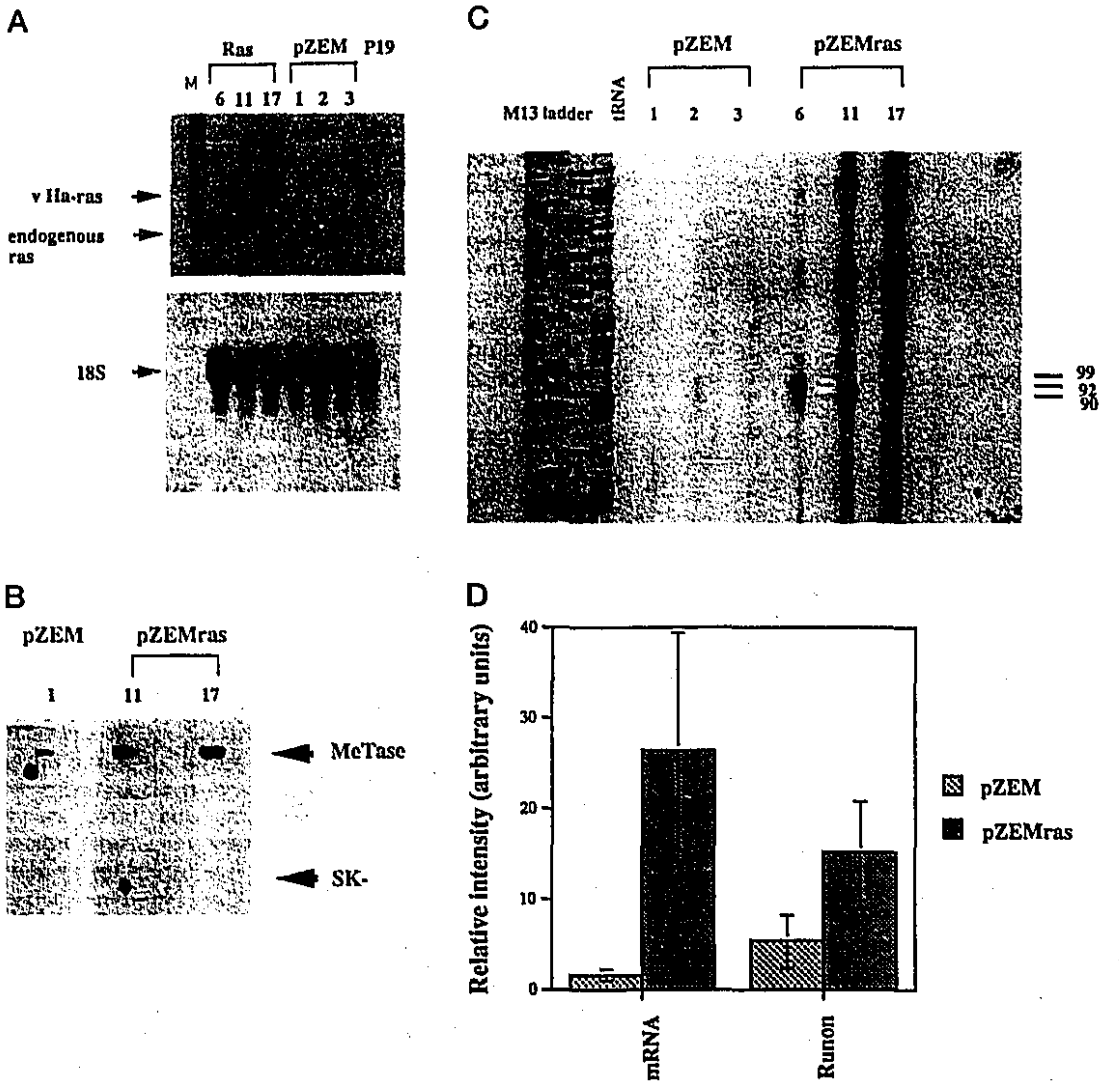

FIG. 5. **Transcription of the endogenous DNA MeTase gene is induced by forced expression of exogenous Ha-*ras*.** P19 cells were stably transfected with either (10 μg) pZEM or (10 μg) pZEM Ha-*ras* encoding the Ha-*ras* oncogene which contains activation mutations in codon 12 (Gly to Arg) and 59 (Ala to Thr) and (1 μg) pUCSVneo as a selectable marker. G418 (0.5 mg/ml) resistant colonies were selected and propagated. Preliminary Southern blot analysis using a ^{32}P-labeled Ha-*ras* probe (0.7 kb *Hin*dIII-*Pst*I fragment) has been performed to identify transfectants and three randomly picked positive clones per each transfected plasmid were used for our further studies. *A*, Northern blot analysis using a *ras* probe. The *ras* transfected panels were exposed for a short time to enable their presentation alongside with the nontransfected controls. The viral *ras* directs a 2-kb transcript that is easily distinguishable from the shorter endogenous *ras* messages (1–1.4 kb). As observed in the figure (*left panel*) the *ras* transfectants express large amounts of *ras* that is probably initiated at multiple sites in addition to the viral initiation site. The filter was stripped from the radioactivity and rehybridized with an 18 S rRNA-specific ^{32}P-labeled oligonucleotide (45) (*bottom panel*). *B*, ^{32}P-labeled RNA (1×10^6 dpm) transcribed in nuclei prepared from *ras*-transfected P19 cells (*lanes 11* and *17*) and pZEM control (*lane 1*) was hybridized with filter immobilized SKmet5′ (10) containing a DNA *MeTase* genomic fragment encoding the 5′ transcribed regions of the gene and SK plasmid as a negative control. *C*, RNase protection assay of DNA *MeTase* mRNA. RNA prepared from three P19 pZEM^{ras} transfectants (*lanes 6*, *11*, and *17*) and pZEM control transfectants was subjected to an RNase protection assay using a 700-bp riboprobe (probe A) (10) encoding the DNA MeTase genomic sequence from −0.39 to +318. The major bands representing the two major initiation sites are indicated (*92* and *90*). The first exon will give a 99-bp protected fragment. Additional minor initiation sites are markedly represented in the two *ras* transfectants that express significant levels of DNA MeTase. The experiment has been repeated twice with similar results. *D*, quantification of transcription rate and steady state mRNA levels in Ras transfectants *versus* controls. 1) Run on assays: a run on assay similar to the one presented in *B* was performed on nuclei prepared from three independent *ras* transfectants (6, 11, 17) and three control transfectants pZEM (lanes 1, 2, and 3). The intensity of the signal obtained following hybridization of the labeled RNA with SKmet5′ was determined by densitometry (Scanalytics Masterscan) and the average for the three lines is presented with the S.D. 2) mRNA- quantification of the signal obtained in the autoradiogram presented in C at the 92-, 90-, and 99-bp fragments hybridizing with DNA MeTase mRNA. The average for the three *ras*-transfected and the three pZEM-transfected lines is presented with the S.D.

strate in this study that when Ras-Jun signaling pathway is activated by either expressing high levels of exogenous Ras or Jun, the DNA *MeTase* promoter is significantly induced (Figs. 1 and 3), that this activation is mediated by AP-1 recognition sequences in the 5′ region of the gene (−175–1640) and that it is inhibited by the glucocorticoid receptor in a ligand dependent manner (Fig. 2). This inhibition is most probably mediated by protein-protein interactions with AP-1 as has been shown for other AP-1 induced promoters since the DNA *MeTase* 5′ region does not contain a full glucocorticoid recognition site (10). Forced expression of an exogenous Ha-*ras* induces transcription and an increase in the steady state levels of endogenous DNA MeTase mRNA (Fig. 5). Induction of Jun appears to be associated with induction of differentiation in P19 cells (40). Since Ras has multiple effects on P19 cells it is hard to assess the relative effect of increased DNA MeTase activity on DNA methylation patterns. Our *ras* transfectants induce high levels of demethylase activity[3] which complicates the analysis of the

effects that induction of DNA MeTase has on the pattern of methylation. Whereas other cell systems should be sought to study the effects of an increase in Ras activity on DNA methylation, P19 cells are an excellent model to dissect AP-1 and glucocorticoid responsiveness of the exogenous promoter because they do not express high levels of c-*jun* (41). Using other cell systems to study the effects induction of Ras and Jun might have on DNA methylation, we have recently shown that inhibition of the Ras-Jun pathway in an adrenocortical tumor cell line Y1 results in inhibition of DNA methylation activity and reduction in methylation levels of CpG dinucleotides.[4] What is the significance of regulation of DNA *MeTase* by Ras and Jun? One documented instance when DNA *MeTase* is markedly up-regulated is cancer; many cancer cell lines and colon cancer cells *in vivo* express significantly higher levels of DNA *MeTase* (11, 12). In this report we suggest a possible molecular route through which oncogenic pathways can lead to induction of DNA *MeTase*. DNA *MeTase* exhibits very low constitutive levels of expression (Figs. 1 and 3). This basal level of expression is responsive to the proliferative state of the cell by a posttranscriptional up-regulation as we have previously shown (24). However, in addition to this basal control it is possible that cellular signaling systems such as the one induced by Ras can induce high activities of DNA *MeTase* in a programmed manner at distinct sites and times in development. When these signaling pathways are aberrantly up-regulated as happens in many cancer cells, DNA *MeTase* promoter is induced, possibly resulting in elevation of DNA MeTase (11, 12) and the hypermethylation of specific genomic regions in cancer cells (17). While it is clear that factors other than the level of activity of DNA MeTase must play a role in shaping the methylation pattern of the genome (43), the level of DNA MeTase activity is most probably an important factor. It is possible however that in addition to AP-1, other pathways control DNA *MeTase* gene expression. What are the changes in DNA methylation that are critical for cellular transformation? Several mechanisms have previously been suggested such as silencing of tumor suppressor genes (13, 16), an increase in spontaneous mutations resulting from deamination of methylated cytosines (44) or altered regulation of replication (23). In contrast to the induction of cellular proliferation by Ras in many cell types, Ras can induce the differentiation of PC12 cells (42). Induction of DNA MeTase might play an important role in these processes as well. Although additional experiments are required to determine whether DNA MeTase plays a critical role in the transformation process as a downstream effector of Ras, the results presented in this report establish a molecular link between cellular signaling pathways and the machinery responsible for controlling the pattern of modification of the genome.

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Chapter 2: Regulation of DNA Methylation by the Ras Signaling Pathway. A.R. MacLeod, J. Rouleau and M. Szyf, *J.Biol.Chem.* **270**, 11327-11337 (1995).

In the first chapter of this thesis we demonstrated that the Ras signal transduction pathway can activate the DNA methyltransferase promoter. The next question we wished to address was whether modulation of the Ras pathway at various levels could lead to stable alterations in genomic DNA methylation patterns, and whether these newly attained methylation states correlated with the transformation state of the cell.

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Regulation of DNA Methylation by the Ras Signaling Pathway*

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We demonstrate that DNA methylation in an adrenocortical tumor cell line, Y1, is controlled by the Ras signaling pathway. Forced expression of a cDNA encoding human GAP^{120} (hGAP), a down-modulator of Ras activity or ∂9-Jun a transdominant negative mutant of Jun, in Y1 cells reverts the transformed morphology of the cells and results in a reduction in the level of DNA methylation, DNA methyltransferase (MeTase) mRNA, and enzymatic activity. Introduction of an oncogenic Ha-*ras* into the GAP transfectants results in reversion to a transformed morphology and an increase in the levels of DNA methylation and DNA MeTase activity. Transient transfection CAT assays demonstrate that the expression of DNA MeTase promoter in Y1 cells is regulated by Ras and AP-1. These results establish a molecular link between a major signaling pathway involved in tumorigenesis and DNA methylation.

Many lines of evidence have established that activation of the Ras signaling pathway is one nodal point in many pathways leading to cellular transformation (1). Recent data have suggested that one possible downstream effector of Ras-triggered cellular transformation is the AP-1 transcription transactivation complex (2). One candidate for playing a role in this pathway is the DNA methyltransferase (MeTase)[1] which bears a number of consensus AP-1 sites in its 5' regulatory region (3, 4) that are responsible for induction of its promoter activity (5). The DNA MeTase is responsible for methylation of cytosine residues located in the dinucleotide sequence CpG (6, 7). The vertebrate genome is methylated at 80–90% of the CpGs (6, 7). Maintaining a pattern of DNA methylation is critical for maintaining the differentiated state of a cell (8) and for completing the normal process of development (9). The DNA MeTase activity is tightly regulated with the growth state of cells (10, 11), and previous models have suggested that the level of DNA MeTase activity can play an important role in determining the pattern of methylation (6, 12, 13). The fact that genes can be inactivated by methylation as well as that wide areas of the genome, such as CpG islands, are methylated in cancer cells is well established (14, 15). A CpG island-rich area on chromosome 17p, which is reduced to homozygosity in lung and colon cancer, as well as regions of chromosome 3p, that are consistently reduced to homozygosity in lung cancer, are hypermethylated (16). One possible explanation for these changes in the DNA methylation pattern of cancer cells is the dramatic elevation of DNA methyltransferase (DNA MeTase) activity observed in many cancer cells (17, 18). Recent evidence suggests that forced expression of an exogenous DNA MeTase cDNA can transform N1H 3T3 cells (19), and we have shown that inhibition of DNA methylation by expression of a DNA MeTase antisense RNA results in reversion of the transformed phenotype of Y1 adrenocortical carcinoma cells (20). What is the molecular link between the known triggers of cellular transformation and DNA hypermethylation? This paper tests the hypothesis that the level of methylation of DNA in cancer cells is controlled by Ras.

Y1 is an adrenocortical tumor cell line that was isolated from a naturally occurring adrenocortical tumor in an LAF1 mouse (21) which bears a 30- to 60-fold amplification of the cellular protooncogene c-Ki-*ras* (22). It stands to reason that c-Ki-*ras* plays an important role in triggering the transformed state of Y1 cells. This cell line expresses many adrenal specific genes but has specifically repressed, by a *cis*-modification event, the *21-hydroxylase* gene (C21) (23). This repression is associated with heavy methylation of the C21 gene locus and the cells still possess the capacity to specifically *de novo* methylate a transfected C21 gene (24). Y1 can therefore serve as an excellent model system for dissecting the molecular mechanisms that are responsible for hypermethylation and gene repression events that are associated with the oncogenic process. In this paper we demonstrate that down-regulation of Ras by forced expression of human Ras-GTPase activating protein (GAP) (25) and down-regulation of Jun by expressing the Jun transdominant mutant ∂9-Jun (26) results in inhibition of DNA methylation. This inhibition is reversed when an oncogenic Ha-*ras* (27) is introduced into GAP transfectants.

MATERIALS AND METHODS

Cell Culture, DNA-mediated Gene Transfer, and Tumorigenicity Assays—Y1 cells (21) were a gift from Dr. B. Schimmer (23) and were maintained as monolayers in F-10 medium which was supplemented with 7.25% (v/v) heat-inactivated horse serum and 2.5% (v/v) heat-inactivated fetal calf serum (Immunocorp, Montreal). All other media and reagents for cell culture were obtained from Life Technologies, Inc. Y1 cells (1×10^6) were plated on a 150-mm dish (Nunc) 15 h before transfection. The CDM8-hGAP^{120} expression vector encoding the cDNA of the human Ras-GTPase activating protein (26) (10 μg) or the lexA-∂9-Jun expression plasmid (a gift from Dr. Wasylik) (26) were cointroduced into Y1 cells with pUCSVneo (1 μg) as a selectable marker by DNA-mediated gene transfer using the calcium phosphate protocol, and G418 resistant cells were cloned in selective medium (0.25 mg/ml G418) (29). To generate the double GAP and Ha-Ras transfectants, GAP transfectants were cotransfected with pZEM-*ras* (encoding the oncogenic Ha-*ras* (27) under the metallothionein promoter in the pZEM vector (30)) and pGK-*hyg* encoding the hygromycin resistance gene (a kind gift from Dr. McBurney (31)) and double hygromycin (0.5 μg/ml, Calbiochem) and G418-resistant cells were cloned in selective medium. Anchorage independent growth in soft agar (1×10^3 cells) was analyzed as described previously (32).

DNA and RNA Analyses—Genomic DNA was prepared from pelleted nuclei, and total cellular RNA was prepared from cytosolic supernatants using standard protocols (29, 33). To quantify the relative abun-

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[1] The abbreviations used are: MeTase, methyltransferase; GAP, GTPase activating protein; CAT, chloramphenicol acetyltransferase; kb, kilobase(s); bp, base pair(s).

dance of DNA MeTase mRNA, total RNA (3 μg) was blotted onto Hybond-N⁺ using the Bio-Rad slot blot apparatus. The filter-bound RNA was hybridized to a ^{32}P-labeled 1.31-kb cDNA probe encoding the putative catalytic domain of the mouse DNA MeTase (3170–4480) (34) and exposed to XAR film (Kodak). The relative amount of total RNA was determined by measuring the signal obtained after hybridization to an 18 S rRNA-specific ^{32}P-labeled oligonucleotide (23). The autoradiograms were scanned with a Scanalytics scanner (one D analysis), and the signal at each band was determined and normalized to the amount of total RNA at the same point. Three determinations were performed per each RNA sample.

DNA Analyses and Demethylation Assays—Two μg of DNA were incubated at 37 °C for 15 min with 0.1 unit of DNase, 2.5 μl of [α-^{32}P]dGTP (3000 Ci/mmol from Amersham), and 2 units of Kornberg DNA polymerase (Boehringer) were then added and the reaction was incubated for an additional 25 min at 30 °C (35). Fifty μl of water were then added to the reaction mixture, and the nonincorporated nucleotides were removed by spinning through a Microspin S-300 HR column (Pharmacia Biotech Inc.). The labeled DNA (20 μl) was digested with 70 μg of micrococcal nuclease (Pharmacia) in the manufacturer's recommended buffer for 10 h at 37 °C. Equal amounts of radioactivity were loaded on TLC phosphocellulose plates (Merck), and the 3′ mononucleotides were separated by chromatography in one dimension (isobutyric acid:H_2O:NH_4OH in the ratio 66:33:1). The chromatograms were exposed to XAR film (Eastman Kodak), and the autoradiograms were scanned by scanning laser densitometry (Scanalytics one-dimensional analysis). Spots corresponding to cytosine and 5-methylcytosine were quantified. To study demethylation of specific genes, genomic DNA (10 μg) was extracted from the transfected lines and subjected to digestion with *Bam*HI (Boehringer Mannheim, 25 units) followed by digestion with either 25 units of *Msp*I(M) which is insensitive to CC*GG methylation or *Hpa*II(H) which is sensitive to methylation, for 8 h at 37 °C. At least three different DNA preparations isolated from three independent passages were assayed per transfectant.

Assay of DNA MeTase Activity—To determine nuclear DNA MeTase levels, cells were maintained at the exponential phase of growth and fed with fresh medium every 24 h for at least 3 days prior to harvesting, and DNA MeTase activity was assayed as described previously (11).

CAT Assays—Y1 cells were transiently transfected and CAT assays were performed on 50 μl of cellular extract (~10 μg of protein) using [^{3}H]acetyl coenzyme A (0.5 μCi per assay (3.8 Ci/mmol)) essentially as described (36) and modified as in Ref. 3. The plasmid pMET CAT⁺ bearing the 2.3-kb 5′ upstream region of the DNA MeTase gene was described previously (3). The constructs bearing the two consensus AP-1 sites at −1744 and −1650 were described (5).

Detection of Guanine Nucleotides Bound to Ras—Assays were performed as described in Ref. 28, except for some modifications. TLC plates (polyethyleneimine, Brinkman) were prerun in distilled water before loading of samples. Samples were purified on Microcon columns (Amicon) before loading, and the chromatograms were developed in 1.3 M LiCl.

Gel Retardation Assays—A double-stranded consensus AP-1 recognition sequence (5) (Promega) was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Boehringer Mannheim) and was incubated with 1–3 μg of nuclear extracts (11). The AP-1 complexes formed were analyzed by gel electrophoresis and autoradiography as described in Ref. 29.

RESULTS

Forced Expression of hGAP, ∂9-Jun, and Ha-Ras + GAP in Y1 Cells—To test the hypothesis that the hypermethylation of DNA in Y1 cells is controlled by the Ras signaling pathway, we generated Y1 transfectants expressing the human GAP^{120} (GAP) under the direction of cytomegalovirus regulatory sequences (28) or the transdominant mutant of c-Jun, ∂9-Jun (26). To verify that the changes induced by GAP result from down-regulation of Ras, we cointroduced an oncogenic Ha-*ras* expression vector (27) into a GAP transfectant (clone number 7). Nontransfected Y1 cells and Y1 cells that were transfected with pUCSVneo only or with another expression vector (pZEM) (30) served as controls. The presence of hGAP, ∂9, and hGAP + Ha-Ras in these clones and its expression was determined using Southern (data not shown) and Northern blot analysis (Fig. 1, *A*, *B*, and *C*). Western blot analysis for hGAP (using a rabbit polyclonal antibody against hGAP) and Ha-*ras* (using a monoclonal antibody against v-Ha-Ras (Y13-259) was used to correlate the level of transfected protein with the levels of mRNA (by scanning the Western blot fluorograms). GAP and GAP + Ras transfectants showed a 3- (GAP 7, GAP 14, and G/R1) to 5-fold (G/R2) increase in GAP levels, and, as expected from the mRNA analysis (Fig. 1*A*), the total Ras protein in the Ras transfectants was 3-fold the level observed in the control Y1 and GAP transfectants (data not shown).

All of the seven GAP positive clones showed distinct morphological changes, while none of the six controls Y1, Y1neo, or Y1 pZEM lines showed similar changes. We focused our attention on two clones that showed high levels of GAP expression. To determine the biological implications of hGAP expression in Y1 cells, the morphological and growth properties of the Y1 GAP transfectants were determined (Fig. 1). While control Y1 cells exhibit limited contact inhibition and form multilayered foci, the GAP transfectants exhibit a flat morphology and grow exclusively as monolayers (Fig. 1, *right panel*). The ability of cells to grow in an anchorage-independent fashion is considered to be an indicator of tumorigenicity (32), the Y1 GAP transfectants demonstrate a distinct reduction in their ability to form colonies in soft agar. Whereas Y1 and Y1 pZEM formed large colonies on soft agar: 166, 153 for Y1, and 120;126 for Y1 pZEM, the GAP transfectants formed less colonies that contained only few cells: 39;45 for GAP 7 and 34;26 for GAP 14. The transcription factor Jun is an important downstream effector of the Ras signaling pathway (37, 38). Forced expression of the transdominant negative mutant of Jun, ∂9 in Y1 cell, results in similar changes in morphology to those observed with GAP (Fig. 1, *right panel*). Oncogenic *ras* is not modulated by GAP and should therefore restore Ras hyperfunction to GAP transfectants (25). All positive Ras transfectants exhibited a transformed morphology. Two lines bearing both GAP and Ha-*ras* were used for our study (Fig. 1, *right panel*).

Modulation of the Ras Signaling Pathway—To determine whether the Ras signaling pathway was affected in the different transfectants, we measured two determinants of the Ras signaling pathway: guanine nucleotide binding to Ras and AP-1 DNA binding activity. Ras is biologically active when bound to GTP and inactive when it is bound to GDP (25). The GTP/GDP ratio was determined following labeling of Y1 pZEM, Y1 GAP 7, and Y1 GAP + Ras (G/R1 and -2) transfectants with [^{32}P]orthophosphate, immunoprecipitation of Ras, and analysis of GTP/GDP by thin layer chromatography (25, 28) and autoradiography. The percentage of GTP bound(activated) Ras was determined by scanning. Whereas 15.9% of the guanine nucleotide bound to Ras is GTP in the Y1 pZEM controls, only 4.8% of total Ras is GTP-bound in the GAP 7 transfectants. Forced expression of Ha-Ras in the GAP transfectants results in a significant increase in GTP-bound Ras (29.1% and 29.7% for 1 and 2, respectively) (data not shown).

To determine whether AP-1 activity was modulated in our transfectants, we performed gel retardation assays on nuclear extracts prepared from the different transfectants using a ^{32}P-labeled oligomer bearing the consensus AP-1 recognition sequence (5). As observed in Fig. 2*A*, forced expression of GAP in Y1 cells results in reduction of total AP-1 binding activity as well as a change in the AP-1 complexes formed (complex A and B for pZEM *versus* complexes C, D, and E in GAP 7). Forced expression of Ha-Ras in GAP transfectants resulted in an increase in overall AP-1 binding activity and a change in the complexes formed (Fig. 2*A*, *G/R1* and *G/R2*; complexes D and E similar to GAP and complex C). Forced expression of ∂9 results in a reduction in overall AP-1 binding activity and a change in the ratio of complex A/B to C (Fig. 2*B*). All complexes are specific to AP-1 since they are competed with excess of

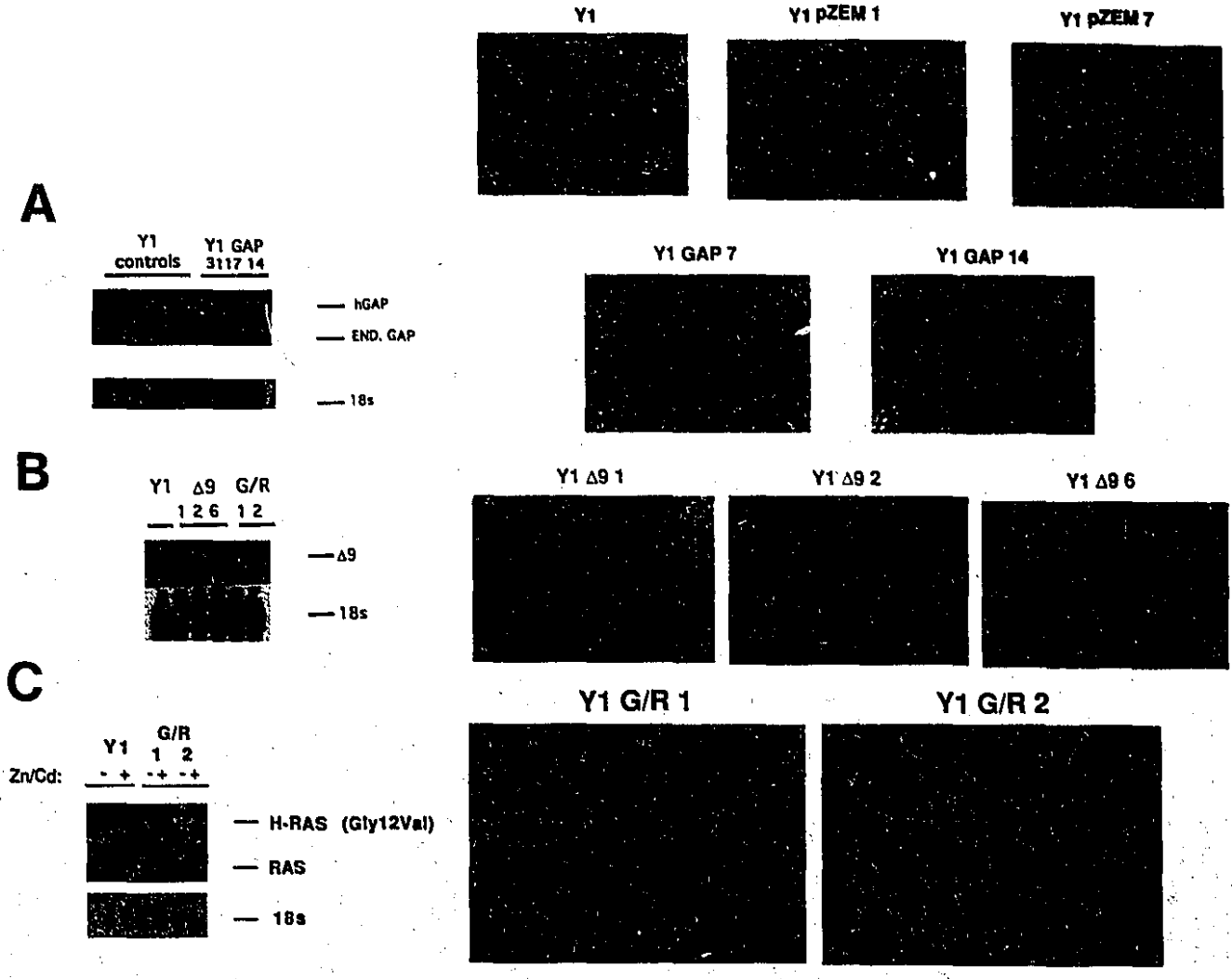

FIG. 1. **Morphological transformation of Y1 cells expressing transfected hGAP^{120},∂9-v-Jun and hGAP^{120} with oncogenic Ha-*ras*.** *A*, *B*, and *C*, expression of transfected constructs in Y1 cells. Total cellular RNA (10 μg) prepared from clonal Y1 transfectants (GAP 7, 14, ∂9 1, 2, 6, and G/R1 and -2) and Y1 cells transfected with the neomycin resistance gene (Y1 controls) or pZEM vector (30) only as controls was subjected to Northern blot analysis and hybridization with ^{32}P-labeled 4.2-kb *Eco*RI fragment encoding hGAP^{120} cDNA (29) (*A*). The human GAP message in the transfectants migrates at 4 kb. *B*, ∂9-v-Jun specific probe (a 447-bp *Sal*I fragment from the v-*jun* domain, Ref. 26. *C*, a Ras-specific probe (0.7-kb *Hin*dII-*Pst*I fragment, Ref. 27). The viral *ras* directs a 2-kb transcript that is easily distinguishable from the shorter endogenous message(s) (1 to 1.4 kb). The filters were stripped from the radioactivity and rehybridized with an 18 S rRNA-specific ^{32}P-labeled oligonucleotide (23). *Right panel*, phase contrast microscopy at × 100 magnification of living cultures of Y1 clonal transfectants with a CMV-hGAP cDNA expression vector (27) (Y1 GAP), ∂9-v-Jun cDNA expression vector (Y1 ∂9) (26), Y1 GAP cells retransfected with pZEM *ras* expression vector (5) (Y1 G/R, × 200 magnification) or Y1 controls transfected with the expression vector pZEM (Y1 pZEM) (30).

nonlabeled AP-1 oligomer (Fig. 2, *A* and *B*). Whereas the composition of the different AP-1 complexes has to be determined in future experiments, this set of experiments demonstrates that AP-1 activity was significantly affected upon forced expression of GAP, ∂9, and Ha-Ras.

Expression of hGAP and ∂9-Jun Results in Genome-wide Hypomethylation—To determine whether expression of GAP results in changes in the methylation status of Y1 DNA, Y1 (pZEM transfected) and two GAP transfectants' DNAs were subjected to "nearest neighbor" analysis using [α-^{32}P]dGTP as described previously (35). This assay determines the relative level of each of the possible 5′ neighbors of G in the genome and thus enables us to quantify the relative abundance of nonmethylated and methylated CpG dinucleotide sequences. The level of nonmethylated cytosines in the genome of GAP transfectants 7 and 14 (35% and 45%) is 3- to 4-fold higher than in the controls pZEM 1 and 7 (8% and 9%). The ∂9 transfectants exhibit a 3- to 4-fold increase in the percentage of nonmethylated cytosines in CpG nucleotides in the genome *versus* the pZEM controls (58%, 60%, and 62% for ∂9 1, 2, and 6, respectively). In summary, this experiment demonstrates that forced expression of GAP or ∂9-Jun in Y1 adrenal carcinoma cells result in a genome-wide reduction in DNA methylation.

The C21 and myoD Genes Are Hypomethylated in GAP and ∂9-Jun Transfectants—To further address the question of whether DNA hypomethylation is triggered by a general change in the DNA methylation capacity of the cell or whether it reflects only local changes in gene expression, specific gene sequences were studied. The C21 gene is specifically repressed and *de novo* methylated when it is exogenously introduced into Y1 cells (23, 24). The methylation state of the 5′ of the gene in four independent Y1 GAP transfectants, a Y1 neo transfectant, and a Y1 control was studied with a *Msp*I/*Hpa*II Southern blot analysis (Fig. 3*A*) using a 0.3-kb 5′ probe (see *bottom panel* for physical map). We have been analyzing this sequence in different Y1 populations as well as different transfectants in the last 5 years. We never observed demethylation of this region in Y1 cells. Additionally, we used four independent GAP lines for this

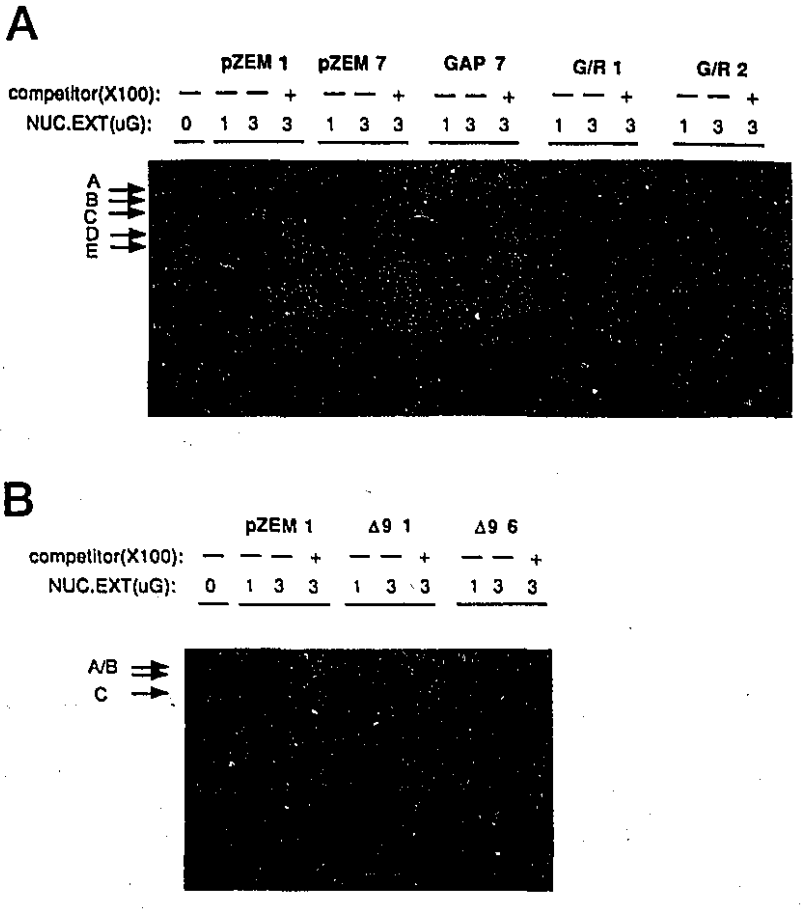

FIG. 2. **Determination of AP-1 binding activities in Y1 GAP, ∂9, and GAP/Ras transfected cells.** *A* and *B*, a double-stranded consensus AP-1 recognition sequence (5) (Promega) end-labeled with [γ-^{32}P]ATP (Amersham) (1 ng, 10^3 cpm) was incubated with 1–3 μg of nuclear extracts (as indicated) from the Y1 transfectants. The first lane of the autoradiograms shows the migration of the free probe alone. An excess of labeled oligonucleotide was used for all assays. AP-1 specificity was demonstrated by competition with an excess (100 ng) of nonlabeled double-stranded AP-1 oligonucleotide. The AP-1 complexes formed were analyzed by gel electrophoresis and autoradiography. The different complexes formed are indicated. AP-1 gel retardation assay performed on nuclear extracts from transfectants (Y1 pZ1 and 7, Y1 GAP 7 and 14, and Y1 G/R1 and -2) as described above (*A*) and ∂9-Jun transfectants (*B*).

Southern blot analysis to rule out the possibility that demethylation is a random event. While both Y1 and the Y1 neo transfectants are heavily methylated and show a diagnostic ~1.9-kb band following *Hpa*II digestion (Fig. 3*A*, *empty arrow*), this band is dramatically reduced in all the GAP transfectants, and new lower molecular weight bands reflecting partial demethylation of the 5′ C21 gene sequence are observed (Fig. 3*A*, *dark arrows*). The fact that four independent transfectants bear a similar pattern of hypomethylation, for example, the relative abundance of the 0.52-kb partial *Hpa*II fragment (Fig. 3*A* and *bottom panel* for map) which is completely absent in the *Hpa*II digests of Y1 cells, and a Y1 neo transfectant demonstrates that this hypomethylation is a direct result of GAP expression rather than clonal variability among Y1 cells. Similar to the 5′ region, the body of the C21 gene exhibits distinct changes in DNA methylation (Fig. 3*B*). There is a relative diminution of the higher molecular weight fragment at 3.8 (*open arrow*) (the DNA was digested with *Bam*HI following the *MspI/Hpa*II analysis which should give a 3.8-kb fragment) and appearance of the 1- and 0.9-kb doublet (see physical map in *bottom panel* indicating the *Hpa*II sites flanking the 1- and 0.9-kb fragments) (Fig. 3*B*). The state of methylation of the C21 gene in ∂9-Jun transfectants is presented in Fig. 4*A*. These results indicate that the *Hpa*II sites flanking the the 1-kb *Hpa*II fragment (see physical map, Fig. 3, *bottom panel*) which are almost fully methylated in Y1 cells and the pZEM controls, as indicated by the absence of the 1-kb *Hpa*II fragment, are almost completely hypomethylated in the ∂9 transfectant (Fig. 4*B*, *top empty arrow*, 1-kb fragment in the *Hpa*II lane). Other sites are less readily hypomethylated as implied by the absence of the lower molecular fragment at 0.8 and 0.4 kb. Some faint bands at this molecular weight range are present in the ∂9 −6 transfectant suggesting that demethylation of the other sites also occurs in a subset of ∂9 transfectants.

Whereas Y1 cells contain the machinery needed for C21 gene expression (23), the hypomethylation observed in the 5′ region of the gene might be a result of local changes in gene accessibility rather than a primary hypomethylation event that is centrally regulated. To address this question, we tested the pattern of methylation of other genes such as the gene for the 5HT1A receptor, a gene specifically expressed in the hippocampus and not in the adrenal or the ubiquitously expressed p53 gene. Demethylation was observed in both gene sequences (data not shown). Another locus that was studied is the *myoD* gene. This locus is heavily methylated in Y1 cells as indicated by the high molecular weight fragments ranging from 3.35 to 7 kb in the *Hpa*II digest of the Y1 control transfectant (Fig. 4*B*, *pZ1*, *empty arrow*). The sites flanking the 3′ 1.06-kb *Hpa*II fragment are, however, partially methylated in Y1 cells. The ∂9 transfectants exhibit complex changes in the pattern of methylation at this locus. The 3.5–~7-kb hypermethylated partial *Hpa*II fragment is partially demethylated resulting in two lower molecular fragments around the 3.35-kb marker (Fig. 4*B*, *dark arrow*). However, the 3′ 1-kb fragment is methylated and absent in the ∂9 transfectants. The GAP transfectants exhibit a distinctly similar pattern (Fig 4*B*, *right panel*) except that they show some demethylation of the 0.58-kb *Hpa*II fragment (*sites 8* and *9* in the physical map). These results indicate that even under conditions when the genome is generally hypomethylated, some specific sites behave differently and become hypermethylated (Fig. 4*B*, the 1.06 fragment). The mechanism responsible for this site-specific response is unclear to us.

GAP and ∂9 Transfectants Express Lower DNA MeTase Activity than Control Transfectants—One possible mechanism through which the Ras signaling pathway can control DNA methylation is by regulating DNA MeTase activity in the cell's

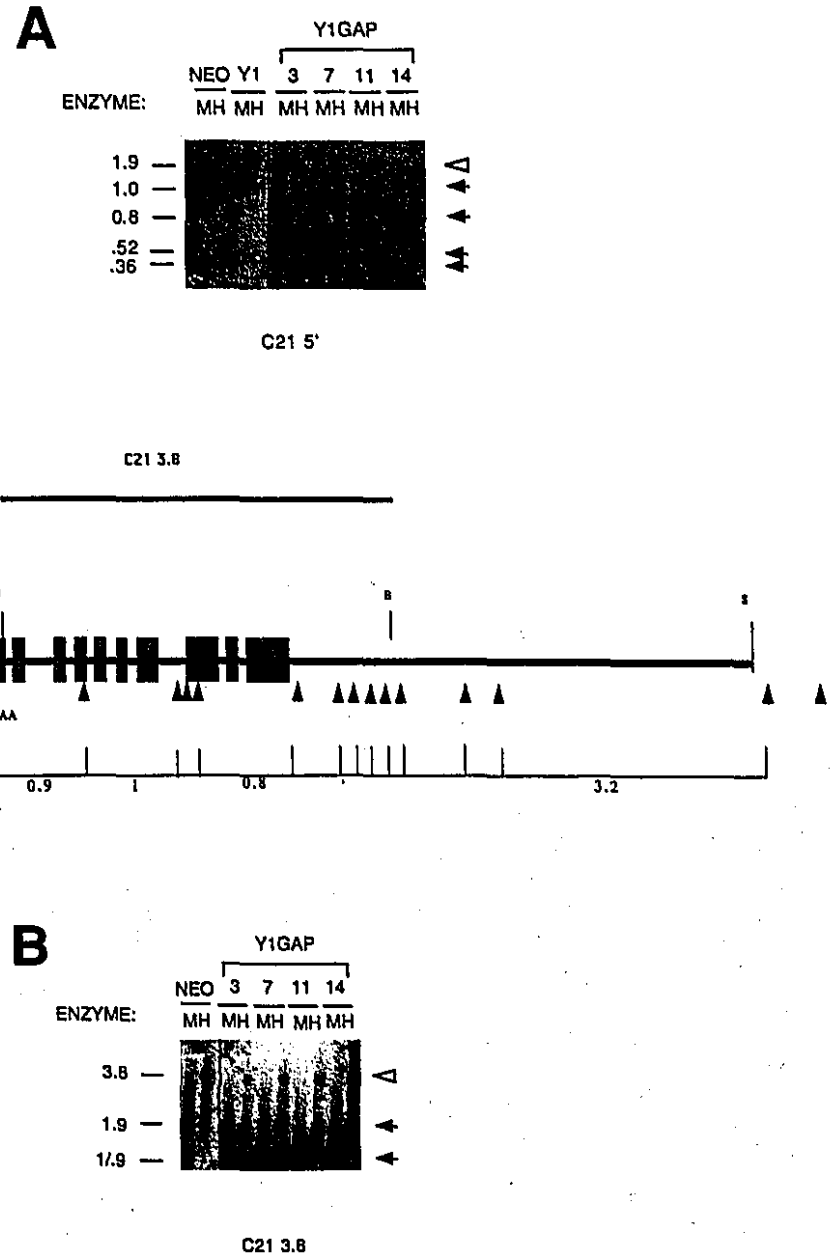

FIG. 3. **Hypomethylation of specific gene sequences in Y1 GAP transfectants.** *A*, C21 gene 5', *top panel*, genomic DNA (10 μg) was extracted from the transfected lines and subjected to digestion with *Bam*HI followed by digestion with either *Msp*I (*M*) or *Hpa*II (*H*), Southern blot transfer, and hybridization with a ^{32}P-labeled DNA probe to the 5' region of the mouse C21 gene (23) (see *bottom panel* for physical map of *Hpa*II sites). The *open arrow* indicates the position of the 1.9-kb band seen in *Hpa*II lanes when this site is methylated. *Msp*I cleavage of these sites is partial because they are nested within a *Hae*III site which is usually resistant to *Msp*I cleavage when the internal cytosine is methylated (42). The *filled arrows* indicate expected *Hpa*II fragments resulting from demethylation of different sites. *Bottom panel*, physical map. The location of CCGG sites and the probes used are indicated relative to the physical map of the C21 gene. The expected *Hpa*II/*Msp*I fragments are indicated by a *line*. (*B*, *Bam*HI). *B*, C21 gene (3.8-kb fragment), genomic DNA (10 μg) extracted from the indicated lines was subjected to *Msp*I/*Hpa*II restriction followed by digestion with 20 units of *Bam*HI, Southern blot transfer, and hybridization with a ^{32}P-labeled DNA probe for the mouse C21 gene (3.8-kb *Bam*HI fragment) (23). The *open arrow* indicates the position of the fully methylated 3.8-kb fragment. The *dark arrows* indicates the position of fully hypomethylated 1.0/0.9/0.8 group of bands and the 1.9-kb partially hypomethylated fragment.

nucleus. We therefore compared the level of enzymatic DNA MeTase activity in GAP and ∂9 transfectants *versus* controls. DNA MeTase activity is regulated with the state of growth of cells (10, 11), therefore, all cultures were maintained at the exponential phase of growth and fed with fresh medium every 24 h for 3 days prior to harvesting. DNA MeTase activity in the different nuclear extracts was assayed using S-[*methyl*-^{3}H]adenosylmethionine as the methyl donor and a hemimethylated double-stranded oligonucleotide as a substrate as described under "Materials and Methods." Results of a representative experiment presented in Fig. 5 shows that both GAP and ∂9 transfectants bear lower levels of DNA MeTase activity than the pZEM controls (20–30% of controls) ($p < 0.05$ for GAP *versus* pZEM and $p < 0.005$ for ∂9 *versus* pZEM). These lines also express lower levels of DNA MeTase mRNA as quantified by a slot blot analysis using a DNA MeTase 3' probe and normalizing the signal to that obtained with an 18 S RNA oligonucleotide probe (Fig. 7*B*). In summary, these results are consistent with the hypothesis that Ras and Jun are important for maintaining high levels of DNA MeTase mRNA and enzymatic activity in Y1 cells.

Forced Expression of an Oncogenic Ha-ras in GAP Transfec-

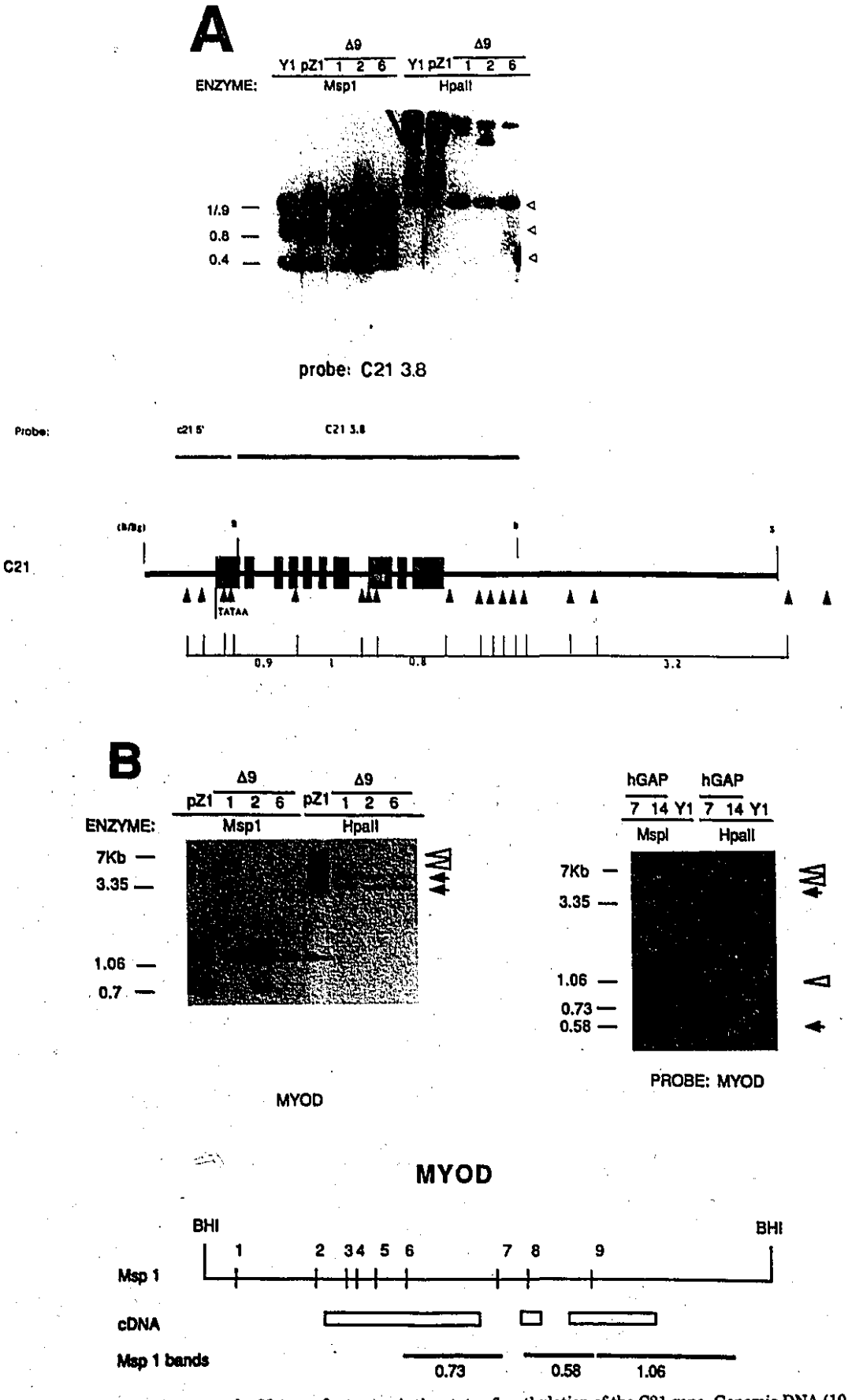

FIG. 4. **Demethylation of specific genes in ∂9 transfectants.** *A*, the state of methylation of the C21 gene. Genomic DNA (10 μg) extracted from the indicated lines was subjected to *MspI/HpaII* followed by *Bam*HI digestion, Southern blot transfer, and hybridization with a ^{32}P-labeled DNA probe for the mouse C21 gene (3.8-kb *Bam*HI fragment). The *open arrows* indicates the position of the demethylated *Hpa*II fragments. *B*,

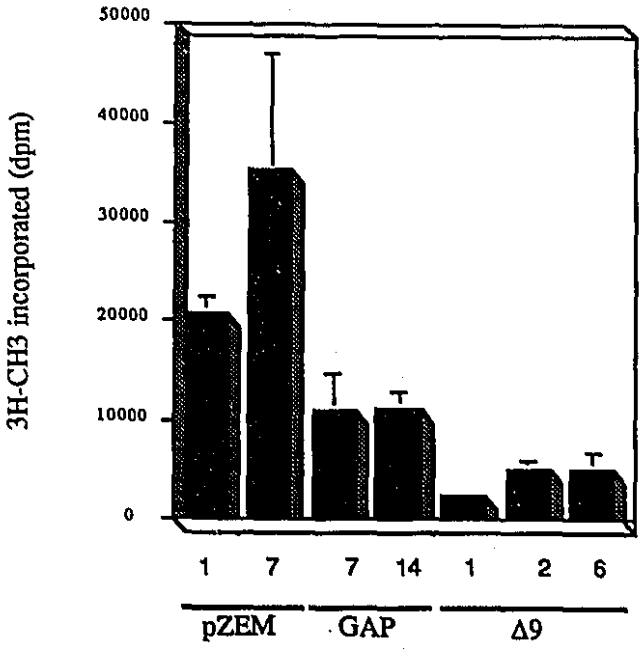

FIG. 5. **DNA MeTase activity in GAP and ∂9 transfectants and Y1 controls.** DNA MeTase activity in nuclear extracts prepared from GAP and ∂9 transfectants and pZEM controls was determined using a synthetic hemimethylated double-stranded oligonucleotide substrate as described under "Materials and Methods." The results are expressed as disintegrations/min of methyl-^{3}H incorporated into the substrate oligonucleotide per 3 μg of nuclear protein for 3 h at 37 °C. The results are presented as averages of three determinations ± S.D. The statistical significance of the difference between the GAP and pZEM and the ∂9 and pZEM was determined by a *t* test: GAP, $p < 0.05$; ∂9, $p < 0.005$.

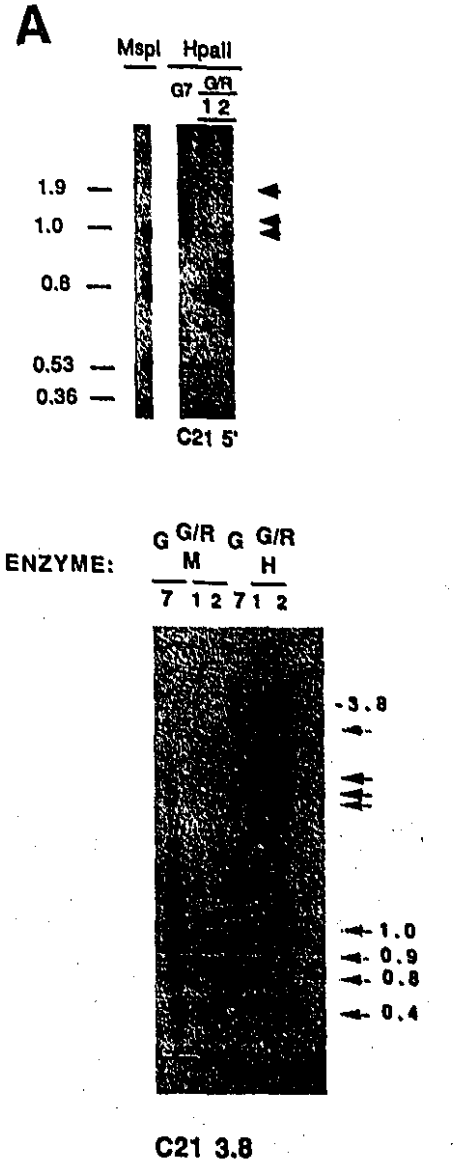

FIG. 6. **Methylation of Y1 GAP DNA following forced expression of Ras.** *A*, C21 gene 5', a duplicate of the filter described above was hybridized with the C21 5' probe (see Fig. 3*A* for physical map). The sizes of the *Msp*I fragments are indicated. The *arrows* point to the partially hypomethylated fragments observed in the GAP transfectants. *B*, C21 gene (3.8-kb fragment), genomic DNA (10 μg) extracted from the indicated lines was subjected to *MspI/Hpa*II followed by digestion with 20 units of *Bam*HI, Southern blot transfer, and hybridization with a ^{32}P-labeled DNA probe for the mouse C21 gene (3.8-kb *Bam*HI fragment) (23). The 3.8-kb line indicates the position of the fully methylated fragment. The *shaded arrows* indicate the position of fully hypomethylated 1.0/0.9/0.8 group of bands, bands hypermethylated in GAP + Ras (G/R) transfectants are indicated by *dark arrows*.

tants Reverses the Hypomethylation Induced by GAP Expression—To verify that the hypomethylation induced by GAP directly results from down-regulation of Ras activity, and to test the hypothesis that overexpression of Ras can induce hypermethylation of a cell's genome, we determine the state of methylation of genomic DNA of GAP + Ras (G/R) transfectants by a "nearest neighbor" analysis. The level of nonmethylated cytosines in CpG sequences in the DNA of the GAP + Ras transfectants is 3–4-fold lower (8% and 12% for G/R1 and -2, respectively) than in the GAP parental lines (35% and 42% for GAP 7 and GAP 14, respectively) and similar to the level observed in control Y1 transfectants. The state of methylation of three specific gene sequences was studied by an *MspI/Hpa*II analysis. The C21 gene (hybridizing to the 3.8-kb C21 probe, see Fig. 3) is significantly hypomethylated in the GAP transfectants as implied by the relative abundance of the fully nonmethylated *Hpa*II fragments at 1.0, 0.9, 0.8, and 0.4 (Fig. 6*A*). The relative abundance of the nonmethylated fragments decreases significantly in both GAP + Ras transfectants. The fully methylated 3.8-kb fragment (the DNA was digested with *Bam*HI following *Hpa*II/*Msp*I digestion, a fully methylated C21 gene will migrate as a 3.8-kb *Bam*HI fragment) which is absent in the GAP transfectants is present in the GAP + Ras transfectants. The C21 gene is not as fully methylated in the GAP + Ras transfectants as it is in the Y1 controls (Fig. 3*B*), suggesting that the *de novo* methylation of this gene in response to Ras overexpression is a slow process. We next studied the state of methylation of the 5' of the C21 gene using a C21 5' probe (see Fig. 3 for physical map). The autoradiogram was scanned and the relative intensity of the different fragments was determined (Fig. 6*B*). The partially hypomethylated *Hpa*II fragments at 1.0 kb and 1.9 kb which are observed in the GAP lane are reduced in intensity or absent in the GAP + Ras digests (Fig. 6*B*); on the

myoD. Genomic DNA (10 μg) extracted from the indicated lines as well as two GAP transfectants (7 and 14) were isolated and treated as above and subjected to Southern blot transfer and hybridization with a cDNA probe encoding mouse *myoD* (43, a kind gift of Dr. Weintraub). The *open arrow* indicates the position of methylated DNA fragment above the 7 kb marker. The dark arrow indicates the position of the partially hypomethylated fragment observed in both GAP and ∂9 transfectants. The expected 0.5-kb *Hpa*II fragment resulting from demethylation of sites 8 and 9 is observed in the GAP transfectants but not the ∂9 transfectants. A physical map of the *myoD* gene and the probe used are depicted in the *bottom panel*.

other hand, the partially hypermethylated *Hpa*II fragments at 1.2 and 4.5 are relatively intensified in the GAP + Ras lanes. In summary, these analyses support the hypothesis that expression of an exogenous Ha-Ras results in hypermethylation of the genome. The fact that one could revert the effects of GAP expression by Ha-Ras supports the conclusion that hypomethylation is induced by attenuation of Ras activity.

GAP + Ras Transfectants Express Higher Levels of DNA MeTase mRNA and Enzymatic Activity than GAP Controls—To determine whether expression of Ha-Ras in the GAP transfectants will result in induction of DNA MeTase activity, we determined DNA MeTase activity in nuclear extracts prepared from GAP and GAP + Ras transfectants as described above and under "Materials and Methods." As observed in Fig. 7*A*, GAP + Ras transfectants express higher levels of DNA MeTase activity relative to the parental GAP line ($p < 0.05$). The level of activity observed in GAP + Ras lines is similar to the level of activity observed in Y1 cells ($p > 0.4$) and the control transfectants (pZEM). Similar changes were observed when the level of DNA MeTase mRNA was quantified using a slot blot analysis and hybridization with the 3′ DNA MeTase probe (Fig. 7*B*) ($p < 0.001$ for GAP + Ras *versus* GAP). These data are consistent with the hypothesis that expression of GAP inhibits DNA MeTase expression by inhibiting Ras activity. Expression of an oncogenic Ha-*ras* (Gly^{12}-Val) by-passes GAP inhibition and reverses the inhibition of DNA MeTase expression. In summary, our results are consistent with the hypothesis that DNA MeTase activity and hypermethylation of the genome in Y1 cells is controlled by Ras.

Expression of a DNA MeTase Promoter-CAT Construct in Y1 Cells Is Dependent on AP-1 and Inhibited by Inhibitors of the Ras Signaling Pathway—The DNA MeTase promoter bears a number of consensus AP-1 recognition sequences (3). Jun, Fos, or Ras can transactivate the DNA MeTase promoter, and this transactivation is dependent on these AP-1 sites (5). One possible mechanism for explaining the inhibition of DNA methylation upon inhibition of Ras or Jun in Y1 cells is that expression of DNA MeTase promoter in Y1 cells is dependent on activation of AP-1 by Ras. To test this hypothesis, we cotransfected the DNA MeTase 5′ region-CAT construct into Y1 cells with hGAP, ∂9-Jun, or Asn^{17}-Ras (39). As observed in Fig. 8*A*, hGAP which down-regulates Ras activity, Asn^{17}-Ras which inhibits Ras, and ∂9 which inhibits Jun also inhibit the activity of the DNA MeTase promoter. Cotransfection of pMET CAT^+ with Ras or Jun did not alter the activity of the construct suggesting that Ras activity in Y1 cells is saturated even without adding it exogenously (data not shown). To determine whether the promoter activity is dependent on AP-1, we tested the ability of a construct bearing the two AP-1 sites at −1744 and −1650 juxtaposed to the minimal DNA MeTase promoter (WT/WT) (5) and constructs bearing a site-specific mutation in either of the AP-1 sites (−1744 MUT/WT; −1650 WT/MUT) or both sites (MUT/MUT) to direct CAT transcription. The CAT assay presented in Fig. 8*B* demonstrates that expression of pMET CAT in Y1 cells is inhibited when either AP-1 site is mutated. In summary, the CAT assays presented above support the hypothesis that the DNA MeTase promoter is activated by AP-1 in Y1 cells and is inhibited by down-regulators of the Ras signaling pathway.

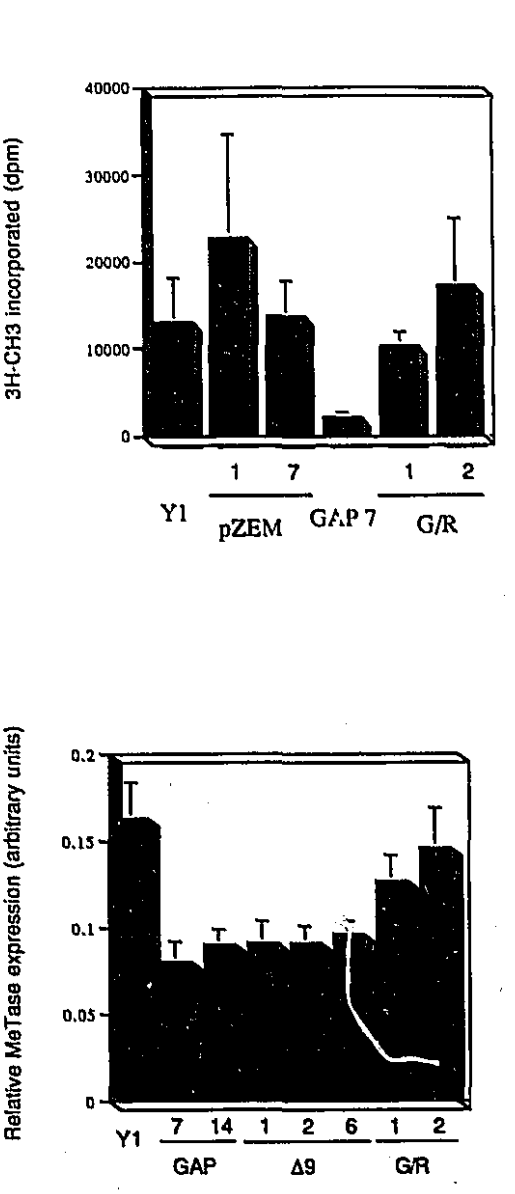

FIG. 7. **DNA MeTase activity and steady state mRNA levels in Y1 controls, GAP, ∂9, and GAP + Ras transfectants.** *A*, DNA MeTase activity in nuclear extracts prepared from Y1, pZEM (pZ) transfectants, GAP 7, and GAP + Ras (G/R) transfectants was determined using a synthetic hemimethylated double-stranded oligonucleotide substrate as described under "Materials and Methods." The results are expressed as disintegrations/min of methyl-^{3}H incorporated into the substrate oligonucleotide per 3 μg of nuclear protein for 3 h at 37 °C. The results are an average of three determinations ± S.D. The statistical significance of the difference between the different groups was determined using a *t* test (Systat). GAP *versus* pZEM, $p < 0.05$; ∂9 *versus* pZEM, $p < 0.005$; GAP *versus* GAP + Ras, $p < 0.05$. However, there is no statistically significant difference between pZEM and Y1 or pZEM and GAP + Ras. *B*, quantification of DNA MeTase mRNA levels by a slot blot analysis. RNA samples as in *A* were spotted in triplicate onto a Hybond-N N^+ filter using a Bio-Rad slot blot apparatus. The filter was sequentially hybridized with the MET 3′ probe, stripped of radioactivity, then rehybridized with an 18 S rRNA probe. The signals were quantified by densitometry, and the abundance of DNA MeTase mRNA relative to 18 S rRNA at each point was calculated. The values are presented as an average of three determinations ± S.D. Statistical significance of the difference between the Y1 group and the GAP group ($p < 0.01$), ∂9 *versus* Y1 ($p < 0.05$), and GAP + Ras *versus* GAP ($p < 0.001$) was determined by a *t* test.

DISCUSSION

While activation of the methylation machinery in cancer cells is well established (17, 18), and it has been shown that overexpression of an exogenous DNA MeTase can lead to cellular transformation (19), the molecular mechanisms responsible for this change are unclear. This paper tests the hypothesis that has been recently suggested that the DNA methylation pattern of tumor cells is controlled by an oncogenic signal transduction pathway (5) using the adrenocortical tumor cell line Y1 as a model. Y1 is a well studied and established cell line

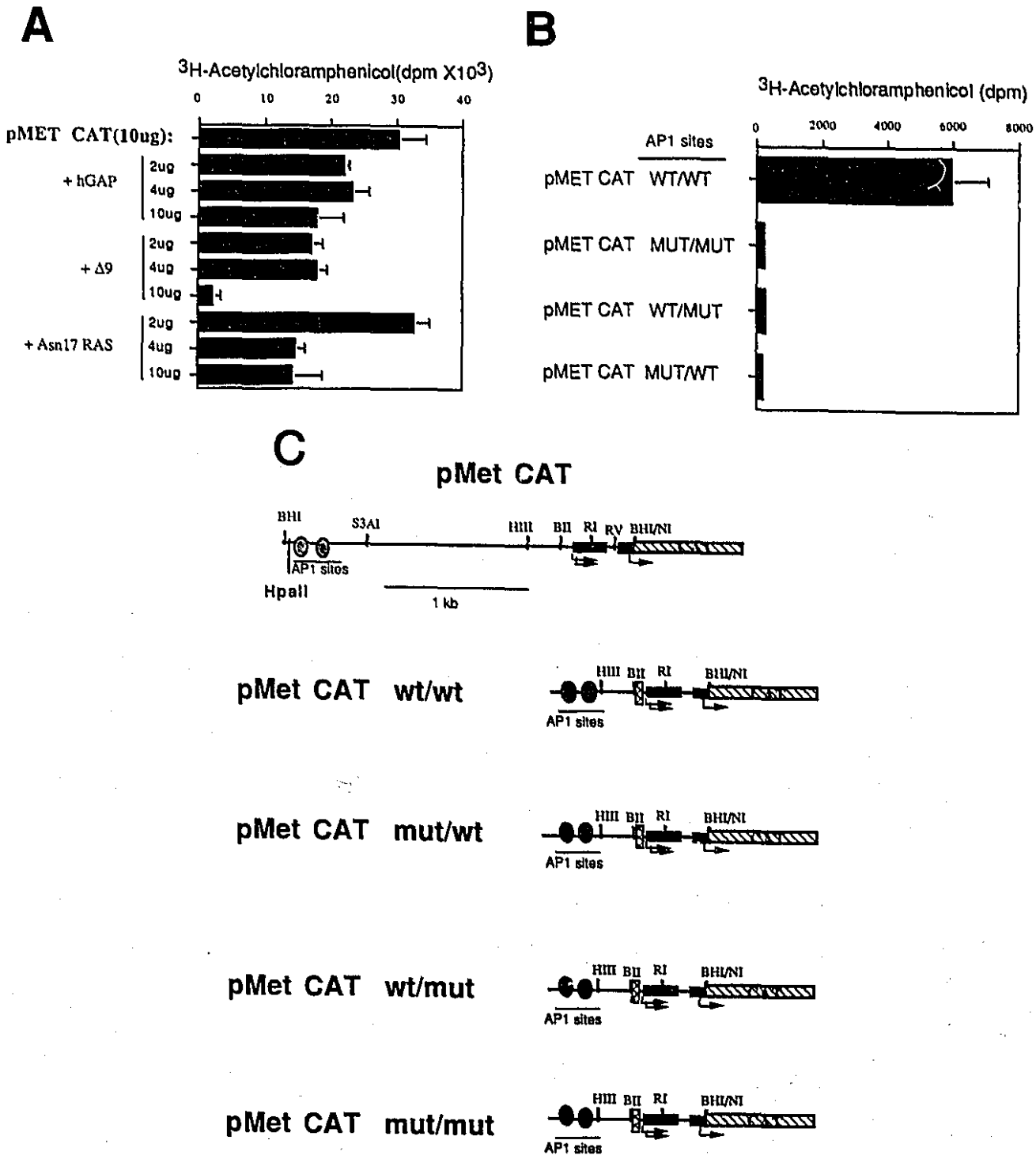

FIG. 8. **DNA MeTase promoter activity is dependent on the presence of a functional Ras signaling pathway.** *A*, Y1 cells were transfected with 10 μg of pMET CAT$^+$ (4) (see *C* for physical map) and increasing concentrations of expression vectors expressing hGAP (28), ∂9-Jun (26), or a transdominant Ras mutant Asn^{17}-Ras (39). The total concentration of DNA transfected in all conditions was maintained constant at 20 μg by adding SK (Stratagene) to rule out nonspecific effects of differences in DNA concentration. Cells were harvested after 48 h, and CAT activity was determined as described below. Each experiment was repeated at least twice. Each value is presented as mean of three determinations ± S.D. *B*, CAT activity expressed in Y1 cells that were cotransfected with 4 μg of CAT constructs bearing the 118-bp sequence AP-1 region of the DNA MeTase promoter (−1744 and −1650) juxtaposed to the *Hin*dIII-*Nae*I fragment (encoding the DNA MeTase promoter) (4, 5). WT/WT bears both sites in the wild type form, (TGACCTCA at −1744 and TGACTGA at −1650), MUT/MUT bears both AP-1 sites in a mutated form (TGAGGCA), WT/MUT bears the 3′ site in a mutated form, and MUT/WT bears the 5′ site in a mutated form. A detailed description of the constructs and their activity is described in Ref. 5.

which has the advantage that it is derived from a naturally occurring tumor, bears an amplification of an endogenous *ras* protooncogene (22) rather than a virally induced oncogene, and exhibits a stable DNA methylation pattern in culture. Whereas it is generally hard to extrapolate from events occurring in tumor cell lines in culture to the situation *in vivo*, a well characterized model such as Y1 enables one to dissect mechanisms and pathways in a manner that is almost impossible to

accomplish *in vivo*, especially in the human. The predictions obtained from such an analysis could be then analyzed in the context of observations derived from *in vivo* situations. The induction of DNA MeTase activity and hypermethylation of specific loci in cancer *in vivo* is a very well established phenomenon (16–18) as well as the fact that Ras is activated in many cancer cells. Our *ex vivo* model enabled us to dissect the possibility that these events are related and establish potential sites through which Ras might control DNA methylation. The DNA MeTase promoter bears a number of AP-1 sites (3) which are responsible for its induction by the Ras-Jun signaling pathway (5) raising the possibility that Ras can control the pattern of DNA methylation (4). This paper tests this hypothesis. First, we demonstrate that expression of GAP in Y1 cells, an established down-modulator of cellular Ras activity (25), leads to a reduction in the state of transformation of the cells (Fig. 1) supporting the hypothesis that transformation of Y1 cells is dependent on high activity of the endogenous Ras. Expression of GAP^{120} also results in a general change in DNA methylation which supports the hypothesis that the methylation pattern is controlled by a signaling pathway that triggers tumorigenesis. Second, changes in DNA methylation occur in genes of different cell type specificity: muscle (*myoD*) and adrenal (C21) (Fig. 3), excluding the model that demethylation is exclusively an effect of local cell-specific changes in gene expression. Third, introduction of a transdominant negative mutant of Jun into Y1 cells (26) (Fig. 1) results in reduction of genomic and gene-specific DNA methylation levels (Fig. 4). Fourth, inhibition of Ras by GAP and Jun by ∂9 results in a reduction in DNA MeTase activity and mRNA levels (Fig. 5 and 7). Fifth, introduction of an oncogenic Ha-*ras* into hypomethylated GAP Y1 transfectants induces morphological transformation (Fig. 1), hypermethylation (Fig. 6), and induction of DNA MeTase activity (Fig. 7). Sixth, the DNA MeTase promoter activity in Y1 cells, as determined by CAT assays, is inhibited by GAP, ∂9, and Asn^{17}-Ras (Fig. 8*A*) and dependent on the presence of functional AP-1 sites (Fig. 8*B*). Our results are consistent with the hypothesis that Ras controls DNA methylation by activating Jun which in turn transactivates the DNA MeTase promoter by interacting with AP-1 sites in the promoter (Fig. 9).

The broad changes in methylation in the GAP and ∂9 transfectants' DNA and the reversal of this hypomethylation in the Ha-Ras-GAP transfectants is consistent with a model suggesting that the Ras signaling pathway controls the general methylation capacity of the cell. Since it is clear that *in vivo* specific regions are hypermethylated in cancer cells (16), this raises the interesting question: why are different sites hypomethylated to a different extent if hypomethylation is a general process? While there is no clear explanation for this phenomenon, some models have been suggested previously (13). It has been suggested that under conditions of a general reduction in the methylation capacity of the cell, different sites will show different levels of hypomethylation because of differences in accessibility to the methylation machinery (13). The results obtained in this paper with inhibitors of the Ras pathway are consistent with the model that even when the general methylation capacity of a cell is reduced, some site selectivity is observed. For example, the *Hpa*II sites flanking the 0.36-kb C21 5′ fragment (Fig. 3*A*) are more extensively hypomethylated than the sites flanking the MyoD 1.06-kb fragment which undergoes some *de novo* methylation in both GAP and ∂9 transfectants (Fig. 4*B*). Similar conclusions can be drawn from the GAP + Ras transfectants (Fig. 8). Whereas the general level of methylation in these transfectants is similar to the Y1 controls, the pattern of methylation of specific genes indicates that not all genes returned to the original pattern observed in parental

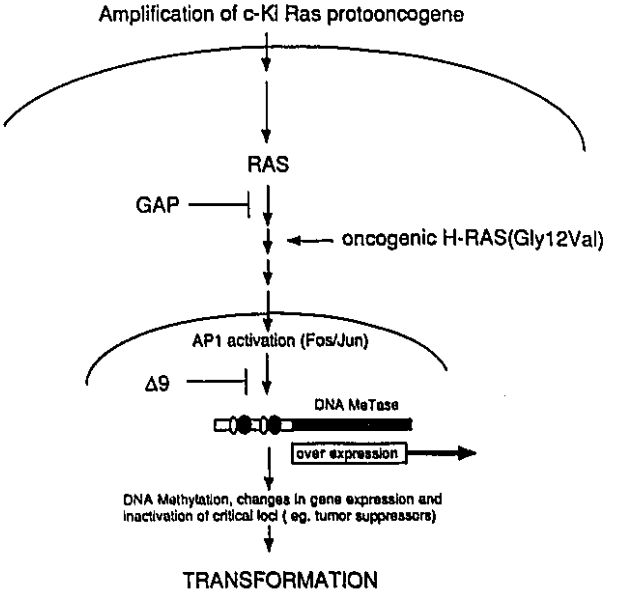

FIG. 9. **Regulation of DNA MeTase in Y1 cells by the Ras signaling pathway: a model.** Amplification of the *ras* gene results in overexpression of Ras (23), which in turn results in induction of a cascade of protein kinases (for Ras pathway) resulting in turn in activation and phosphorylation of Jun. Activation of Jun results in activation of the DNA MeTase promoter (Fig. 8), induction of DNA MeTase activity (Fig. 7), and an increase in the DNA methylation capacity of the cell. A change in the methylation capacity results in aberrant methylation patterns which are involved in establishing or maintaining a transformed state by a yet unknown mechanism. To test this hypothesis, we have used inhibitors of the pathway at different nodal points: GAP which activates Ras-GTPase activity and ∂9 which is a transdominant negative mutant of Jun. Both modulators cause hypomethylation, inhibition of DNA MeTase activity, and reversal of transformation. Introduction of an oncogenic *ras* which by-passes GAP regulation into GAP cells results in hypermethylation and induction of DNA MeTase activity. In a separate work, we show that inhibition of DNA MeTase by an antisense can reverse tumorigenesis (20).

Y1 lines. For example, the C21 (3.8-kb fragment, Fig. 6*B*) shows an intermediate level of methylation between the parental Y1 (Fig. 3*B*) and GAP (Fig. 3*B* and Fig. 8*B*). Our results are consistent with previous observations showing that even when the methylation capacity of 10T1/2 cells is significantly inhibited by sequential rounds of azaC treatment, certain sites will remain heavily methylated while most of the genome will undergo extensive demethylation (40). A tenacious site of methylation in the p53 gene that has been recently analyzed by Jones and his group (41) is another example of a site that exhibits an inherent tendency for hypermethylation. Whereas no specific factor that can modulate the methylation level of a specific site has been yet identified, the data cited above are consistent with the existence of such factors.

Are the changes in DNA methylation observed following attenuation of Ras signaling critical for the reversal of the transformation process in Y1 cells? Recent evidence from our laboratory indicates that inhibition of DNA methylation by either an antisense to the DNA MeTase or 5-azadeoxycytidine reverses the transformation of Y1 cells (20). Whereas additional experiments are required to determine whether activation of Ras in tumors *in vivo* can induce the DNA MeTase and whether this induction plays a role in the oncogenic state, the data presented in this report establish a potential link between DNA methylation, oncogenesis, and the Ras signaling pathway. One should also emphasize that induction of Ras might have different effects on DNA methylation in different cell types since other genes which can also influence DNA methylation might be induced in different cell systems. While addi-

tional experiments will be required to address these questions, the results presented above demonstrate that the pattern of DNA methylation can be controlled by a cellular signal transduction pathway that can lead a cell toward a transformed state.

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Chapter 3: Expression of Antisense to the DNA Methyltransferase mRNA induces DNA Demethylation and Inhibits Tumorigenesis. A.R. MacLeod and M. Szyf, J. Biol. Chem. **270**, 8037-8043 (1995).

In the previous chapter we showed the Ras signaling pathway can activate the DNA MeTase as a downstream target, leading to the stable epigenetic modification of the genome. This suggests a mechanistic link between known oncogenic signals, DNA hypermethylation and the transformed state. The critical question remaining to be answered is whether indeed the level of expression of the endogenous DNA MeTase plays a causal role in oncogenic transformations induced by naturally occurring oncogenic signal transduction pathways. To address this question, we developed an antisense approach allowing us to specifically modulate the DNA MeTase levels in cells derived from a naturally occurring mouse tumor and to study the effect of this modulation on the oncogenic potential of these cells.

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Expression of Antisense to DNA Methyltransferase mRNA Induces DNA Demethylation and Inhibits Tumorigenesis*

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Many tumor cell lines overexpress DNA methyltransferase (MeTase) activity; however it is still unclear whether this increase in DNA MeTase activity plays a causal role in naturally occurring tumors and cell lines, whether it is critical for the maintenance of transformed phenotypes, and whether inhibition of the DNA MeTase in tumor cells can reverse transformation. To address these basic questions, we transfected a murine adrenocortical tumor cell line Y1 with a chimeric construct expressing 600 base pairs from the 5′ of the DNA MeTase cDNA in the antisense orientation. The antisense transfectants show DNA demethylation, distinct morphological alterations, are inhibited in their ability to grow in an anchorage-independent manner, and exhibit decreased tumorigenicity in syngeneic mice. *Ex vivo*, cells expressing the antisense construct show increased serum requirements, decreased rate of growth, and induction of an apoptotic death program upon serum deprivation. 5-Azadeoxycytidine-treated cells exhibit a similar dose-dependent reversal of the transformed phenotype. These results support the hypothesis that the DNA MeTase is actively involved in oncogenic transformation.

Vertebrate DNA is methylated at the 5-position of the cytosine residues in the dinucleotide sequence CpG (1, 2). Twenty percent of the CpG sites are nonmethylated, and these sites are distributed in a nonrandom manner to generate a pattern of methylation that is site-, tissue-, and gene-specific (1–3). Methylation patterns are formed during development: establishment and maintenance of the appropriate pattern of methylation is critical for development (4) and for defining the differentiation state of a cell (5–7). The pattern of methylation is maintained by the DNA MeTase[1] at the time of replication (8), and the level of DNA MeTase activity and gene expression is regulated with the growth state of different primary (8) and immortal cell lines (9). This regulated expression of DNA MeTase has been suggested to be critical for preserving the pattern of methylation (8–10).

An activity that has a widespread impact on the genome such as DNA MeTase is a good candidate to play a critical role in cellular transformation. This hypothesis is supported by many lines of evidence that have demonstrated aberrations in the pattern of methylation in transformed cells. While many reports show hypomethylation of total genomic DNA (11) as well as individual genes in cancer cells (12), other reports have indicated that hypermethylation is an important characteristic of cancer cells (13). First, large regions of the genome such as CpG-rich islands (14) or regions in chromosomes 17p and 3p that are reduced to homozygosity in lung and colon cancer, respectively, are consistently hypermethylated (15, 16). Second, the 5′ region of the retinoblastoma (Rb) and Wilms Tumor (WT) genes are methylated in a subset of tumors, and it has been suggested that inactivation of these genes in the respective tumors resulted from methylation rather than a mutation (17). Third, the short arm of chromosome 11 is regionally hypermethylated in certain neoplastic cells (15). Several tumor suppressor genes are thought to be clustered in that area (18). If the level of DNA MeTase activity is critical for maintaining the pattern of methylation as has been suggested before (8–10), one possible explanation for this observed hypermethylation is the fact that DNA MeTase is dramatically induced in many tumor cells well beyond the change in the rate of DNA synthesis (13, 19). The observation that the DNA MeTase promoter bears AP-1 sites (20) and is activated by the Ras-AP-1 signaling pathway (21) is consistent with the hypothesis that elevation of DNA MeTase activity is an effect of activation of the Ras-Jun signaling pathway (22).

It has recently been demonstrated that forced expression of exogenous DNA MeTase cDNA causes transformation of NIH 3T3 cells supporting the hypothesis that overexpression of DNA MeTase can cause cellular transformation (23). The critical question that remains to be answered is whether indeed the level of expression of the endogenous DNA MeTase plays a causal role in tumors that are induced by naturally occurring oncogenic signal transduction pathways. To address this question, we have chosen the adrenocortical carcinoma cell line Y1 as a model system. Y1 is a cell line that is derived from a naturally occurring adrenocortical tumor in LAF1 mice (24). Y1 cells bear a 30–40-fold amplification of the *ras* proto-oncogene (25). If the level of expression of DNA MeTase activity is critical for the oncogenic state, then the transformed state of a cell should be reversed by partial inhibition of DNA methylation. We have previously demonstrated that forced expression of an "antisense" mRNA to the most 5′ 600 bp of the DNA MeTase message (pZαM) can induce limited DNA demethylation in 10T1/2 cells (7). To directly test the hypothesis that the tumorigenicity of Y1 cells is controlled by the DNA MeTase, we transfected either pZαM or a pZEM control into Y1 cells. We demonstrate that inhibition of DNA MeTase activity causes demethylation of Y1 DNA and results in reversal of the tumorigenic phenotype suggesting that DNA MeTase plays a critical role in tumorigenesis.

MATERIALS AND METHODS

Cell Culture and DNA-mediated Gene Transfer—Y1 cells were maintained as monolayers in F-10 medium which was supplemented with 7.25% heat-inactivated horse serum and 2.5% heat-inactivated fetal

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[1] The abbreviations used are: MeTase, methyltransferase; 5-azaCdR, 5-azadeoxycytidine; bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction.

calf serum (Immunocorp, Montreal) (23). 5-azaCdR was from Sigma, all other media and reagents for cell culture were obtained from Life Technologies, Inc. Y1 cells (1×10^6) were plated on a 150-mm dish (Nunc) 15 h before transfection. The pZαM expression vector (7) encoding the 5' of the murine DNA methyltransferase cDNA (10 μg) was co-introduced into Y1 cells with 1 μg of pUCSVneo as a selectable marker by DNA-mediated gene transfer using the calcium phosphate protocol, and G418-resistant cells were cloned in selective medium (0.25 mg/ml G418) (26). Anchorage-independent growth in soft agar (1×10^3 cells) was as described previously (27).

DNA and RNA Analyses—Genomic DNA was prepared from pelleted nuclei, and total cellular RNA was prepared from cytosolic fractions according to standard protocols (26). *Msp*I or *Hpa*II restriction enzymes (Boehringer Mannheim) were added to DNA at a concentration of 2.5 units/μg for 8 h at 37 °C. At least three different DNA preparations isolated from three independent passages were assayed per transfectant. To quantify the relative abundance of DNA MeTase mRNA, total RNA (3 μg) was blotted onto Hybond N^+ using the Bio-Rad slot-blot apparatus. The filter-bound RNA was hybridized to a ^{32}P-labeled 1.31-kb cDNA probe encoding the putative catalytic domain of the mouse DNA MeTase (3170–4480) (28) and exposed to XAR film (Koduk). The relative amount of total RNA was determined by measuring the signal obtained after hybridization to an 18 S RNA-specific ^{32}P-labeled oligonucleotide (29). The autoradiograms were scanned with a Scanalytics scanner (one-dimensional analysis), and the signal at each band was determined and normalized to the amount of total RNA at the same point. Three determinations were performed per RNA sample.

Nearest Neighbor Analysis—Two μg of DNA were incubated at 37 °C for 15 min with 0.1 unit of DNase, 2.5 μl of [α-^{32}P]dGTP (3000 Ci/mmol from Amersham), 2 Kornberg units of DNA polymerase (Boehringer) were then added, and the reaction was incubated for an additional 25 min at 30 °C. Fifty μl of water were then added to the reaction mixture, and the nonincorporated nucleotides were removed by spinning through a Microspin S-300 HR column (Pharmacia). The labeled DNA (20 μl) was digested with 70 μg of micrococcal nuclease (Pharmacia) in the manufacturer's recommended buffer for 10 h at 37 °C. Equal amounts of radioactivity were loaded on TLC phosphocellulose plates (Merck), and the 3' mononucleotides were separated by chromatography in one dimension (isobutyric acid:H_2O:NH_4OH in the ratio 66:33:1). The chromatograms were exposed to XAR film (Eastman-Kodak), and the autoradiograms were scanned by scanning laser densitometry (Scanalytics one-dimensional analysis). Spots corresponding to cytosine and 5-methylcytosine were quantified.

Assay of DNA MeTase Activity—To determine nuclear DNA MeTase levels, cells were maintained at a nonconfluent state and fed with fresh medium every 24 h for at least 3 days prior to harvesting, and DNA MeTase activity was assayed as described previously (9).

Strand-specific Reverse-transcribed PCR—Total RNA (1 μg) prepared from each transfectant was reverse-transcribed with either a sense primer corresponding to bases 1–30 in the published mouse DNA MeTase cDNA sequence (28), 5' GCAAACAGAAATAAAAAGCCAGTTGTGTGA 3' to detect antisense RNA or an antisense primer corresponding to bases 475–451, or 5' CCACAGCAGCTGCAGCACCACTCT 3' to detect sense DNA MeTase RNA using the conditions described above. RNA incubated with reverse transcriptase in the absence of primers was used as a control. The reaction was terminated by heating to 95 °C for 10 min. The reverse-transcribed cDNA was subjected to amplification in the presence of both primers using the Hot Tub amplification protocol conditions described above. The DNA was amplified for 40 cycles of 2 min at 95 °C, 2 min at 60 °C, and 0.5 min at 72 °C. The reaction products were separated on an agarose gel, Southern blotted onto Hybond N^+ filter, and hybridized with a ^{32}P-labeled internal oligonucleotide corresponding to bases 190–211: 5' AAATGGCAGACTCAAATAGAT 3'. The conditions used (40 cycles of amplification) do not provide a quantitative assessment of the level of mRNA, but were used to exclude the possibility that small levels of antisense mRNA is present in the control transfectants.

Tumorigenicity Assays—LAF-1 mice (Bar Harbor) (6–8-week-old males) were injected subcutaneously (in the flank area) with 10^6 cells. Mice were monitored for the presence of tumors by daily palpation. Mice bearing tumors of greater than 1 cm in diameter were sacrificed, while tumor-free mice were kept for 90 days.

Electron Microscopy—Cells were fixed in glutaraldehyde (2.5%) in cacodylate buffer (0.1 M) for 1 h and further fixed in 1% osmium tetroxide. The samples were dehydrated in ascending alcohol concentrations and propylene oxide followed by embedding in Epon. Semithin sections (1 μm) were cut from blocks with an ultramicrotome and counterstained with uranyl acetate and lead citrate. Samples were analyzed using a Philips 410 electron microscope (30).

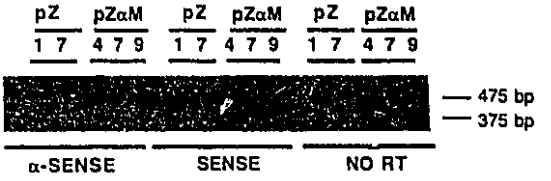

FIG. 1. **Expression of pZαM in Y1 adrenocortical cells.** To determine the strand specificity of the RNA transcribed by the pZαM vector, we employed a reverse transcriptase-PCR analysis using a sense-specific primer (*lanes labeled SENSE*), an antisense-specific primer (*lanes labeled α-SENSE*), or no primers (*lanes labeled NO RT*). Total RNA (1 μg) was reverse-transcribed with either a sense-specific primer, an α-sense-specific primer, or no primers. Resulting cDNA was then subjected to PCR with the complementary oligonucleotide (sense or α-sense) as described under "Materials and Methods," one-tenth of the PCR reaction was Southern-blotted and hybridized with a ^{32}P-labeled oligonucleotide encoding a sequence included in the amplified mRNA region (see "Materials and Methods" for description of the sequences of the primers). Sense DNA MeTase (*SENSE*) is observed in both pZαM and control pZEM transfectants as expected (475-bp product). An antisense transcript is seen only in pZαM transfectants. An unexpected additional sense amplification product of 375 bp is seen in all the pZαM transfectants.

RESULTS

Expression of Antisense to the DNA Methyltransferase mRNA in Y1 Cells Results in Limited Inhibition of DNA Methylation—To directly inhibit DNA methylation in Y1 cells, we introduced either the DNA MeTase antisense expression construct pZαM (encoding 600 bp from the 5' of the DNA MeTase cDNA in the antisense orientation) or a pZEM control vector (7) into Y1 cells by DNA-mediated gene transfer as described under "Materials and Methods." Six G418-resistant colonies were isolated and propagated for both constructs. All antisense transfectants (determined by a preliminary Southern blot analysis) exhibited distinct morphological differences from the pZEM transfectants or nontransfected Y1 cells. Based on Northern blot analysis of the antisense mRNA expression, three independent pZαM transfectants (4, 7, 9) were selected for further characterization.

To verify that the antisense mRNA strand is transcribed in the pZαM transfectants, we employed reverse-transcribed PCR analysis using strand-specific primers as described under "Materials and Methods." The experiment presented in Fig. 1 demonstrates that when the sense oligonucleotide is used for reverse transcription (transcribing the antisense mRNA strand), the expected 0.475- kb amplification product is observed only in pZαM transfectants. As expected, the endogenous DNA MeTase sense mRNA is amplified in both pZEM and pZαM transfectants when the antisense oligonucleotide is used for reverse transcription (sense). Interestingly, a smaller amplification product (0.375 kb) is also seen in the pZαM lines suggesting that another splice variant of DNA MeTase mRNA is transcribed in these transfectants. The biological significance of the induction of the smaller variant in the pZαM transfectants is unclear.

The mechanism responsible for inhibition of gene expression by antisense is still unclear; however, some models suggest that degradation of the hybrid RNA by RNase H might be involved. To determine whether expression of an antisense to the DNA MeTase can lead to a reduction in the steady state level of endogenous DNA MeTase mRNA, we isolated total RNA from the antisense transfectants and the pZEM controls. DNA MeTase activity is regulated with the state of growth of cells (8, 9); therefore, all cultures were maintained at the logarithmic phase of growth and fed with fresh medium every 24 h for 3 days prior to harvesting. Total RNA isolated from the

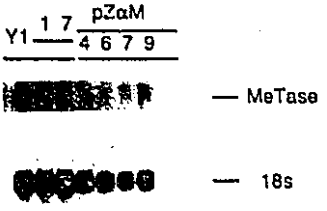

FIG. 2. **DNA MeTase expression, activity, and genomic methylation levels of pZαM transfectants.** Total cellular RNA (10 μg) prepared from pZαM lines (*4*, *6*, 7, and *9*), pZEM transfectants (*1* and *7*) and from Y1 controls was subjected to Northern blot analysis and hybridization with a 1.3-kb DNA MeTase 3'-cDNA probe (encoding bases 3170–4480 from the cloned mouse cDNA (27)). The filter was stripped and rehybridized with an 18 S rRNA probe. Relative MeTase expression was determined by densitometric analysis (see text).

transfectants was subjected to a Northern blot analysis and sequentially hybridized with a probe to the putative catalytic domain of the mouse DNA MeTase mRNA (MET 3') and an 18 S rRNA-specific ^{32}P-labeled oligonucleotide probe as described under "Materials and Methods." A result of such an analysis is presented in Fig. 2. Scanning of the autoradiogram indicates that the relative abundance of the 5-kb DNA MeTase mRNA (Fig. 2, *top panel*) relative to 18 S rRNA (Fig. 2, *bottom panel*) is reduced 2-fold in the three antisense transfectants. To quantify expression of DNA MeTase, the different RNA samples were subjected to a slot-blot analysis and sequential hybridization with the DNA MeTase and 18 S rRNA probes. The relative level of DNA MeTase mRNA in the different samples was determined by scanning densitometry. The results of such an analysis show that the pZαM transfectants exhibit an average decrease of 45% and a maximal decrease of 58% in the abundance of DNA MeTase mRNA relative to the pZEM controls ($p < 0.001$). The mean value for the control group was 0.480, S.D. = 0.104, the mean for the antisense group was 0.280, S.D. = 0.066.

We next compared the DNA MeTase enzymatic activity present in nuclear extracts prepared from antisense transfectants relative to control pZEM transfectants using *S*-[*methyl*-^{3}H]adenosyl-L-methionine as the methyl donor and a hemimethylated double-stranded oligonucleotide as a substrate. The results of two experiments with triplicate determinations each indicate that the three pZαM transfectants express a lower level of DNA MeTase activity than the control transfectants with an average inhibition of DNA MeTase activity of 42% and a maximum of 48% relative to control ($p < 0.05$).

Whereas our experiments demonstrate that the DNA MeTase antisense transfectants bear a lower level of DNA MeTase activity than the control transfectants, it is important to note that we measured only steady state levels in the transfectants. It is hard to assess the actual level of inhibition of DNA MeTase activity at the time of transfection, when a higher copy number of DNA MeTase antisense RNA might have been present in the cell. The steady state level of DNA MeTase mRNA might reflect an equilibrium of different cellular regulatory controls over the level of DNA MeTase activity in the cell. To directly demonstrate that expression of the DNA MeTase antisense leads to inhibition of DNA methylation activity in the cell, we determined whether it leads to a general reduction in the level of methylation of the genome. We performed a "nearest neighbor" analysis using [α-^{32}P]dGTP as described previously (6). This assay enables one to determine the percentage of methylated and nonmethylated cytosines residing in the dinucleotide sequence CpG (6). The results of three such experiments show that the mean value for the pZEM controls as a group was 9.7% nonmethylated cytosines, S.D. = 2.13, the mean value for the antisense lines as a group was 23.83% cytosine, S.D. = 5.88. $p < 0.001$.

In summary, our experiments demonstrate that expression of an antisense to the DNA MeTase mRNA leads to partial inhibition of DNA MeTase mRNA and DNA MeTase enzymatic activities and a significant reduction in the level of genomic cytosine methylation.

Demethylation of Specific Genes in Y1 and pZαM Transfectants—To further verify that expression of pZαM results in demethylation and to determine whether specific genes were demethylated, we resorted to a *Hpa*II/*Msp*I restriction enzyme analysis followed by Southern blotting and hybridization with specific gene probes. *Hpa*II cleaves the sequence CCGG, a subset of the CpG dinucleotide sequences, only when the site is unmethylated, while *Msp*I will cleave the same sequence irrespective of its state of methylation. By comparing the pattern of *Hpa*II cleavage of specific genes in cells expressing pZαM with that of the parental Y1 or cells harboring only the vector, we determined whether the genes are demethylated in the antisense transfectants. We first analyzed the state of methylation of the steroid 21-hydroxylase gene (C21) (29, 31). This gene is specifically expressed and hypomethylated in the adrenal cortex, but is inactivated and hypermethylated in Y1 cells (29, 31). We have previously suggested that hypermethylation of C21 in the Y1 cell is part of the transformation program that includes the shutdown of certain differentiated functions (29). DNA prepared from Y1, pZαM (4, 7, 9), and pZEM (1 and 7) transfectants was subjected to either *Msp*I or *Hpa*II digestion, Southern blot analysis, and hybridization with a 3.8-kb *Bam*HI fragment containing the body of the C21 gene and 3' sequences (Fig. 3, *bottom panel*, for physical map). Full demethylation of this region should yield a doublet at ~1 kb, an 0.8-kb fragment, and a 0.4-kb fragment, as well as a number of low molecular weight fragments at 0.1–0.4 kb. As observed in Fig. 3, the C21 locus is heavily methylated in Y1 cells, as well as the control transfectant, as indicated by the high molecular weight fragments. Only a relatively weak digestion product is seen at 1.9 kb (Fig. 3). This pattern of hypermethylation of C21 which is observed in Y1 cells and different control transfectants, that were analyzed in our laboratory in the last 5 years, is markedly stable. On the other hand, the antisense transfectant's DNA is significantly hypomethylated at this locus as indicated by the relative diminution of the high molecular weight fragments and relative intensification of the partial fragment at 1.9 kb. The appearance of new partial fragments in the lower molecular weight range between 1 and 0.4 kb indicates partial hypomethylation at a large number of *Hpa*II sites contained in the 3' region of the C21 gene (see physical map) (29, 31). The pattern of demethylation, indicated by the large number of partial *Hpa*II fragments, is compatible with a general partial hypomethylation rather than a specific loss of methylation in a distinct region of the C21 gene.

To determine whether demethylation is limited to genes that are potentially expressible in Y1 cells such as the adrenal cortex-specific C21 gene (29) or if the demethylation is widely spread in the genome, we tested the methylation state of the MyoD (32) and p53 5' locus. Specific demethylation of MyoD and the p53 fragment was seen in the pZαM transfectants (data not shown).

Morphological Transformation Loss of Anchorage-independent Growth and Inhibition of Tumorigenicity of Y1 Cells Expressing Antisense to the DNA MeTase—As the level of DNA MeTase activity is regulated with the state of growth and is induced in transformed cells and in tumors *in vivo* (8, 9, 13, 19), we determined whether expression of the DNA MeTase antisense construct results in a change in the tumorigenic potential of Y1 cells. A comparison of pZαM transfectants and controls showed a small

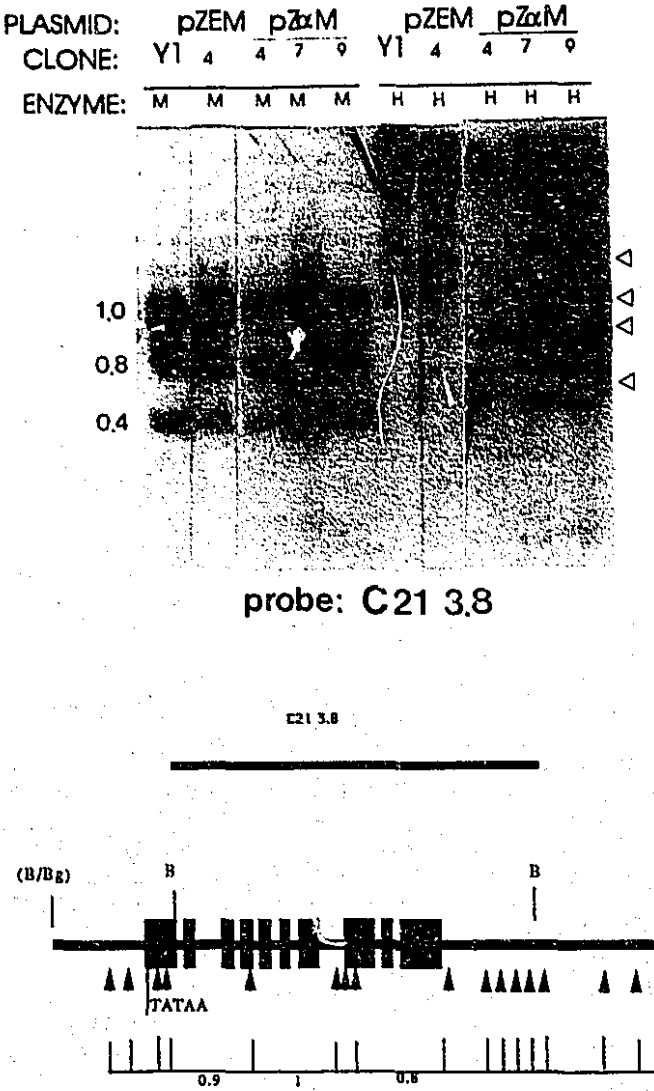

FIG. 3. **The pattern of methylation of C21 hydroxylase in pZαM transfectants and pZEM controls.** Genomic DNA (10 μg) was extracted from the transfected lines and subjected to digestion with either *Msp*I (*M*) or *Hpa*II (*H*), Southern blot transfer, and hybridization with a ^{32}P-labeled DNA probe (3.8-kb genomic fragment of the C21 gene, see *bottom panel* for physical map). The *open arrows* indicate *Hpa*II fragments resulting from demethylation of the different sites in the C21 gene in pZαM transfectants. Complete digestion of the region will yield 0.36- and 0.16-kb fragments.

but statistically significant reduction in the growth rate of antisense lines relative to the Y1 controls especially at higher densities (which is statistically significant, $p < 0.001$). This may reflect contact-inhibited growth and increased serum requirements of the antisense lines (data not shown). The morphological properties of the pZαM transfectants further support this conclusion (Fig. 4). While control Y1 and pZEM cells exhibit limited contact inhibition and form multilayer foci, pZαM transfectants exhibit a more rounded and distinct morphology and grow exclusively in monolayers, and, in many cases, pZαM cells form distinct cellular processes (Fig. 4).

The ability of cells to grow in an anchorage-independent fashion is considered to be an indicator of tumorigenicity (27). A soft agar assay performed in triplicate showed that the pZαM transfectants demonstrate a significant decrease in their ability to form colonies in soft agar; pZEM 1 and 7 form an average of 38 and 37 colonies, respectively, while pZαM transfectants 4, 7, and 9 formed an average of 12, 15, and 18 colonies, respectively. Moreover, the colonies that do form are significantly smaller and contain fewer cells.

Another indicator of the state of transformation of a cell is its

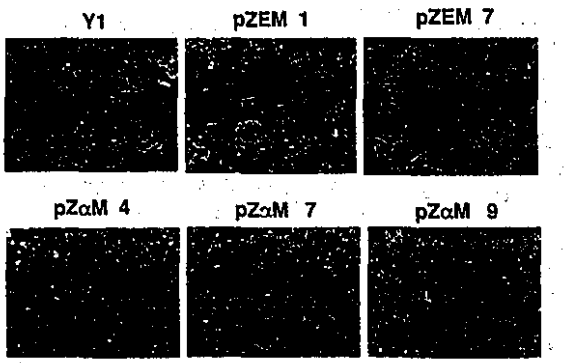

FIG. 4. **Morphological transformation of Y1 cells transfected with pZαM.** Phase contrast microscopy at × 200 magnification of living cultures of Y1 clonal transfectants with pZαM and pZEM controls. Equal numbers of cells were plated (1×10^5 cells per well in a six-well dish), and pictures were taken 72 h after seeding.

serum dependence. Tumor cells exhibit limited dependence on serum and are usually capable of serum-independent growth (33). Factors present in the serum are essential for the survival

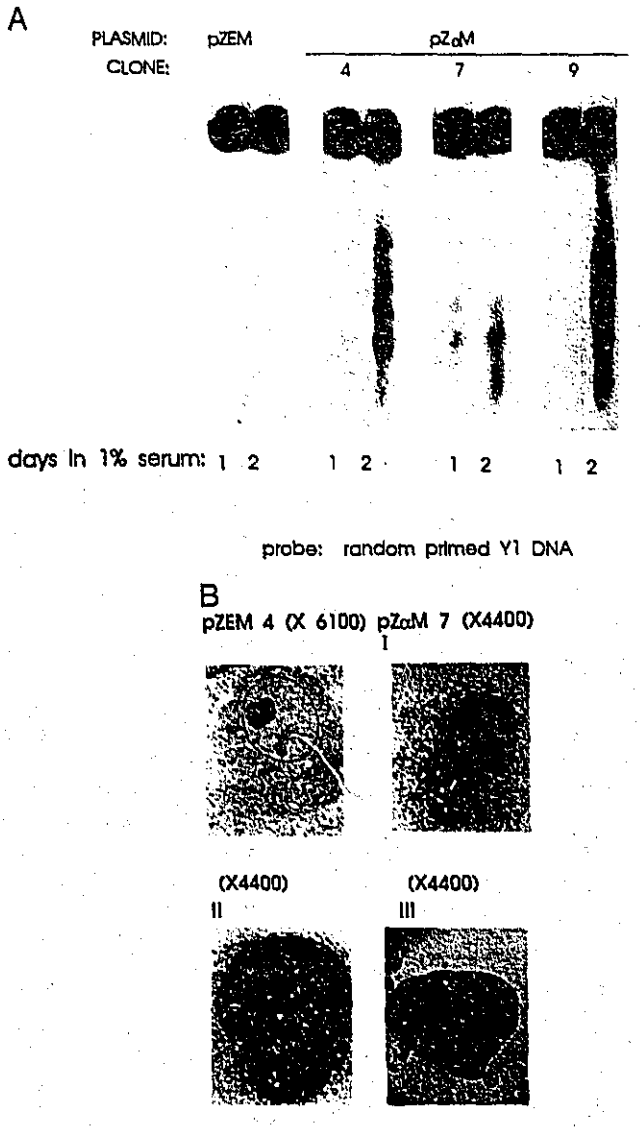

FIG. 5. **Survival and apoptosis of pZEM transfectants in serum-deprived medium.** *A*, the indicated transfectants were plated in 1% serum-containing medium and harvested after 1 and 2 days. Total cellular DNA was isolated, separated by agarose gel electrophoresis, transferred to nitrocellulose membrane, and probed with ^{32}P-labeled Y1 genomic DNA. A 180-bp internucleosomal ladder characteristic to cells dying via apoptosis can be seen in the pZαM transfectants only. *B*, Y1 transfectants were grown in 1% serum medium for 24 h, fixed, and analyzed by electron microscopy for early signs of apoptotic death; *I–III* are various sections (the magnification is indicated) of Y1 pZαM transfectants and pZEM control lines.

of many nontumorigenic cells. As observation of the pZαM transfectants indicated that they expressed enhanced dependence on serum and limited survivability under serum-deprived conditions, we determined whether this limited survivability involved an enhancement or induction of an apoptotic program. While the control cells exhibited almost 100% viability up to 72 h after transfer into serum-deprived medium, all pZαM transfectants showed up to 75% loss of viability at 48 h.

To test whether the serum-deprived pZαM cells were dying as a result of an activated apoptotic death program, cells were plated in starvation medium and harvested at 24-h intervals, and total cellular DNA was isolated from the cells and analyzed by agarose gel electrophoresis. After 48 h in serum-starved conditions, pZαM transfectants exhibit the characteristic 180-bp internucleosomal DNA ladder while the control pZEM transfectants show no apoptosis at this time point (Fig. 5*A*).

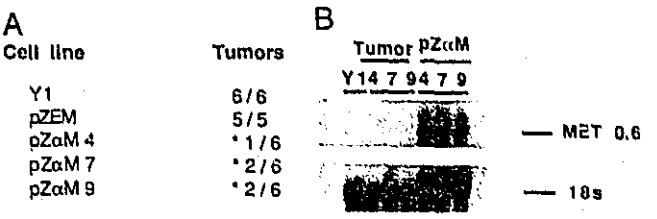

FIG. 6. ***In vivo* tumorigenicity of pZαM transfectants.** *A*, parental Y1 cells, a pZEM control line, and three pZαM transfectants (*4*, *7*, and *9*) were tested for their ability to form tumors in syngeneic LAF-1 mice. Tumor formation was assessed by palpation for 2 months after injection. The number of mice forming tumors is tabulated. The statistical significance of the difference between the control and antisense transfectants was determined using a test; $p > 0.001$. * indicates that these tumors were negative for pZαM expression. *B*, loss of antisense DNA MeTase expression in tumors derived from antisense transfectants. RNA (10 μg) isolated from the indicated tumors was subjected to Northern blot analysis and hybridization with the 0.6-kb MET cDNA probe. Expression of the 1.3-kb antisense message is seen only in the original cell lines pZαM (*4*, *7*, and *9*) and is undetectable in tumors arising from pZαM transfectants or Y1 cell lines even after long exposure. The filter was stripped of radioactivity and rehybridized with a ^{32}P-labeled oligonucleotide corresponding to 18 S rRNA (28).

To determine whether cells expressing antisense to the DNA MeTase exhibit early morphological markers of apoptosis, cells were serum-starved for 24 h, harvested, and analyzed by electron microscopy. Fig. 5*B* shows representative electron micrographs of several blocks of control pZEM and pZαM transfectants at various magnifications (*I–III*). The control cells have a fine uniform nuclear membrane whereas the pZαM cells exhibit the cardinal markers of apoptosis (34): condensation of chromatin and its margination at the nuclear periphery (*panels I* and *II*), chromatin condensation (*panel II*), nuclear fragmentation (*panel III*), formation of apoptotic bodies, and cellular fragmentation. Whereas it is still unclear whether apoptosis upon serum deprivation is directly enhanced by demethylation or is an indirect effect of the change in the transformed state of the transfectants, the serum deprivation-induced cell death is another indicator of the reversal of cellular transformation by DNA MeTase antisense.

To determine whether demethylation can result in inhibition of tumorigenesis *in vivo*, we injected 1×10^6 cells for each of the Y1, pZEM, and pZαM (4, 7, and 9) transfectants subcutaneously into the syngeneic mouse strain LAF-1. The presence of tumors was determined by palpation. While all the animals injected with Y1 or pZEM cells formed tumors, animals injected with the pZαM transfectants had very few tumors arise (Fig. 6*A*; $p > 0.005$).

One possible explanation for the fact that a small number of tumors did form in animals injected with the pZαM transfectants is that they are derived from revertants that lost expression of the antisense to the DNA MeTase under the selective pressure *in vivo*. RNA was isolated from tumors arising from the pZαM transfectants, and the level of expression of the 0.6-kb antisense message was compared with the transfectant lines *in vitro* (Fig. 6*B*). The expression of the antisense message is virtually nonexistent in the tumors derived from pZαM transfectants even after long exposure of the Northern blots, supporting the hypothesis that expression of an antisense message to the DNA MeTase is incompatible with tumor growth *in vivo*.

DNA Demethylation Induced by 5-AzaCdR Results in Reversal of Cellular Transformation ex Vivo—To further verify that inhibition of DNA methylation results in reversal of cellular transformation and to exclude the possibility that the effects observed are nonspecific results of antisense expression we used an inhibitor of DNA methylation 5-azadeoxycytidine (5-

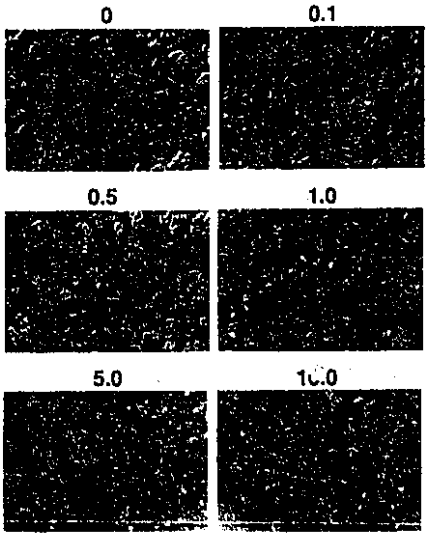

FIG. 7. **Morphological change in Y1 cells treated with 5-azaCdR.** Y1 cells were treated with concentrations of 5-azaCdR ranging from 0–10 μM every 12 h for 72 h. Phase contrast microscopy at × 200 magnification of living cultures of the treated cells is presented.

azaCdR) that acts at a different site than antisense RNA (35). 5-azaCdR is a deoxycytidine analogue that inhibits DNA methylation once it is incorporated into DNA. It has been suggested that an irreversible complex is formed between the DNA MeTase enzyme and the C-6 position of the cytosine moiety (36). We treated Y1 cells with concentrations of 5-azaCdR ranging from 0 to 10 μM every 12 h for 72 h. 5-azaCdR increases the proportion of cytosine to methylcytosine in the DNA by 1.6-fold in a dose-dependent manner (0–5.0 μM) as determined by a nearest neighbor analysis. Over the same concentration range, cell viability in low serum is reduced from 80% to ~40%, and the ability of cells to form colonies in soft agar is reduced by ~50-fold. No differences were seen in the ability of cells to form colonies on regular plastic dishes. The 5-azaCdR-treated cells exhibited dose-dependent morphological changes similar to those observed in the pZαM transfectants (Fig. 7). This experiment suggests that 5-azaCdR treatment reversed the transformed phenotype of Y1 cells but did not affect their viability.

DISCUSSION

This paper tests the hypothesis that overexpression of the DNA MeTase plays a causal role in cellular transformation by expressing an antisense message to the DNA MeTase in an adrenocortical carcinoma cell line. Expression of an antisense DNA MeTase (Fig. 1) leads to: (i) a limited reduction in DNA MeTase steady state mRNA and protein levels (Fig. 2), (ii) a general but limited reduction in the methylation content of the genome (Fig. 2), (iii) demethylation of regions aberrantly methylated in this cell line such as the adrenal specific 21-hydroxylase gene (Fig. 3), (iv) morphological changes indicative of inhibition of the transformed phenotype, (v) inhibition of anchorage-independent growth as determined by soft agar assays, (vi) inhibition of serum-independent survivability and induction of apoptosis under serum-deprived conditions, as well as (vii) inhibition of tumorigenesis in syngeneic mice (Fig. 6) and (viii) inhibition of DNA methylation by 5-azaCdR, which acts at a site completely different from antisense to the DNA MeTase, also results in reversal of transformation indicators *ex vivo*. The fact that a 2-fold inhibition in DNA MeTase expression is sufficient to induce such profound changes in the state of transformation of Y1 cells is in accordance with previously published data showing that a 2–3-fold elevation in DNA MeTase activity by forced expression of an exogenous DNA MeTase in NIH 3T3 can induce cellular transformation of these cells (23). Whereas antisense expression is considered one of the most direct means to inhibit gene expression, no experimental method is devoid of potential complications. 5-azaCdR, which is the most commonly used DNA methylation inhibitor, has side effects (36, 37). However, the fact that both inhibitors had similar effects strongly validates our conclusions. The fact that 5-azaCdR inhibited transformation indicators but not the survival of the cells and their ability to form colonies, and the fact that the reversal of transformed phenotype was expressed weeks after the inhibitor had been removed is consistent with the model that 5-azaCdR triggered a change in the cellular program rather than a cytotoxic or cytostatic effect. It stands to reason that this change in program was triggered by the initial demethylation event caused by the drug.

Our experiments support a previously proposed hypothesis that overexpression of DNA MeTase is an important component of an oncogenic pathway(s) (22). Since Y1 is a line derived from a naturally occurring tumor (24) which bears amplified copies of Ras (25), it is possible that hyperactivation of the DNA MeTase is triggered by the Ras-Jun signaling pathway (21, 22). The DNA MeTase promoter bears a number of AP-1 sites (20), and we demonstrated that the activity of the DNA MeTase promoter is dependent on binding of AP-1 (21) and that down-regulation of the the Ras-Jun pathway in Y1 cells results in inhibition of DNA MeTase activity, hypomethylation, and reversal of the transformed phenotype.[2] Our data might explain previous observations demonstrating an increase in DNA MeTase activity (13, 19) in cancer cells by suggesting that this increase is critical for the transformed state.

What is the possible mechanism by which hypermethylation can cause cellular transformation? The answer to this question is still elusive and could not be resolved by the data presented in this paper; however, several hypotheses have been previously suggested. One plausible explanation that has been previously suggested by Baylin and his colleagues (38) is that methylation may establish abnormalities of chromatin organization which in turn mediate the progressive losses of gene expression associated with tumor development. One interesting class of genes that might be affected are the tumor suppressor genes. There is evidence that ectopic inactivation of tumor suppressor genes by methylation contributes to cancer (39, 40). The promoter region of the RB-1 gene was found to be methylated in 6 of 77 retinoblastomas (17), and the 5′ region of the WT-1 gene was methylated in 2 out of 29 Wilms tumors (40), while the gene methylated was otherwise grossly normal. However, there is no evidence that tumor suppressor genes are the critical targets for hypermethylation in cancer cells or that tumor suppressor genes are selectively demethylated in the DNA MeTase antisense transfectants. Also, our unpublished data do not suggest any induction in the level of expression of these genes in the pZαM transfectants.

Another interesting mechanism that has been suggested by Jones and his colleagues is that methylated CpGs are hot spots for mutations by deamination of the methylated cytosine into thymidine (41). This kind of change induced by methylation will not be reversible, the fact that we could reverse transformation by inhibiting DNA MeTase suggests that other mechanisms must be involved. While inhibition of gene expression by methylation is the best analyzed function of DNA methylation, one should bear in mind that any function of the genome might be modified by methylation. Sites that are especially sensitive to changes in methylation might be controlling DNA functions such as repair, replication, and susceptibility to

[2] A. R. MacLeod, J. Roleau, and M. Szyf, unpublished results.

death program-related endonucleases.

One question that remains to be answered is how to explain the contradiction between the fact that DNA MeTase is overexpressed in cancer cells and the observed regional hypomethylation of the genome of many cancer cells (11, 12). However, there are no data at this stage to resolve this apparent contradiction. While additional experiments will be required to address these questions, this paper demonstrates that inhibition of DNA methylation leads to a reversal of the transformed state and that DNA methylation plays a critical role in cellular transformation.

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Chapter 4: Expression of antisense mRNA to the DNA Methyltransferase inhibits v-Ha-Ras initiated transformation of C2C12 cells. A.R. MacLeod, V. Bozovic, J. Rouleau and M.Szyf. (Submitted manuscript).

The results presented in the previous chapters demonstrate that induction of the DNA MeTase by Ras is critical for maintaining the transformed state of Y1 cells. Here, we address the hypothesis that the DNA MeTase is actively involved in the initiation of transformation of C2C12 myoblast cells by v-Ha-Ras, and that DNA hypermethylation is involved in the inhibition of differentiation of v-H-Ras expressing C2C12 cells.

Expression of antisense mRNA to the DNA Methyltransferase inhibits transformation initiated by v-H-Ras in C2C12 myoblasts.

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ABSTRACT

Many tumor cell lines overexpress DNA methyltransferase (MeTase) activity and forced expression of an exogenous DNA MeTase cDNA leads to transformation of NIH 3T3 cells. Recently it has been shown that the Ras signal transduction pathway can trans-activate the DNA MeTase promoter. Here we test the hypothesis that transformation of C2C12 cells initiated by oncogenic v-H-ras can be inhibited by coexpression of an antisense to the DNA MeTase mRNA. C2C12 cells transformed by v-H-ras expression show increased DNA MeTase mRNA, DNA MeTase enzymatic activity and DNA hypermethylation at both the MyoD and p53 loci. C2C12 cells coexpressing v-H-ras and an antisense to the DNA MeTase have DNA MeTase levels similar to parental C2C12 cells, do not exhibit DNA hypermethylation at either MyoD or p53 locus and retain their non transformed phenotype. C2C12 cells expressing v-H-ras and DNA MeTase antisense also show delay in appearance of tumors as well as reduced tumor load when injected into syngeneic C3H mice compared to v-H-Ras transformed C2C12 cells. These results support the hypothesis that the DNA MeTase is a critical component in oncogenic transformation initiated through the Ras signal transduction pathway.

INTRODUCTION

Terminal differentiation and cellular transformation have been shown in many cases to be a mutually exclusive processes (1-3). In fact, the progression of the transformed state is characterized by sequential changes in normal gene expression profiles and the inactivation of differentiation specific functions as well as by loss of cell cycle control (4).

In muscle cells, terminal differentiation requires the activation of several of muscle-specific genes (including a-actin, myosin heavy and light chains, and acetylcholine receptors). The transcription of some of these muscle-specific genes is activated by the products of the MyoD gene family (including MyoD,MyoH and myogenin) (5). However, expression of the activated ras oncogene in these cells inhibits their differentiation, most probably by transcriptional silencing of regulatory control loci such as MyoD1, myf5 (6).

What are the molecular events initiated by ras that lead to the inactivation of such control loci and ultimately to transformation? Recent data has suggested that one possible downstream effecter of RAS-triggered cellular transformation is the AP-1 transcription transactivation complex (7). We have recently shown that ras can lead to activation of the DNA methyltransferase (DNA MeTase) gene through consensus AP-1 sites contained in its 5' regulatory region (8). The DNA MeTase is responsible for methylation of cytosine residues located in the dinucleotide sequence CpG (9,10). Methylation of CpG sequences in the body or regulatory regions of a gene is generally thought to be a potent transcriptional inactivation signal. The fact that genes can be inactivated by methylation as well as that wide areas of the genome, such as CpG islands, are methylated specifically in cancer cells is well established (11,12). A CpG-island rich area on chromosome 17p, which is reduced to homozygocity in lung and colon cancer, as well as regions of chromosome 3p, that are consistently reduced to homozygocity in lung cancer, are hypermethylated (13). Interestingly a CpG island contained within the first exon of the MyoD gene itself is unmethylated in all somatic tissues but is extensively methylated in several tumor cell lines, except those expressing myogenic genes (14). One possible explanation for these changes in the DNA methylation pattern is the dramatic elevation of DNA MeTase activity observed in many cancer cells (15,16). Recent evidence also

suggests that forced expression of an exogenous DNA MeTase cDNA can transform NIH 3T3 cells (17).

We reasoned that if transformation by the Ras oncogene is mediated by induction of the DNA MeTase, then expression of v-H-Ras in C2C12 cells should lead to induction of the DNA MeTase then DNA hypermethylation and that inhibition of DNA MeTase by expression of antisense to the DNA MeTase in these transformed cells should cause both DNA hypomethylation and suppression of the transformed phenotype.

MATERIALS AND METHODS

Cell culture and DNA mediated gene transfer. C2C12 cells were maintained as monolayers in Dubelcco's modified Eagle's medium which was supplemented with 20% (v/v) heat inactivated fetal calf serum (Immunocorp, Montreal). All other media and reagents for cell culture were obtained from GIBCO-BRL. C2C12 cells (1X106) were plated on a 150 mm dish (Nunc) 15 hours before transfection. The pZEM ras expression (10μg) vector (8) encoding v-Ha-Ras and 10μg of the pZαM expression vector encoding the 5' of the murine DNA MeTase cDNA (27) were cointroduced into C2C12 cells with 1 μg of pUCSVneo as a selectable marker by DNA mediated gene transfer using the calcium phosphate protocol (18). Another set of cells was cotransfected with pZEM ras and pUCSVneo. Selection was initiated 48 hours after transfection by adding 0.4 mg/ml G418 (GIBCO-BRL) to the medium.

Soft Agar assay. Parental C2C12, pZEM, pZEM Ras and pZEM Ras + pZαM transfectants were seeded in triplicate onto 30 mm dishes (Falcon) at a density of 1x103 cells/well with 4 ml of Dubelcco's modified Eagle's medium which was supplemented with heat inactivated fetal calf serum, 0.4 mg/ml G418 (for transfectants) and 0.33% agar solution at 37oC. Cells were fed with 1 ml of fresh medium and G418 every two days. Growth was scored as colonies containing >10 cells, 21 days after plating.

[3H]Thymidine incorporation assay. C2C12 controls, pZEM, pZEM ras and pZEM ras + pZαM were plated at a density of 1X104 cells/well in a 24-well plate (Nunc) in Dubelcco's modified Eagle's medium supplemented with 20% (v/v) heat inactivated fetal calf serum. After 6hrs cells were transferred to Dubelcco's modified Eagle's medium containing 0.5% (v/v) heat inactivated fetal calf serum. 24hrs later 1μCi of 3H-Thymidine (Dupont) was added to each well and left for an additional 6hrs. Cells were washed with PBS and 1ml of 10%TCA was added to each well and left for 1hr at 4oC after which 0.2 ml of the 10% TCA was left on the cells; this procedure was repeated another time. 0.5ml of a 1M NaOH-1%SDS solution was added to each well and incubated at 37oC for 1hr. The supernatant was then assayed for [3H]-Thymidine incorporation by liquid scintillation

counting.

Assay of DNA MeTase activity. To determine nuclear DNA MeTase levels, cells were maintained at a nonconfluent state and fed with fresh medium every 24 hours for at least three days prior to harvesting. Nuclei were isolated from 1x107 cells by incubating the cellular pellet for 15 min. at 4oC in a buffer containing 10 mM Tris-HCl (pH 7.4), 1.5 mM MgCl2, 15mM CaCl2, 0.5 mM DTT, 0.2 mM PMSF and 0.5% Nonidet P40 (BDH). Following centrifugation at 2 000 g for 15 min. the supernatant was separated from the nuclear pellet for total RNA extraction using the RNAzol preparation method (19). Nuclear extract was prepared by incubating the nuclear pellet in a buffer containing 20 mM Tris-HCl (pH 8.0), 25% glycerol, 1,5 mM MgCl2, 0.5 mM PMSF, 0.2 mM EDTA, 0.5 mM DTT and 0.4M NaCl followed by centrifugation at 10 000g for 30 min. DNA MeTase activity (3µg) was assayed by incubating 3 µg of nuclear extract with a synthetic hemimethylated double-stranded oligonucleotide (20) substrate and S-[methyl-3H]-S-adenosyl-L-methionine (78.9 Ci/mmol, Amersham) as a methyl donor for 3 hours at 37oC as previously described (20). To subtract non DNA dependent MeTase activity, each extract was incubated under the same conditions in the absence of the DNA substrate. The level of incorporation of methyl-3H into DNA was determined by 10% TCA precipitation, followed by GFC (Fisher) filtration and liquid scintillation (OptiPhase Hisafe Fison Chemicals) counting. Each assay was performed in triplicate. The no-DNA controls typically gave readings in the range of 1x104 dpm while the DNA samples gave readings in the range of 6x104 per 3 µg.

DNA and RNA analyses. Genomic DNA was prepared from pelleted nuclei and total cellular RNA was prepared from cytosolic fractions according to standard protocols (18). MspI or HpaII restriction enzymes (Boehringer Mannheim) were added to DNA at a concentration of 2.5 units/µg for 8 hrs at 37oC. At least three different DNA preparations isolated from three independent passages were assayed per transfectants to exclude variations between different DNA samples and verify the stability of the DNA methylation patterns. Radionucleotides (3000 mCi/mmol) were purchased from Amersham and the probes were labelled by random prime method (Boehringer Mannheim). To determine expression of Ras in stable transfectants total cellular RNA was prepared from cytosolic

supernatants by the RNAzol preparation method, fractionated on an 1.3% agarose/formaldehyde gel and Northern blotted onto Hybond-N+ (Amersham) in 20xSSC. The filter bound RNA was fixed with 40 mM NaOH as recommended by the manufacturer. The Northern blot was hybridized with a Ras probe generated by cutting pZEM ras with PstI and HindIII (Boehringer Mannheim) to generate a 0.7 kb fragment which was subsequently labelled according to procedures described above, and exposed to XAR film (Kodak). To determine expression of antisense to the DNA MeTase in the pZEM Ras + pZαM transfectants and to quantify the relative abundance of DNA MeTase mRNA, RNAase protection analysis was performed using probe A as previously described (21). The relative amount of total RNA was determined by measuring the signal obtained after RNAase protection against an 18S riboprobe (Ambion) which protects an 82 bp fragment. The autoradiograms were scanned with a Scanalytics scanner (one D analysis) and the signal at each band was determined. The signal obtained at each point was normalized to the amount of total RNA at the same point.

Generation of p53 5' probe by PCR. Oligoprimers for the 5' region of the mouse p53 gene were selected from the published genomic sequence (Accession number: X01235) (22) using the Primer selecting program (PC Gene). The 5' primer corresponding to bases 154-172: 5'TCC GAA TCG GTT TCC ACC C3' and the 3' primer corresponding to bases 472-492: 5'GGA GGA TGA GGG CCT GAA TGC3' were added to an amplification reaction mixture containing 100ng of mouse DNA (from C2C12 cells) using the incubation conditions recommended by the manufacturer (Amersham Hot tub) (1.5 mM MgCl2) and the DNA amplified for 40 cycles of 2 minutes at 95oC, 2 minutes at 55oC and 0.5 minutes at 72oC. The reaction products were separated on a low-melt agarose gel (BRL) and the band corresponding to the expected size was excised and extracted according to standard protocols (18).

Tumorigenicity assays. C3H mice (6-8 week old males, Charles River) were injected subcutaneously (in the flank area) with 106 cells. Mice were monitored for the presence of tumors by daily palpation. Mice bearing tumors of greater than 1 cm in diameter were sacrificed by asphyxiation with CO2, tumors were removed by dissection and weighed.

RESULTS

Morphological transformation and anchorage independent growth of C2C12 cells transfected with pZEM ras is inhibited by antisense to the DNA Methyltransferase. A remarkable feature of transformed cells is their ability to form multilayer foci, to grow in low serum (23,24,25) and in anchorage independent fashion (26). Since RAS has been shown to transactivate the DNA MeTase promoter (8) we determined whether RAS could induce DNA MeTase in C2C12 cells and whether this induction is critical for cellular transformation. C2C12 myoblast cells were transfected with either pZEM ras, an expression vector encoding the oncogenic viral gene V-(gly 12-val) H-ras, or with pZEM ras together with an expression vector encoding an antisense mRNA to the DNA MeTase (pZEMras/pZαM), construction of pZEM ras and pZαM has been described previously (8,27).

Northern blot analysis revealed limited expression of the oncogenic form of H-RAS in pZEM ras clones 5 and 10 as well as in pZEM ras/pZαM 3,6 and 23 (arrow indicates H-RAS expression at 2 kb) endogenous N,K,and H-RAS all hybridize to the probe used and give rise to the bands around 1 kb (Fig. 1C). These clones were selected because the limited expression of H-ras observed was ideal to test the ability of DNA MeTase antisense to inhibit the RAS initiated transformation. RNase protection analysis verified the expression of the antisense mRNA in three independent pZEM ras/pZαM clones (Fig.1D). Quantitative analysis of antisense expression was not determined. In culture, control C2C12 cells grew exclusively as a monolayer and showed the ability to fuse and form multinucleated myotubes spontaneously (Fig.1A), pZEM ras transfectants exhibited limited contact inhibition and formed multilayer foci consistent with a transformed phenotype (6). Cells coexpressing antisense to the DNA MeTase along with oncogenic ras (pZEM ras /pZαM transfectants) did not form multilayer foci but rather grew mostly as a monolayer (fig 1A), and exhibited a myogenic phenotype similar to parental C2C12 cells, suggesting that inhibition of the DNA MeTase by antisense expression could negate the Ras initiated

changes in morphology.

To further test whether expression of antisense to the DNA MeTase could inhibit the transformed phenotype initiated by Ras, we determined the ability of the various transfectants to grow in an anchorage independent fashion, which is considered an in vitro indicator of tumorigenicity. Nontransfected C2C12 and C2C12 cells transfected with vector sequences alone (C2 pZEM) served as controls. Transfectants were plated in triplicate in soft agar and foci were counted visually after 21 days (see materials and methods for details). Representative photomicrographs shown in figure 1B were taken 21 days after plating. As expected non-transfected C2C12 or pZEM controls did not form colonies in soft agar while the pZEM ras transfectants (5 and 10) were able to grow in anchorage independent fashion, while pZEM ras /pZαM transfectants lose their ability to form colonies in soft agar and only aggregates of a few cells were visible when evaluated by light microscopy (Fig.1B) Graphical representation of this data is shown in Figure 2A. Another indicator of the state of transformation of a cell is its serum dependence. Tumor cells exhibit limited dependence on serum and are usually capable of serum independent growth, whereas factors present in the serum are essential for the survival of many nontumorigenic cells. To further test the ability of antisense to the DNA MeTase to inhibit transformation initiated by H-ras in C2C12 cells, we determined the ability of these cells to initiated DNA synthesis in the absence of serum by [3H]-Thymidine incorporation assays. Transfectants and C2C12 cells were transferred from their growth medium containing 10% heat inactivated fetal calf serum to medium containing 0.5% heat inactivated fetal calf serum and assayed for their ability to incorporate [3H]-Thymidine into newly replicating DNA. As expected nontransformed C2C12 and pZEM controls do not initiate DNA synthesis in low serum. The pZEM ras transfectants (5 and 10) show a significant increase in the incorporation of [3H]-Thymidine suggesting that DNA replication occurs despite the lack of serum. However, when C2C12 were transfected with pZEM ras /pZαM, [3H]-Thymidine incorporation was comparable to nontransfected C2C12 controls (Fig.2B). In summary, the above set of experiments show that the expression of an antisense to the DNA MeTase in C2C12 cells can inhibit some

characteristic indicators of the transformed state initiated by oncogenic Ras.

C2C12 cells transfected with Ha-ras show an increase level of DNA Methyltransferase mRNA and activity. The DNA MeTase activity in the cell is limiting and therefore regulates the pattern of DNA methylation (28). Deregulation of DNA MeTase may therefore result in widespread changes in DNA methylation such as those common to cancer cells. The above results show that transformation of C2C12 by Ha-ras can be reverted by an antisense to the DNA MeTase suggesting that DNA MeTase is a downstream effecter of the Ras signalling pathway. Transformation by Ras should therefore lead to an elevated level of DNA MeTase both at the mRNA and enzymatic activity levels. To test this hypothesis we performed both RNAase protection assays and DNA MeTase activity assays on the various C2C12 transfectants. Total cellular RNA was prepared from exponentially growing cell lines and subjected to RNase protection assay for DNA MeTase mRNA using probe A, (see materials and methods). Protection of three fragments ranging from 90 to 99 is consistent with known initiation sites of the mouse DNA MeTase (21) (Fig. 3A). Normalization against the 82 bp protected fragment from an 18S ribonucleotide probe and quantitation of these results demonstrates that pZEM ras transfectants have an elevated level of DNA MeTase mRNA when compared to pZEM controls and that pZEM ras /pZαM transfectants (3 and 6) show DNA MeTase mRNA levels similar to controls (Fig. 3a bottom panel). Relatively high expression of DNA MeTase mRNA in the antisense clone 23 is not inconsistent with a functional antisense effect as the mechanism of antisense action on gene expression remains controversial. Furthermore we demonstrate that the DNA MeTase protein activity in this clone is significantly inhibited (Fig.3B), suggesting that translational arrest and not transcriptional arrest nor RNAse H dependent duplex degradation is the dominant mechanism of action of antisense in this clone. This mechanism of antisense action is supported by several lines of evidence (29,30). To determine if this increase in steady state mRNA level seen in the Ras transfectants translates into an increased level of DNA MeTase activity we resorted to enzymatic assays. DNA MeTase activity is regulated with the state of growth of cells, therefore all cultures were maintained at the exponential phase of growth and fed with fresh

medium every 24 h for three days prior to harvesting. DNA MeTase activity in the different nuclear extracts was assayed using [3H]-S-adenosyl-methionine as the methyl donor and a hemimethylated double stranded oligonucleotide as a substrate as described in Materials and Methods. Results of a representative experiment are shown in Figure 3B. Ras transfectants incorporate significantly more tritiated CH3 into hemimethylated DNA than do the pZEM or C2C12 controls. Expression of antisense to DNA MeTase in the Ras transfectants resulted in a reduction in DNA MeTase activity down to control levels. These results suggest that expression of Ras in C2C12 cells results in an increase in the steady state DNA MeTase mRNA level which translates into increased DNA MeTase activity.

Gene specific hypermethylation in C2C12 transfected with Ha-ras is inhibited in pZEM ras /pZαM transfectants. To further verify that Ha-ras transformation of C2C12 results in an increased activity of DNA MeTase we tested the possibility that specific genes would be hypermethylated in these lines and that this could be reverted by antisense to the DNA MeTase. To determine whether specific genes were hypermethylated, we resorted to MspI/HpaII restriction analysis followed by Southern blotting and hybridization with gene specific probes. HpaII cleaves the sequence CCGG, a subset of the CpG dinucleotide sequences, only when the site is unmethylated while MspI will cleave the same sequence irrespective of its state of methylation. By comparing the pattern of HpaII cleavage of specific genes we determined whether these genes have undergone any changes in their methylation state. The first locus to be analyzed was the muscle specific gene MyoD. The MyoD gene is ideal for this analysis because it contains a CpG island which is heavily methylated only in transformed cells (14). MspI/HpaII southern blot analysis of the MyoD locus in two independent pZEM Ras transfectants (5 and 10) as well as two pZEM ras /pZαM transfectants (3 and 23), revealed hypermethylation in both the pZEM ras transfectants. This hypermethylation is seen as an increase in the relative abundance of high molecular weight fragments in HpaII digested lanes (those above the 0.8 kb fully non methylated fragment) of pZEM ras clones 5 and 10 (Figure 4A). The relative diminution in intensity of the 0.8 kb band in pZEM ras 10 also

indicates that this fragment has undergone hypermethylation thus HpaII digestion gives rise to the higher molecular weight fragments (1.5, 2.0 and 3.0kb respectively). pZEM ras /pZαM transfectants do not exhibit any hypermethylation in the MyoD gene. The diagnostic 0.8 kb fragment is fully demethylated (intensified in the HpaII lanes) in both clone 3 and 23, to a level similar to pZEM controls. Moreover demethylation of the 630 bp fragment was observed in clone 23. Another class of genes that might have undergone hypermethylation includes the tumor suppressor genes. Loss of these gene products has been shown to lead to deregulation of the cell cycle and neoplasia (31). We therefore determined the state of methylation of the p53 5' region (Fig 4B). The p53 locus was studied using a 0.3 kb fragment from the 5' region 300 bp upstream to the initiation site as a probe as described in Materials and Methods. Cleavage of the C2C12 p53 loci (the mouse genome also contains a p53 pseudogene) with MspI and BamHI yields fragments with molecular weight of 0.4, 1.9, 6.0, 8.0 and 9.4 kb (Fig 4B. MspI lanes). Expression of pZEM ras in C2C12 cells results in hypermethylation of this locus as can be seen by loss of the 6.0 kb band, diminution in intensity of the 8.0 kb band and the relative intensification of the high molecular weight 9.4 kb band in the pZEM ras 5 and 10 HpaII lanes. Coexpression of antisense to the DNA MeTase results in a decreased hypermethylation of the p53 loci initiated by Ras as indicated by the reappearance of the 6.0 kb band and the loss in intensity of the 9.4 kb fully methylated band. Although not all transfectants showed the same degree of hypermethylation of the MyoD and p53 loci, possibly due to clonal variability, all Ras transfectants analysed were hypermethylated when compared to controls, consistent with the idea that Ras causes DNA hypermethylation through superinduction of the DNA MeTase.

C2C12 cells transfected with pZEM ras /pZαM show decreased tumorigenicity in vivo when compared to pZEM ras transfectants. To determine whether demethylation can result in inhibition of tumorigenesis initiated through the Ras signalling pathway, we injected 1x106 cells of the C2C12, pZEM, pZEM Ras or pZEM ras /pZαM transfectants subcutaneously into the syngeneic mouse strain C3H. The

presence of tumors was determined by palpation. While none of the animals injected with C2C12 or pZEM cells formed tumors, animals injected with pZEM ras transfectants did form tumors. Animals injected with pZEM ras /pZαM transfectants did form tumors but showed a delay in appearance compared to tumors in the pZEM Ras animals. Animals injected with pZEM Ras transfectants showed tumors within 4 to 10 days of injection whereas animals injected with C2C12 cells transfected with pZEM ras/pZαM were tumor free up to between days 32 and 36 after injection. In addition the tumors that did form in pZEM Ras/pZαM were all of smaller size than their pZEM ras counterparts (mean of 0.84 grams, SD= 0.28 for pZEM Ras, and mean of 0.246 grams, SD= 0.21 for pZEM Ras/pZαM).

One possible explanation for the fact that a small number of tumors did form in animals injected with pZEM ras/pZαM transfectants is that they are derived from revertants that lost expression of the antisense to the DNA MeTase under strong negative selective pressure in vivo. In fact we recently showed that tumors isolated from LAF-1 mice injected with Y1 adrenocortical tumor cells expressing antisense to the DNA MeTase lost the expression of the antisense message (32), supporting the hypothesis that expression of an antisense message to the DNA MeTase is incompatible with tumor growth in vivo.

DISCUSSION

Activating mutations in the Ras protooncogene gene are seen in approximately 50% of all human cancers (33). The induction of DNA MeTase activity and hypermethylation of specific loci is also a very common feature of cancer cells. Recent work by our laboratory suggests that a mechanistic link exists between these two observed phenomena (8,34). Therefore this study was designed to determine whether the DNA methylation machinery is an essential downstream component of Ras mediated oncogenic transformation using C2C12 cells as a model system. Although it is generally hard to extrapolate from events occurring in tumor cell lines in culture to the in vivo situation, it remains that cell lines offer the best possibility to dissect mechanisms and pathways in a manner that is almost impossible to accomplish in vivo, especially in the human. To test the hypothesis that DNA MeTase was causal to the active transformation of C2C12 myoblasts we transfected these cells with an expression vector encoding viral H-Ras, (pZEM Ras) while a second set of C2C12 cells were cotransfected with v-H-Ras and an expression vector encoding an antisense to the DNA MeTase (pZEM ras/pZαM). Morphologically, pZEM Ras transfected cells grow as multilayer foci while the pZEM Ras/pZαM cells grew exclusively as monolayers and do not form foci. C2C12 cells transfected with pZEM Ras/pZαM retain morphological features similar to their nontransformed myogenic parental line whereas pZEM Ras transfectants have lost this phenotype. This study focuses on the role of DNA methylation in oncogenic transformation therefore its potential role in myogenic differentiation however interesting is open to investigation. Soft agar assays demonstrated that coexpression of antisense to the DNA MeTase and Ras results in cells that have lost the ability to grow in an anchorage independent fashion. To further support the claim that DNA MeTase is involved in initiation of transformation, [3H]-Thymidine incorporation assays were performed and demonstrated that the pZEM Ras but not pZEM Ras/pZαM transfectants nor controls have the ability to initiate DNA synthesis in low serum. Next we asked whether Ras could induce an increase in the steady state level of DNA MeTase. RNAase protection analysis demonstrated a 2 fold increase in the level of DNA MeTase

mRNA in pZEM Ras transfectants when compared to controls, this level of induction is consistent with levels required to transform NIH 3T3 fibroblasts by forced expression of exogenous DNA MeTase (17) . Expression of antisense to the DNA MeTase resulted in a decreased DNA MeTase mRNA level in all but clone 23. This could be explained by the fact that antisense is not a complete Knockout and variability exists with this method of gene modulation. Several observations have shown dramatic inhibition of gene expression at the protein level without inhibition at the mRNA level when using antisense technology suggesting a mechanism of translational arrest (29,30). Other antisense strategies have been shown to inhibit gene expression simultaneously by independent mechanisms acting at different points in the expression of a gene (35). The inhibition seen in pZEM Ras/pZαM clone 23 seems to be working by a mechanism a translational arrest since the DNA MeTase enzymatic activity is significantly inhibited while mRNA levels remain unaltered. To determine if the increase in steady state mRNA, resulting from forced expression of Ras, resulted in increase activity of the DNA methylation machinery we measured the rate of incorporation methyl group, using [3H]-S-adenosyl-methionine as the methyl group donor, into a hemimethylated double stranded oligonucleotide substrate. All Ras transfectants showed an increase DNA MeTase activity when compared to pZEM while pZEM Ras/pZαM cells had activities significantly reduced from the Ras lines. Taken together these experiments demonstrate that Ras has the ability to upregulate steady state levels of DNA MeTase mRNA as well as enzymatic activity of the DNA methylation machinery and since antisense to the DNA MeTase can alleviate these effects it strongly suggests that DNA MeTase is a downstream effecter of the Ras signalling pathway and is actively involved in the transformation process.

To demonstrated that this increase in steady state mRNA levels and enzymatic activity of DNA MeTase results in hypermethylation of specific genes we tested the muscle specific MyoD and the 5' region of the ubiquitously expressed tumor suppressor p53. The MyoD is unmethylated in all somatic tissues but is extensively methylated in several tumor cell lines except those expressing myogenic genes, the MyoD gene also becomes denovo methylated during immortalization of cells (14). The p53 locus is of obvious important in

light of the facts that inactivation of this gene predisposes one to neoplastic disease (31) and that DNA methylation is one mechanism suggested in the inactivation of the tumor suppressor gene Rb (36). Both of these genes showed hypermethylation in the Ras transformed C2C12 lines and this hypermethylation could be inhibited by the coexpression of antisense to the DNA MeTase (Fig 4A and B).

To test the ability of an antisense to the DNA MeTase to revert initiation of Ras transformation in vivo, we injected mice with the different clones and demonstrated that although all transfectants (with the exception of pZEM controls) eventually formed tumors, the pZEM ras /pZαM formed tumors significantly later than their pZEM Ras counterparts. The fact that tumors appear in the pZEM ras /pZαM transfectants although these clones don't have the ability to grow in soft agar can be due to the fact that their exists a strong negative selective pressure in vivo against expression of the antisense whereas in tissue culture positive selective pressure is maintained by the addition of selection media containing neomycin.

Results presented above demonstrate that the DNA methylation machinery can be activated by the Ras signal transduction pathway and that activation of this downstream target of Ras is a critical initial event involved in leading a cell towards the transformed state.

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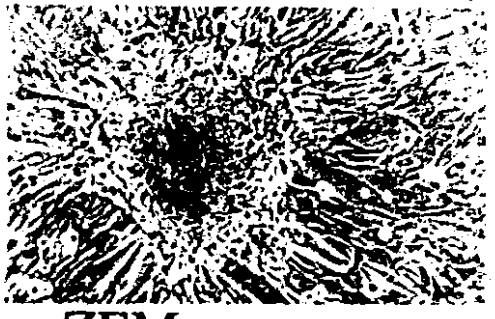
Fig. 1. Morphological transformation of C2C12 cells transfected with oncogenic ras is reversed by antisense to the DNA MeTase. A. Phase contrast microscopy at 200X and 100X magnification of living cultures of C2C12 cells and C2C12 clonal transfectants with pZEM ras and pZEM ras /pZαM. B. Anchorage independent growth assay: C2C12 controls, pZEM ras (clones 5 and 10) and pZEM ras+ pZαM (clones 3 and 6) transfectants were plated in triplicate at a density of 1X103 cells per well and grown in 0.33% soft agar for 21 days as described in material and methods. Photos show representative regions of the plates 21 days after plating cells.

C. Northern blot analysis of C2C12 cells transfected with pZEM ras or pZEM ras/pZαM. Total cellular RNA was prepared from the indicated clones and subjected to northern blot analysis and hybridized with o 0.7 kb Ras probe described in materials and methods. Arrow indicates the limited expression of v-H ras in these transfectants. D. Antisense expression to the DNA MeTase was verified using RNase protection assay. Protection of the expected fragment was seen in three independent pZαM lines.

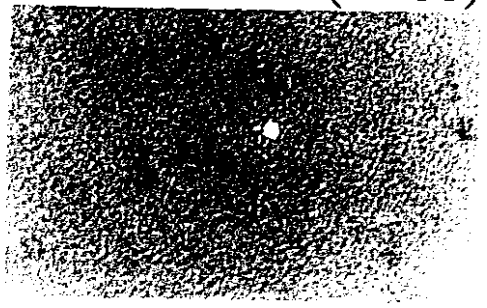
A. C_2C_{12} (X200)

pZEM ras (X100)



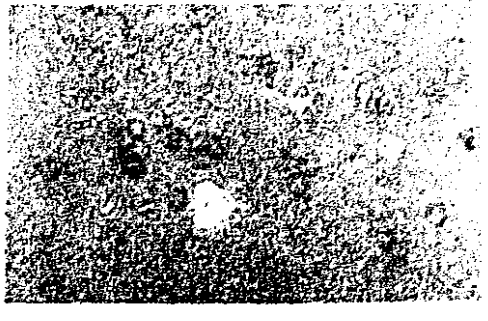
pZEM ras +
pZαM (X100)

B. (X200)



clone 5 (X100)

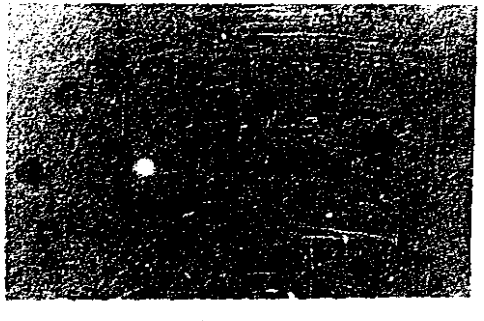
clone 3 (X200)



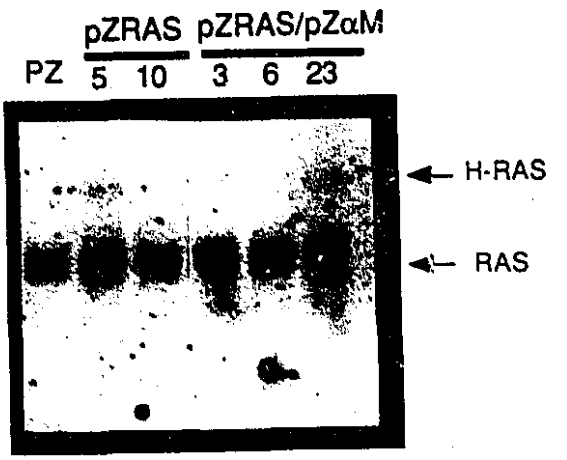
(X200)

clone10 (X100)

clone 6 (X200)



C


pZRAS
pZRAS/pZαM
PZ
5
10
3
6
23
H-RAS
RAS

D

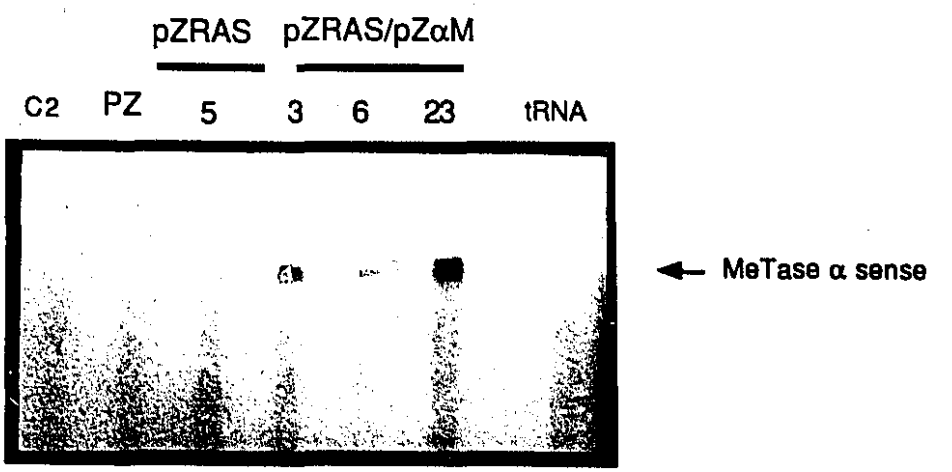
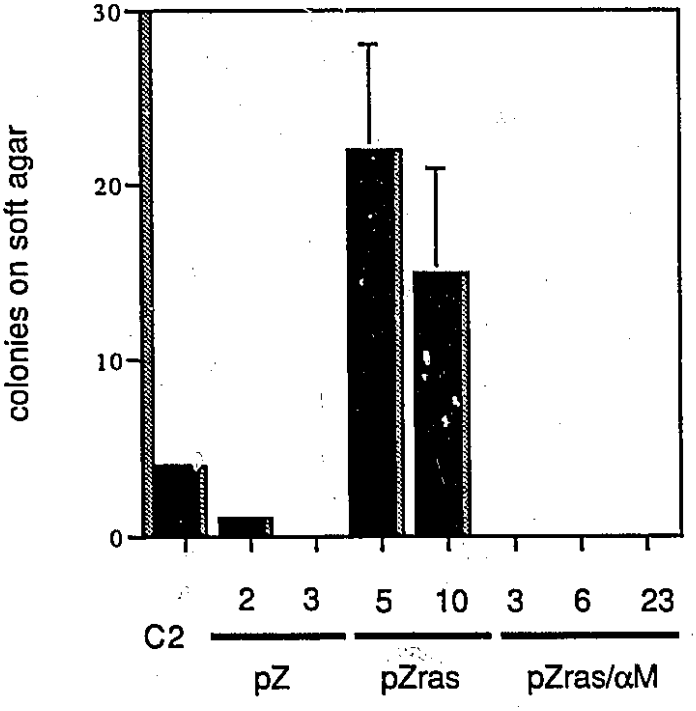

pZRAS
pZRAS/pZαM
C2
PZ
5
3
6
23
tRNA
MeTase α sense

Fig. 2. in vitro indicators of the transformed phenotype.

A. Graphical representation of anchorage independent growth assay shown in fig.1A.

B. Graphical representation of Thymidine incorporation assay. C2C12 controls, pZEM, pZEM ras and pZEM ras + pZαM were plated at a density of 1X104 cells/well in a 24-well plate in medium containing 0.5% (v/v) heat inactivated fetal calf serum and assayed for their ability to incorporation [3H]-Thymidine into newly replicated DNA (see materials and methods for details).

A


colonies on soft agar
30
20
10
0
2
3
5
10
3
6
23
C2
pZ
pZras
pZras/αM

B

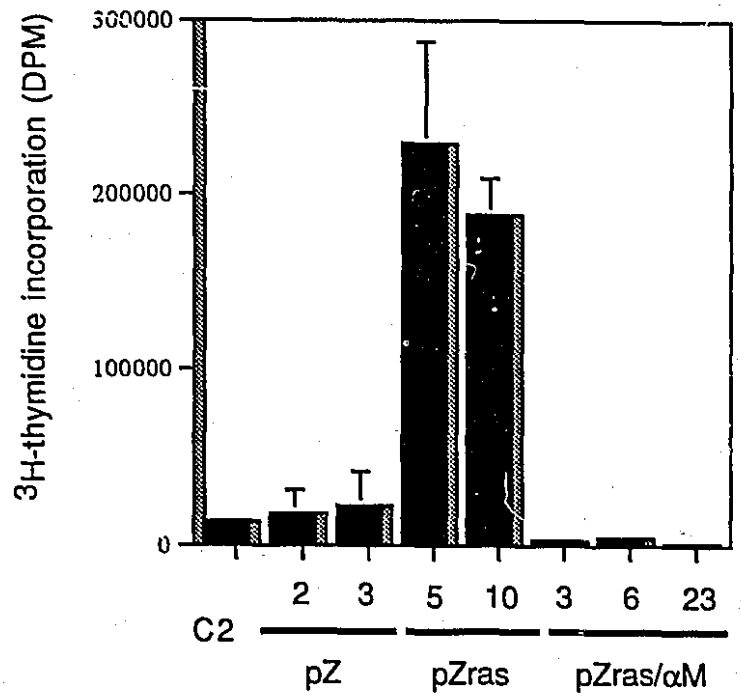
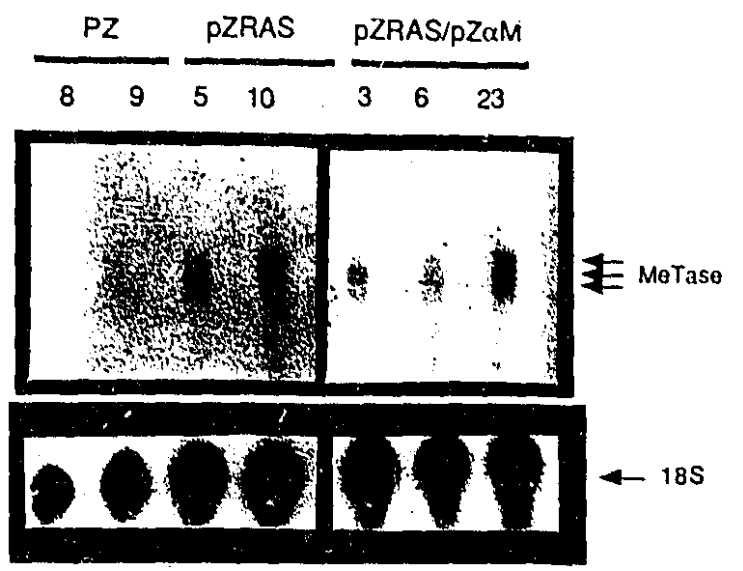
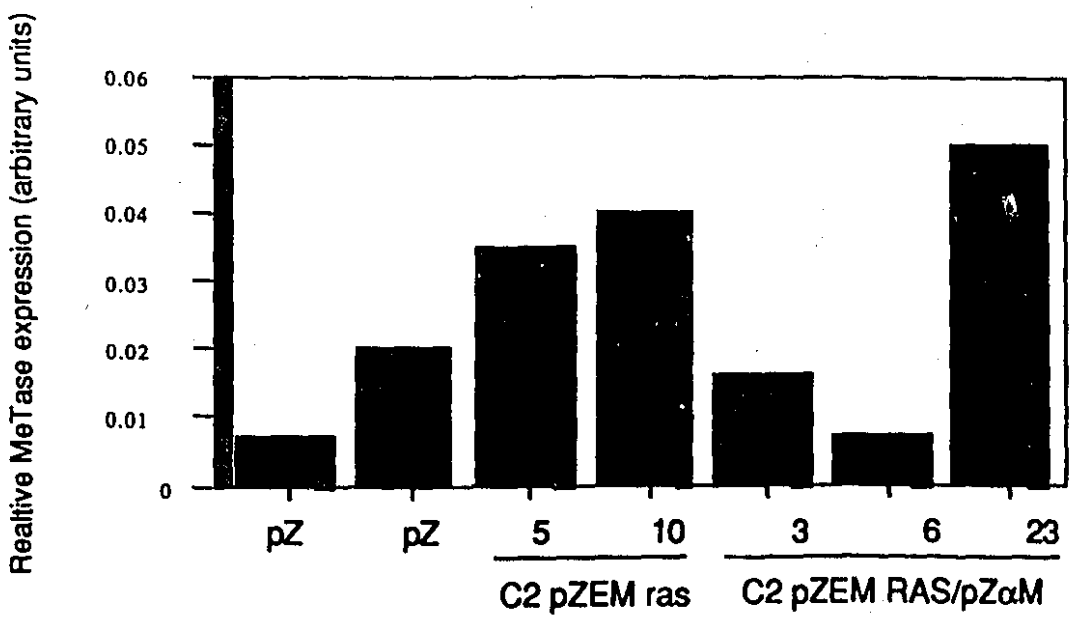

^{3}H-thymidine incorporation (DPM)
300000
200000
100000
0
2
3
5
10
3
6
23
C2
pZ
pZras
pZras/αM

Fig. 3.

A. Analysis of DNA MeTase message in C2C12 cells transfected with pZEM ras or pZEM ras+ pZαM. RNAse protection assays were performed on total cellular RNA from exponentially growing C2C12 clones. The probes were derived from mouse DNA MeTase genomic clone (300 bp NaeI/HindII fragment) and mouse 18S genomic clone (Ambion) see materials and methods for details. DNA MeTase arrows indicate the protected fragments corresponding to the known initiation sites for the mouse DNA MeTase (21). Protection by 18S probe was done simultaneously in the same reaction to control for amount of RNA used. B. DNA MeTase activity in nuclear extracts prepared from the indicated C2C12 transfectants was determined using a synthetic hemimethylated double-stranded oligonucleotide substrate (see materials and methods for details). The results are expressed as dpm [methyl-3H] incorporated into the substrate oligonucleotide per 3 μg nuclear protein for 3 h at 37oC.

A


PZ
pZRAS
pZRAS/pZαM
8
9
5
10
3
6
23
MeTase
18S


Realive MeTase expression (arbitrary units)
0.06
0.05
0.04
0.03
0.02
0.01
0
pZ
pZ
5
10
3
6
23
C2 pZEM ras
C2 pZEM RAS/pZαM

B

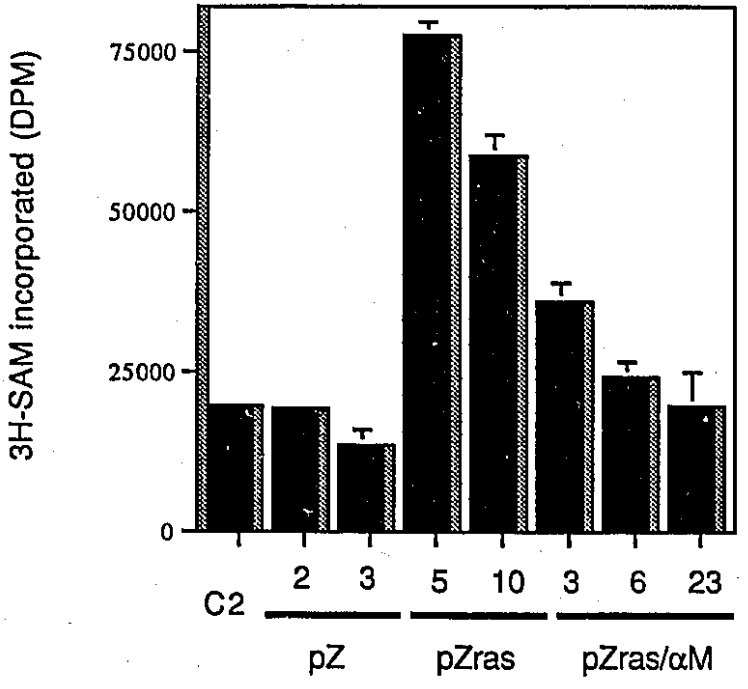
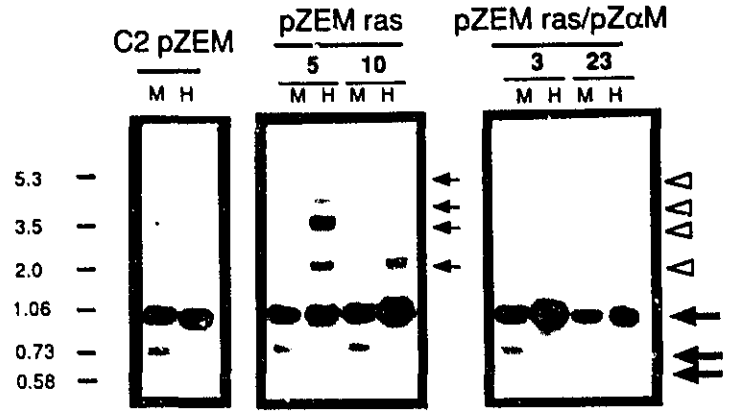
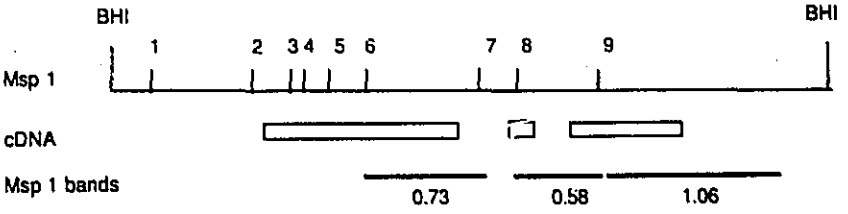

3H-SAM incorporated (DPM)
75000
50000
25000
0
C2
2
3
5
10
3
6
23
pZ
pZras
pZras/αM

Fig. 4. The pattern of methylation of specific genes in pZEM ras and pZEM ras+pZαM transfectants. A. State of methylation of the MyoD gene. Genomic DNA (10ug) extracted in duplicate from the indicated lines was subjected to MspI/HpaII digestion followed by digestion with BamHI, Southern blot transfer and hybridization with a 32P labelled DNA probe encoding the complete mouse MyoD cDNA (14) (an EcoRI fragment isolated from the plasmid pEMC11s a kind gift of Dr. Weintraub). The filled arrows indicates the position of a fragments in HpaII lanes that change in relative intensity due to hypermethylation, the open arrow indicates bands arising from demethylation events. B. State of methylation of the p53 5' region. Genomic DNA (10ug) from the indicated lines was isolated in duplicate and digested with MspI/HpaII and Bam HI as above, subjected to Southern blot transfer and hybridization with a 32P labelled PCR fragment corresponding to the 5' region of the p53 gene (154-472 from the sequence published in reference 22 and amplified as described in the Materials and Methods). The filled arrows indicate a loss of lower molecular weight bands or the intensification of the higher molecular weight bands due to hypermathylation in the pZEM ras clones. Open arrows indicate the reappearance of demethylated bands in the pZEM ras/pZαM transfectants.

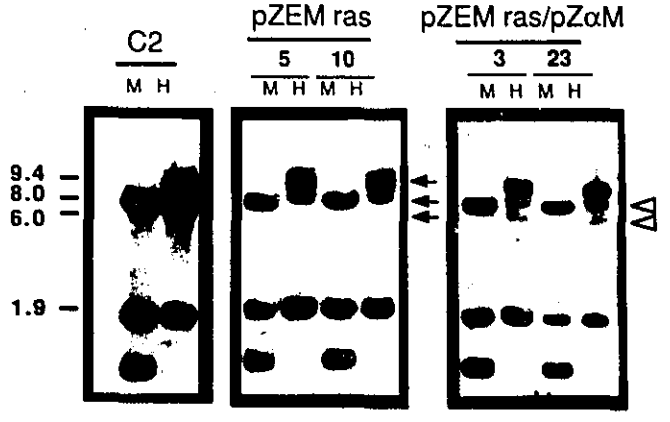
A


C2 pZEM
pZEM ras
5
10
pZEM ras/pZαM
3
23
M H M H M H M H M H
5.3
3.5
2.0
1.06
0.73
0.58

MYOD


BHI
BHI
1
2
3
4
5
6
7
8
9
Msp 1
cDNA
Msp 1 bands
0.73
0.58
1.06

B


C2
pZEM ras
5
10
pZEM ras/pZαM
3
23
M H M H M H M H M H
9.4
8.0
6.0
1.9

p53 5'

Chapter 5: Antisense oligonucleotides to the DNA Methyltransferase inhibit tumor growth *in vivo*. A.R. MacLeod, V. Bozovic, E. Van Hoffe and M Szyf. (manuscript in preparation).

The previous chapters have defined DNA methylation and the DNA MeTase itself as causal components to certain oncogenic transformations. This raises the possibility that specific inhibitors of the DNA MeTase can be used as anticancer drugs. To test the therapeutic potential of specific inhibition of the DNA MeTase, we designed a series of DNA MeTase antisense and control phosphorothioate oligonucleotides that could be administered subcutaneously to tumor bearing animals.

Antisense oligonucleotides to DNA Methyltransferase mRNA induce DNA demethylation and inhibit tumor growth *in vivo*.

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Many tumor cell lines overexpress DNA methyltransferase (MeTase) activity. Recently we have shown that expression of 600 bp from the 5' of the DNA MeTase cDNA in the antisense orientation could render Y1 adrenocortical cells non-tumorigenic. To determine the therapeutic potential of antisense to the DNA MeTase we designed modified antisense oligonucleotides that could be delivered to tumor bearing mice by subcutaneous injection. Cells treated with the DNA MeTase antisense oligonucleotides in vitro demonstrate a reduction in DNA MeTase activity, a general decrease in genomic methylation and diminished ability to grow in an anchorage independent manner. Tumor bearing mice treated with DNA MeTase antisense oligonucleotides showed a significant decrease in tumor growth compared to mice treated with control oligonucleotides.

The methylation of vertebrate DNA at the 5 position of the cytosine residues in the dinucleotide sequence CpG is the most common DNA modification known (1,2). Twenty percent of CpG sites however are nonmethylated and these sites are distributed in a non-random manner forming patterns of methylation that are both tissue and gene specific (1-3). Establishment and maintenance of the appropriate pattern of methylation is critical for development (4) and for maintaining the differentiation state of a cell (5-7). DNA methylation is known to influence gene expression such that an inverse correlation exists between the level of DNA methylation and transcriptional activity of a gene (8). The enzyme that catalyse the methylation of cytosine in DNA is the DNA Methyltransferase (DNA MeTase). The DNA MeTase activity is tightly regulated with the growth state of cells (9,10) and previous models have suggested that the cellular level of DNA MeTase activity can play an important role in determining the pattern of methylation (11,12). An enzyme with such a widespread impact on genome function as the DNA MeTase is a good candidate to play a critical role in cellular transformation. This hypothesis is supported by many lines of evidence that have demonstrated aberrations in the pattern of methylation in cancer cells. While many reports show hypomethylation of both total genomic DNA (13) as well as individual genes in cancer cells (14), other reports have indicated that regional hypermethylation is an important feature of cancer cells (15-18). Consistent with the role of hypermethylation in cancer are the findings that tumor suppressor loci such as the 5' region of the retinoblastoma (Rb) and Wilms Tumor (WT) genes are methylated in a subset of tumors, and that inactivation of these genes in the respective tumors seems to result from methylation rather than from mutation (19). The short arm of chromosome 11, where several tumor suppressor genes are thought to be clustered (20), is also regionally hypermethylated in certain neoplastic cells (17).

One possible explanation for the changes in DNA methylation patterns is the dramatic elevation of DNA MeTase activity seen in many cancer cells (15,21). Recent work from our lab shows that overactivation of the Ras signal transduction pathway can trans-activate the DNA MeTase gene (22) and lead to DNA hypermethylation and transformation (23). It has been shown that forced expression of an exogenous DNA MeTase cDNA can

transform NIH 3T3 cells (24) and recently that inhibition of DNA methylation by transfection of a construct expressing 600 bp from the 5' of the DNA MeTase in the antisense orientation into Y1 adrenocortical carcinoma cells results in reversion of their transformed phenotype (25). In accordance with this model, mice heterozygous for the multiple intestinal neoplasia (Min) mutation of the adenomatosis polyposis coli (Apc) gene show a dramatic inhibition in the incidence of intestinal neoplasia when crossed with mice heterozygous for the DNA MeTase null mutation (26). Suggesting that high levels of DNA MeTase are required for the development of the Min phenotype.

Here we explore the possibility that modified antisense oligonucleotides to the DNA MeTase can be used to effectively inhibit DNA MeTase activity and to inhibit tumorigenesis of Y1 adrenocortical carcinoma cells *in vivo*.

Y1 is an adrenocortical tumor cell line that was isolated from a naturally occurring adrenocortical tumor in an LAF1 mouse (27) and bears a 30 to 60 fold amplification of the cellular protooncogene c-*Ki-ras* (28). This cell line also shows tumor specific DNA hypermethylation (23,25,29) and therefore serves as an excellent model system to test the antitumorigenic potential of DNA MeTase antisense oligonucleotides.

Y1 cells growing in culture were treated with 20μM of either DNA MeTase antisense oligonucleotide or control (scrambled sequence) oligonucleotide (30) every 24 hrs for 9 days. Y1 cells treated with antisense oligo grew to be much larger than control treated cells and adopted a very flat morphology (Fig.1A) that has been shown to be consistent with growth arrested-non transformed cells (31). Control treated cells remained morphologically unchanged from untreated cells. Electronmicroscopic analysis of oligonucleotide treated cells (Fig.1B) reveals the dramatic increase in size and the flat cell morphology of the antisense treated cells as well as the presence of lipid vacuoles unique to the antisense group (one hundred cells were scanned by electron microscopy most of the antisense treated cells had large accumulation of lipid vacuoles whereas non of the controls show this phenotype).

The ability of cells to grow in an anchorage independent fashion is considered as an *in vitro* indicator of tumorigenicity (32). A soft agar assay performed in triplicate showed

that the antisense treated cells showed a dose dependent decrease in their ability to form colonies in soft agar (Fig.1C) with the most dramatic effect seen at 20μM where antisense treated cells were inhibited by 94 % in their ability to grow under anchorage independent conditions over control oligo treated cells. To determine whether the *in vitro* treatment of Y1 cells with the antisense oligonucleotide leads to a reduction in DNA MeTase steady state mRNA levels we performed RNase protection assays (33) . Total cellular RNA was isolated from cells treated for 9 days with concentrations of antisense or control oligos ranging from 0-20μM. The RNA samples were protected simultaneously with two probes, one for the DNA MeTase and one for 18S ribosomal RNA as an internal control for amount of RNA loaded (33). Results of the RNase protection analysis indicate a maximal reduction of 75% in the steady state level of DNA MeTase mRNA when cells were treated with 20μM of antisense oligo for 9 days (as determined by densitometric scanning) (Fig.2A). Prolonged treatment up to 13 days with 20μM of oligo further inhibited the DNA MeTase mRNA to an almost undetectable level (Fig.2B). DNA MeTase mRNA levels remained relatively constant at all concentrations of control oligo. We next compared the DNA MeTase enzymatic activity present in nuclear extracts prepared from cells treated with control oligo (scrambled) (30) or the antisense oligos using a hemimethylated double-stranded oligonucleotide as a substrate as described previously (25). DNA MeTase activity was inhibited by as much as 84% in cells treated with 20μM antisense oligo for 13 days relative to cells treated with control oligos (Fig.2C). To determine whether the inhibition of DNA MeTase activity observed led to demethylation of the Y1 genome and to explore the nature of these demethylation events we resorted to MspI/HpaII restriction enzyme analysis followed by Southern blotting and hybridization with gene specific probes. HpaII cleaves the sequence ccgg only when the CpG dinucleotide is unmethylated, while MspI will cleave the same site irrespective of its state of methylation. We first analysed the state of methylation of the MyoD gene using a MyoD cDNA probe in cells treated with antisense or scrambled oligo (0-20μM as above). This

gene is unmethylated in all somatic tissues but is extensively methylated in several tumor cell lines, except those expressing myogenic genes (34). The MyoD gene is heavily methylated in Y1 cells as indicated by the presence of the high molecular weight bands at 2.8 and 2.0 kb in HpaII digested lanes (see map for MspI/HindIII, and HpaII/HIII patterns). The treatment of Y1 cells with antisense oligonucleotides to the DNA MeTase at concentrations of 10 μM and 20μM results in the demethylation of the MyoD gene as can be seen by the diminution in intensity of the methylated 2.8 and 2.0 kb fragments (open arrows) and the relative intensification of the 0.73 kb band and the appearance of the fully demethylated 0.58 and 0.3 kb bands (filled arrows) (Fig.2D).

The above findings indicate that antisense oligonucleotides to the DNA MeTase can inhibit the DNA Methyltransferase and lead to DNA demethylation and cause a reversion of the transformed phenotype in Y1 adrenocarcinoma cells *in vitro*. The therapeutic potential of such an antisense approach however relies on its ability to exert antitumorigenic effects *in vivo*. To test this *in vivo* potential, we injected 15 LAF-1 mice (5 per group) with $2X10^6$ of the syngeneic Y1 cells subcutaneously in the flank to generate controlled tumor bearing animals. Oligonucleotides (20 mg/kg) of either antisense or two control sequences (scrambled, reverse) were administered intraperitoneally every 48 hrs until tumors were visible in the flank of the animal then every 48 hrs subcutaneously at the site of the tumor. Animals were sacrificed 30 days after injection of the cells when tumor burden was determined and toxicity studies were performed. Two of the 15 mice failed to form any visible tumor. Because this study attempts to determine the effect of the treatments on existing tumors these mice were excluded from further analysis. Animals treated with DNA MeTase antisense oligonucleotide showed a statistically significant reduction in tumor load when compared to control groups ($p<0.001$) (Fig.3). Reverse group had an average tumor weight of 2.91g (SD=0.218), scrambled 2.98 g (SD= 0.98) and antisense group 0.693 g (SD=.797). Toxicological studies revealed no adverse effects due to DNA MeTase antisense or control oligo treatments: mouse body weight remained stable throughout the experiment (data not shown). Hematological evaluation demonstrated higher Hematocrit and % hemoglobin levels, lower platelet counts and higher red blood cell

counts in the antisense treated mice compared to the control groups (table I). These results are consistent with antisense treated animals being of generally better health than control groups, probably due to decreased tumor load. It is not surprising that inhibition of DNA MeTase by antisense oligonucleotides does not shows toxic effects as mice heterozygous for the DNA MeTase null mutation have decreased DNA MeTase yet are indistinguishable from wild type mice (4).

The results presented here support the recent hypothesis that deregulation and overexpression of the DNA MeTase and DNA hypermethylation is causal to certain oncogenic transformations (22, 23, 24) and demonstrates that inhibition of this target by antisense oligonucleotides has strong therapeutic potential. Future efforts will be aimed at developing antisense oligonucleotides with greater affinity to the DNA MeTase and the inhibition of DNA MeTase in various human cancers.

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30. The modified phosphorothioate oligonucleotides used were synthesized by Hybridon Inc. Antisense oligonucleotide: 5'-TCT ATT TGA GTC TGC CAT TT-3' corresponding to bases -2 to +18 of the murine DNA Methyltransferase, Reverse oligonucleotide contains the same sequence of bases as the antisense but the 5' end has been made the 3' and vice versa, Scrambled oligonucleotide contains all the same bases as the above two with the sequence randomized.
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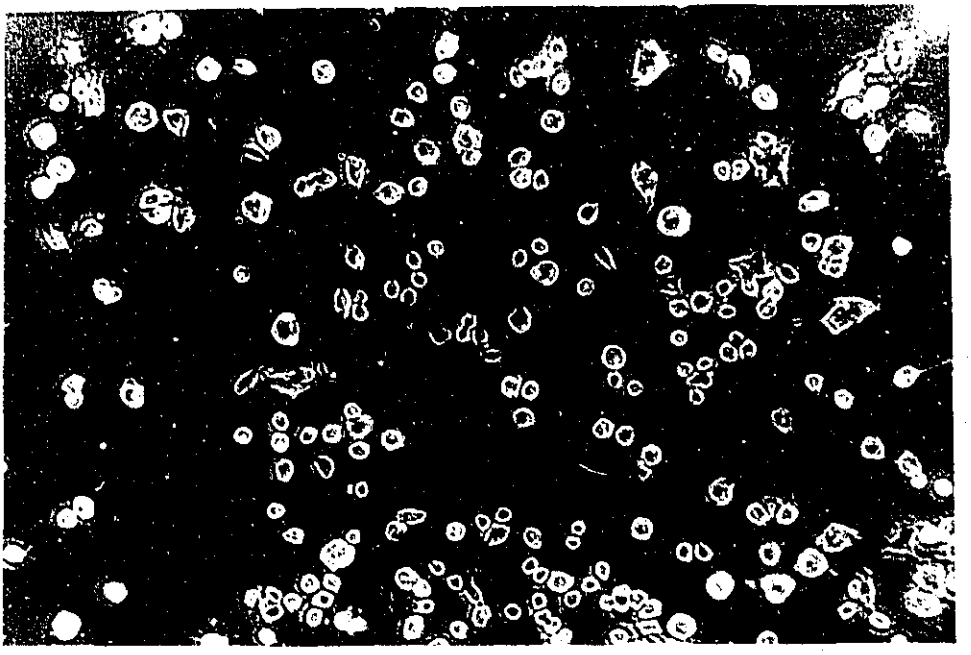
35. We thank Hybridon Inc. for financial support and synthesis of the oligonucleotides used, M.Ballak for her expertise with the electron microscopy, and M.Pinard for many helpfull suggestions and critical analysis of the manuscript.

FIGURE LEGENDS

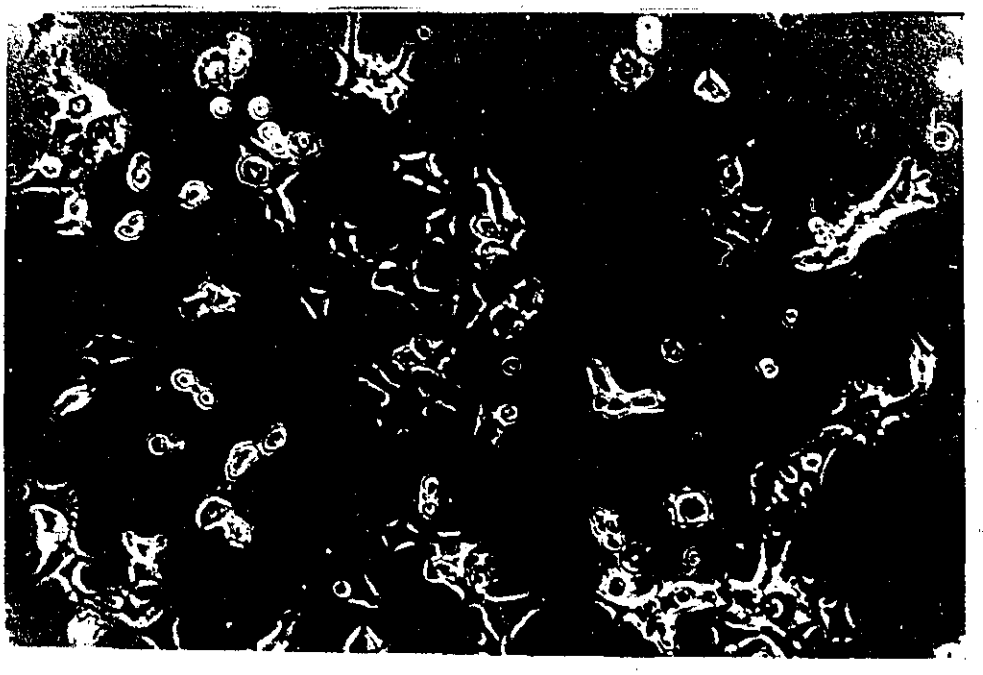
Fig.1. Morphological changes in Y1 cells treated with DNA MeTase antisense oligonucleotides . (A) Phase contrast microscopy at X200 magnification of living cultures of Y1 cells treated for 48 hrs with 20μM antisense or scrambled control oligo. (B) Electron micrographs at X2124 magnification of Y1 cells treated for 48 hrs with 20μM antisense or scrambled control oligo. Arrow indicates the presence of vacuoles specific to the antisense treated group. (C) Anchorage independent growth assay (25): Y1 cells ($1X10^3$) treated with the indicated concentration of oligonucleotide for 9 days were then plated in triplicate in soft agar and colonies were counted visually after 21 days of growth. The data points represent an average of three determinations.

A

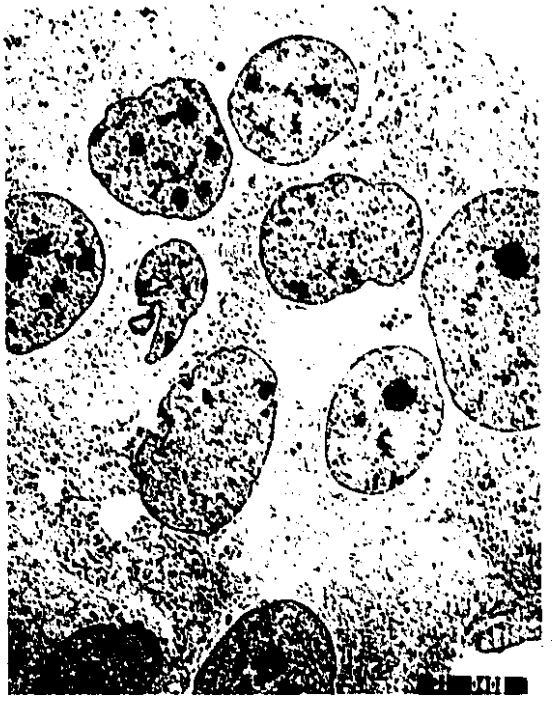
20 μM scrambled oligo



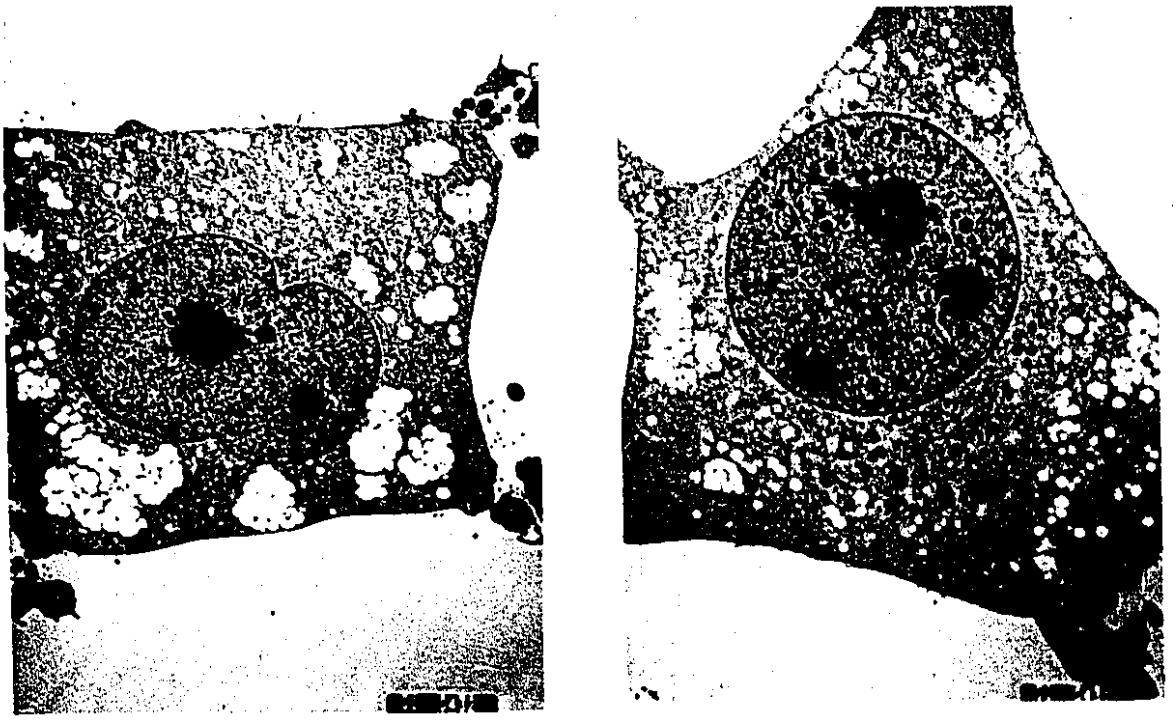
20 μM antisense oligo



20μM Scrambled (X2120)



20μM Antisense (X2120)



C

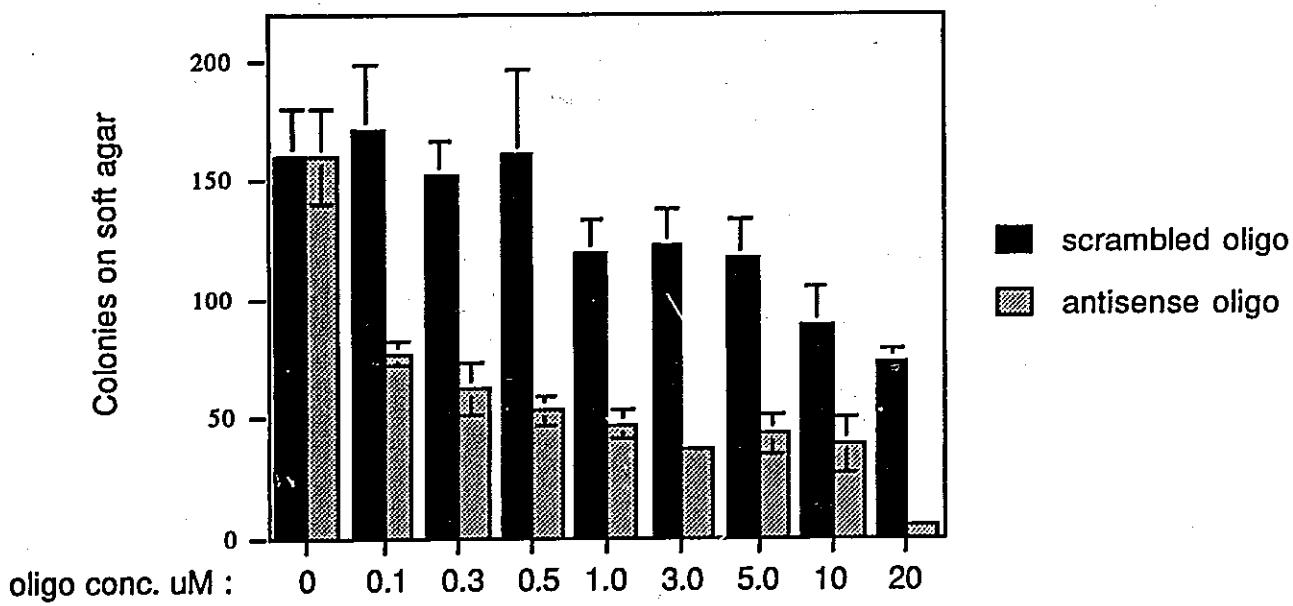
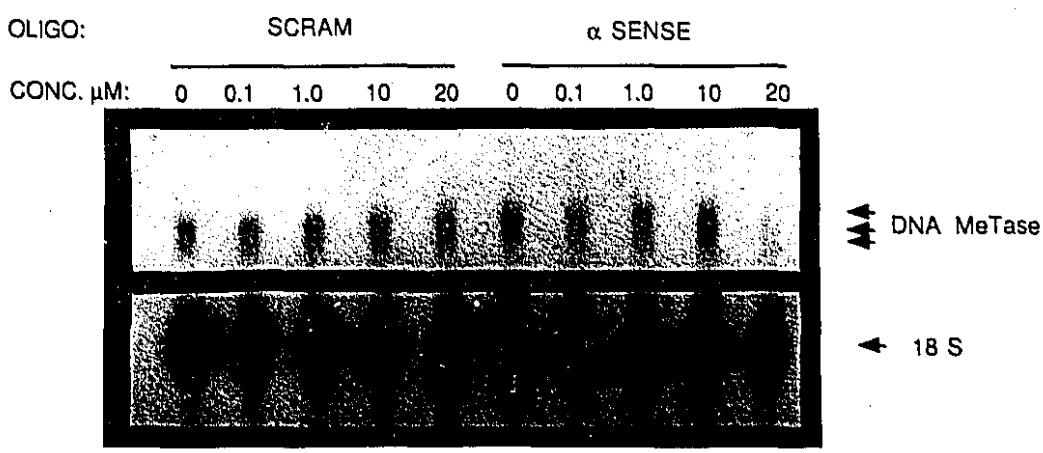
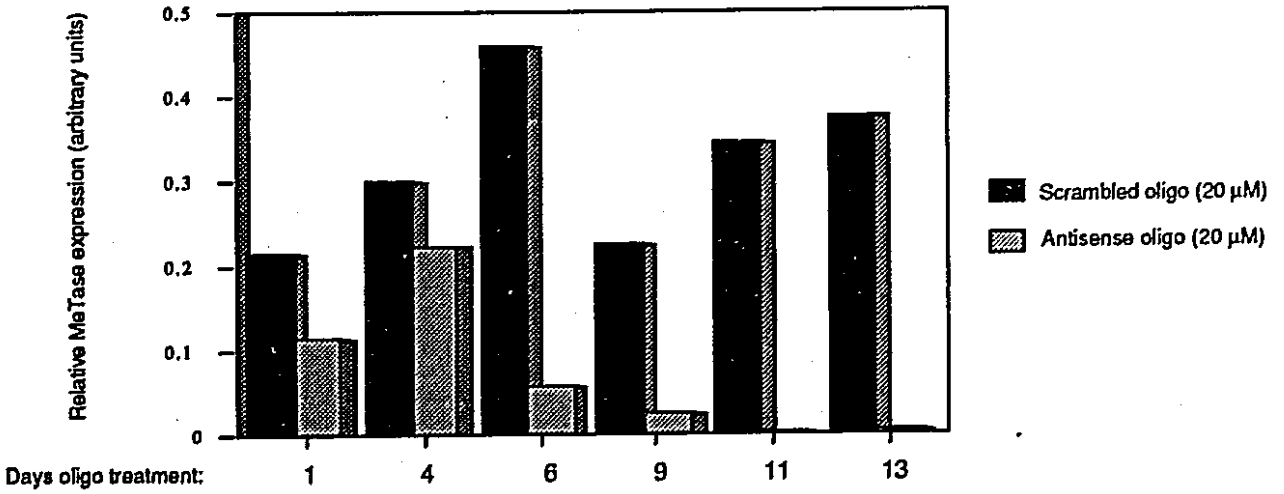

Colonies on soft agar
200
150
100
50
0
oligo conc. uM :
0
0.1
0.3
0.5
1.0
3.0
5.0
10
20
scrambled oligo
antisense oligo

Fig.2.(A) Analysis of DNA MeTase mRNA, activity and DNA methylation in Y1 cells treated with antisense or control oligo. RNAse protection assays (33) were performed on total cellular RNA from Y1 cell treated with the indicated concentration of oligo for 9 days. DNA MeTase arrows indicate the protected fragments corresponding to the known initiation sites for the mouse DNA MeTase (22). Protection by 18S probe was done simultaneously in the same reaction to control for the amount of RNA used. (**B**) Quantification (by densitometric analysis of autoradiograms using Scanalytics MasterScan software) of relative MeTase expression (MeTase/18S) in Y1 cells treated with 20μM oligo for the indicated times. (**C**) DNA MeTase activity in nuclear extracts prepared from Y1 cells treated with 20μM of the indicated oligo for the indicated times, using a synthetic hemimethylated double-stranded oligonucleotide substrate (25). The results are expressed as dpm [methyl-^{3}H] incorporated into the substrate oligonucleotide per 3 μg nuclear protein after 3 h at 37°C.(**D**) State of methylation of the MyoD gene. Genomic DNA (10 μg) extracted from cells treated with the indicated oligo was subjected to MspI or HpaII digestion followed by digestion with HindIII, Southern blot transfer and hybridization with a ^{32}P labelled DNA probe encoding the complete mouse MyoD cDNA (33) (an EcoRI fragment isolated from the plasmid pEMC11was a kind gift of the late Dr. H. Weintraub). The open arrows indicates the position of bands that have been lost or decrease in relative intensity due to demethylation events, the filled arrows indicates new bands or relative intensification of bands arising from demethylation events.

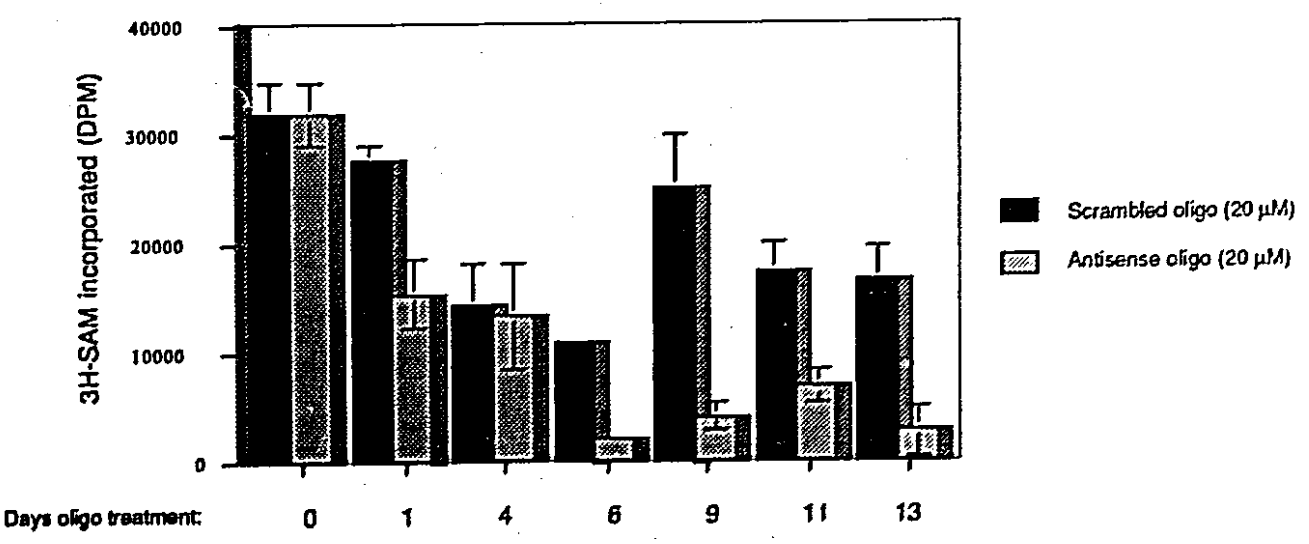
A


OLIGO:
SCRAM
α SENSE
CONC. μM:
0 0.1 1.0 10 20 0 0.1 1.0 10 20
DNA MeTase
18 S

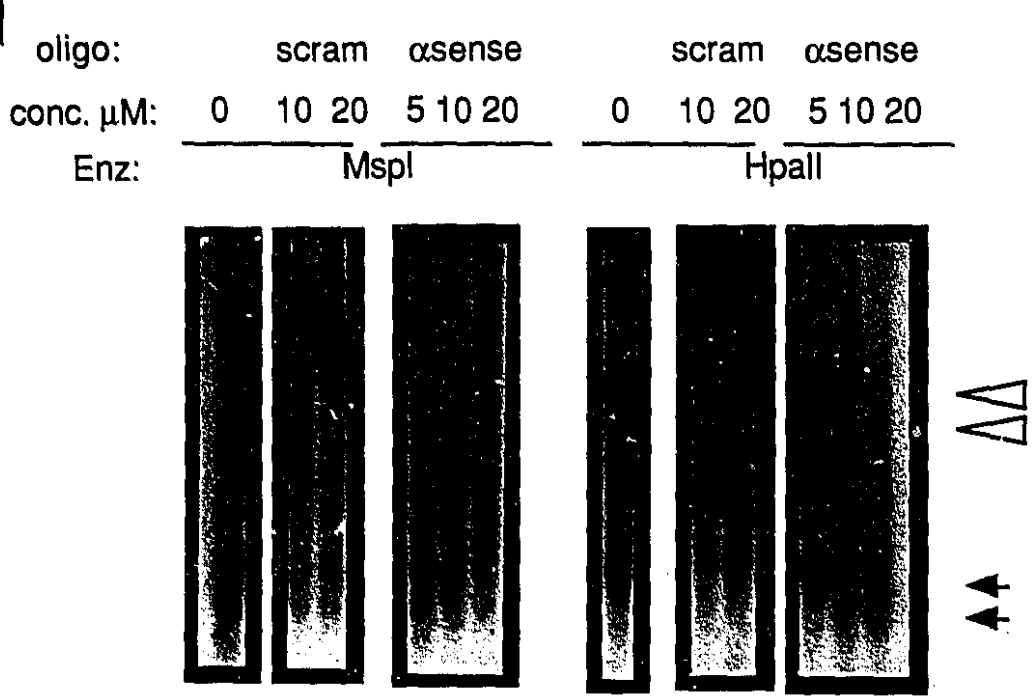
B


Relative MeTase expression (arbitrary units)
0.5
0.4
0.3
0.2
0.1
0
Days oligo treatment:
1
4
6
9
11
13
Scrambled oligo (20 µM)
Antisense oligo (20 µM)

C


3H-SAM incorporated (DPM)
40000
30000
20000
10000
0
Days oligo treatment:
0
1
4
6
9
11
13
Scrambled oligo (20 µM)
Antisense oligo (20 µM)

d


oligo:
scram
αsense
scram
αsense
conc. μM:
0
10 20
5 10 20
0
10 20
5 10 20
Enz:
MspI
HpaII

MYOD

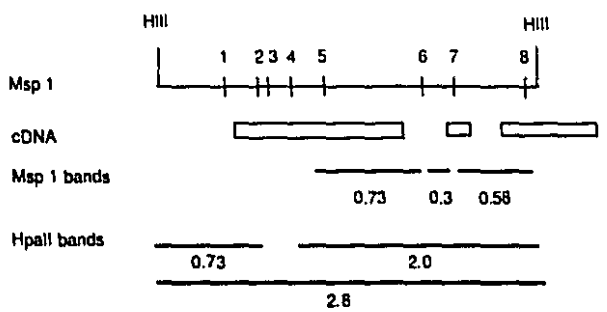

HIII
HIII
1
2 3 4
5
6
7
8
Msp 1
cDNA
Msp 1 bands
0.73
0.3
0.58
HpaII bands
0.73
2.0
2.8

Fig.3. Inhibition of tumor growth by subcutaneous injection of DNA MeTase antisense oligonucleotide. $2X10^6$ Y1 cells were injected subcutaneously in the flank of syngeneic LAF1 mice (10 week old males). 100µL of a 8mg/ml solution (20mg/kg of mouse body weight) of the various oligos was injected intraperitoneally every 48 hrs until tumors were visible in the flank of the animal then every 48 hrs subcutaneously. Graphical representation of tumor weights is shown.

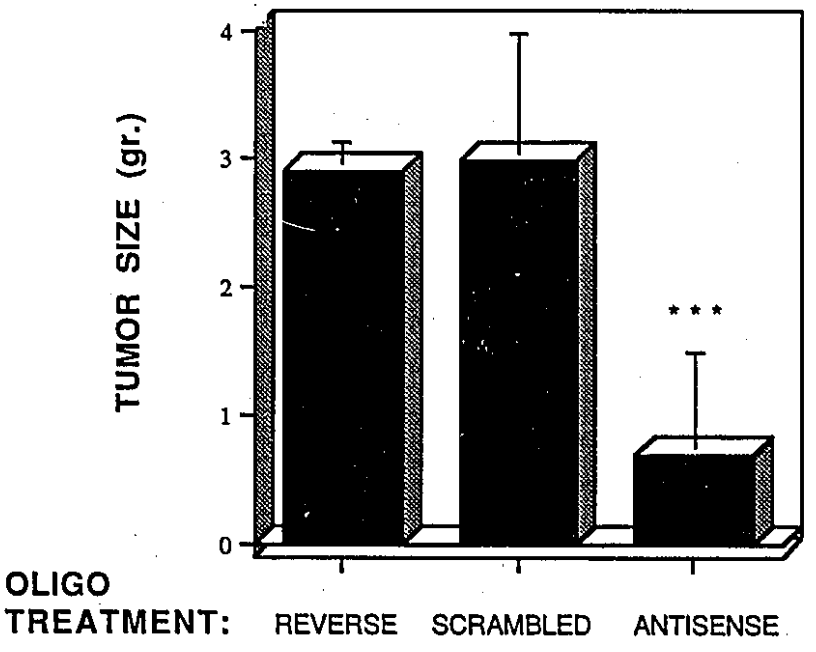

TUMOR SIZE (gr.)
4
3
2
1
0
***
OLIGO
TREATMENT:
REVERSE
SCRAMBLED
ANTISENSE

Table 1. Hematological analysis of LAF-1 mice treated with antisense or control oligonucleotides (20 mg/kg) for 30 days. The numbers represent the mean and standard deviations in brackets, within the groups for the various parameters.

Table I

Treatment	Hematocrit	%Hemoglobin	WBC	RBC	Platelets
REVERSE	17.2 (9.2)	6.40 (3.3)	59.6 (29.8)	3.40 (2.19)	514.0 (291.0)
SCRAMBLED	16.1 (2.5)	6.16 (0.9)	71.8 (21.9)	2.99 (.450)	503.2 (104.0)
ANTISENSE	21.9 (9.5)	7.44 (3.8)	50.7 (33.0)	4.40 (1.80)	302.0 (95.0)

White blood cell count (WBC), red blood cell count (RBC), hematocrit in g/dcl.

Summary and General Discussion

In vertebrates, DNA methylation is the covalent modification of the cytosine moiety in the dinucleotide sequence CpG. This methylation of DNA is catalysed by the enzyme DNA methyltransferase (DNA MeTase). This enzyme is highly conserved in evolution however its biological importance has only recently gained significant attention. The finding that mice homozygous for the DNA MeTase null mutation are non viable (Li et al., 1992) demonstrates its importances for proper development and has sparked renewed interest in the field. Other important observations include the aberrant DNA methylation patterns and the superinduction of DNA MeTase activity common to many cancer cells, suggesting that it may be a player in oncogenic transformation. Although interesting, these findings do not provide conclusive evidence for the role of DNA methylation in the generation of cancer. To truly understand a biological system one has to be able to intervene in a controlled and specific manner, make predictions and interpret the results in light of those predictions. My work was designed with this in mind.

The data presented in this thesis is consistent with the hypothesis that overexpression of the DNA MeTase and hypermethylation of DNA is causal to oncogenic transformations, it also provides a mechanistic link between known oncogenic signal transduction pathways and DNA hypermethylation and demonstrates the potential of DNA MeTase inhibitors as possible cancer therapeutics. In addition to the therapeutic implications of DNA MeTase inhibitors it was clear that such specific inhibitors would yield definitive answers to basic biological questions that were elusive for some time. As mentioned above, much of the data regarding the biological role of DNA methylation was based on correlations suchs as those between methylation state and the state of activity of a gene. Attempts to modulate DNA MeTase activity relied on the nucleoside analog 5-azaC to inhibit the DNA MeTase, but because of its many other known and unknown side effects, results were often difficult to interpret. In an attempt to achieve the greatest specificity possible we employed an antisense strategy to downregulate the DNA MeTase mRNA and thus DNA MeTase activity. The strategy was successful and allowed us to ask questions about the role this

enzyme was playing in the genesis of cancer and the mechanisms involved.

The discovery of specific short DNA sequences elements (AP1 elements) in the promoter of the mouse DNA MeTase gene was striking in that these are known to be regulated by mitogenic stimuli, and suggested the possibility that oncogenic stimuli might transactivate the DNA MeTase gene, and induce cellular transformation. This was in direct conflict with existing theories that proposed inhibition of the DNA MeTase resulting in DNA hypomethylation would be oncogenic. These short DNA elements provided the clues which led us to test the biology and then further to testing potential therapeutics in animal models. Obviously the DNA is not the only level of regulation in biological systems, however the genome holds many important clues to cellular functions that if pursued can lead to greater understanding of biology and to effective therapeutics. It is for these reasons that Molecular Biology and initiatives like the Human Genome Project are gaining much interest in the fields of drug discovery and modern pharmacology.

Summary of results, implications and future directions.

Chapter 1: Regulation of the DNA Methyltransferase by the Ras-AP-1 signaling Pathway. J. Rouleau, A.R.MacLeod and M.Szyf, J. Biol. Chem , 270, 1595-1601 (1995).

This study began with the sequence analysis of the mouse DNA MeTase promoter. This analysis revealed several potential cis-elements that could play a role in the transcriptional regulation of this gene. The DNA MeTase 5' region was found to have three consensus binding sites for the AP-1 transactivation complex. The AP-1 complex can be composed of either Fos/Jun heterodimers or Jun/Jun homodimers and is activated by the Ras signal transduction pathway (Angel and Karin, 1991). This observation led us to propose the transactivation of the DNA MeTase by this cellular signaling pathway and to test the biological function of these sites. Deletion analysis and site directed mutagenesis combined with reporter gene experiments as well as other experimental approaches demonstrate that:

1) The DNA MeTase can be transcriptionally activated by exogenously expressed Ras or Jun.

2) Transactivation of the DNA MeTase promoter is mediated by a direct physical interaction of the AP-1 complex on the promoter.

3) AP-1 transactivation can be inhibited by the glucocorticoid receptor in a ligand dependent manner although DNA MeTase promoter does not contain a consensus GRE. Inhibition is probably the result of the formation of inactive AP-1-GR complexes (Muller and Renkawitz, 1991).

4) Ha-Ras expression induces transcription and an increase in the steady state level of DNA MeTase mRNA.

Regulated expression of DNA MeTase has been suggested to be critical for preserving the pattern of methylation (Szyf, 1991). Aberrant induction of DNA MeTase is a good candidate to play a role in cellular transformation as cancer cells have altered methylation patterns. This hypothesis is supported by many lines of evidence demonstrating high levels of DNA MeTase activity in transformed cells (Kautiainen and Jones, 1986) and the hypermethylation of specific genes (Antequera, 1990, Makos et al., 1992). Thus regulation of the DNA MeTase by a major oncogenic signaling pathway provides the first mechanistic link between oncogenic signaling pathways and the dramatic elevation of DNA MeTase activity seen in many cancer cells.

DNA MeTase has been shown to be regulated in a cell cycle dependent manner, this regulation is at the post transcriptional level (Szyf et al., 1991). It is clear from this study that the DNA MeTase can also be regulated at the transcriptional level. Therefore, DNA MeTase gene expression is regulated at a number of levels to respond to different classes of signals. Expression is maintained at limiting levels under normal conditions while responding to needs to increase DNA methylation in response to other stimuli such as proliferative or differentiation signals .

Activation of the Ras pathway can induce cellular proliferation and transformation but it can also induce cellular differentiation in some cell lines such as PC12. (de Groot, R.P., et al 1990). Interestingly then, DNA methylation may also be involved in cellular

differentiation as well as transformation. Consistent with this idea are the findings that inhibition of DNA methylation by antisense to the DNA MeTase can induce differentiation of 10T1/2 fibroblasts into myocytes and adipocytes (Szyf et al, 1992) and as mentioned previously that inhibition of DNA methylation by 5-azaC can induce terminal differentiation of malignant T cells. Nerve growth factor can activate the Ras pathway through interaction with its cell surface receptor trkA, in PC12 cells this induces differentiation to a neuronal phenotype (Haubruck and McCormick, 1991). I initiated experiments to investigate the role of DNA Methylation in NGF induced differentiation of PC12 cells however time constraints did not allow me to complete this study.

How can the Ras signalling pathway induce both differentiation and transformation? The answer clearly lies downstream of AP-1 activation as this transcription complex is induced in both processes. An interesting model to explain the "switch" from proliferation to differentiation was proposed by Szyf (1993) and postulates that at least two classes of AP-1 responsive genes exist. Namely, those associated with mitogenic response and those induced under differentiating stimuli. Nuclear factors that modulate the accessibility of the activated AP-1 complexes to these genes would determine whether differentiation or proliferation AP-1 responsive genes are transactivated. Such nuclear factors could interact with the AP-1 complex directly modulating its binding to DNA or might act by binding sequences juxtaposed to AP-1 elements themselves to the alter gene expression program of the cell. Another interesting possibility is the finding that Ras can induce a DNA demethylase activity (Szyf et al., 1995). The selective activation of a demethylase versus a methylase may help explain the dual action of Ras.

The Ras-AP-1 signal transduction pathway is not likely to be the only one to regulate the DNA MeTase. A more detailed analysis of the promoter is currently been done in the lab by Marc Pinard. This work involves linker scanning mutagenesis of the promoter to uncover important regulatory elements controlling this gene.

Chapter 2: Regulation of DNA Methylation by the Ras Signaling Pathway. A.R. MacLeod, J. Rouleau and M. Szyf , J.Biol.Chem. 270, 11327-11337 (1995).

The progression of the transformed state has two defining characteristics: first is the loss of cell cycle control leading to uncontrolled proliferation and second is the sequential changes in normal gene expression profiles and the inactivation of differentiation specific functions (Nowell, P.C., 1986). Consistent with this is the observation that terminal differentiation and cellular transformation have been shown in many cases to be a mutually exclusive processes (Beug H. et al 1982, Copola J.A. et al 1986, Freytag S.Q. et al 1988). Could a single transforming event trigger both these alterations seen in cancer cells? The Y1 adrenocortical cell line is ideal to study this question because it is an aggressive cancer and it exhibits well characterized tissue specific gene inactivation.

To test the hypothesis that the Ras signal transduction pathway controls DNA MeTase activity and that this controls the state of methylation of the genome and the state of cellular transformation, we used Y1 cells as a model system. Y1 is an adrenocortical tumor cell line that was isolated from a naturally occurring adrenocortical tumor from an LAF1 mouse (Yasumura et al., 1966) which bears a 30 to 60 fold amplification of the cellular protooncogene c-Ki-ras (Schwab et al., 1983) . It stands to reason that c-Ki-ras plays an important role in triggering the transformed state of Y1 cells. This cell line expresses many adrenal specific genes but has specifically repressed, by a cis-modification event, the 21-hydroxylase gene (C21) (Szyf et al.,1990). This repression is associated with heavy methylation of the C21 gene locus and the cells possess the capacity to specifically de novo methylate a transfected C21 gene (Szyf et al., 1989). We therefore suggested that DNA methylation may be involved in both the proliferation and gene repression events associated with the oncogenic process (see Fig.4) and used the Y1 system to dissect the molecular mechanisms responsible for these events.

In this paper I demonstrate that:

1) Downregulation of the Ras pathway by forced expression of human Ras GTPase activating protein , or downregulation of Jun by expressing the Jun transdominant mutant ∂D9-Jun in the Y1 adrenocortical carcinoma cell line leads to inhibition of expression of DNA MeTase mRNA and decreased DNA MeTase activity.

2) Downregulation of the Ras-AP-1 pathway in Y1 cells leads to stable genome wide DNA hypomethylation aswell as hypomethylation of specific genes.

3) Inhibition of DNA MeTase resulting in DNA hypomethylation was coincident with the inhibition of cellular transformation.

4) Overexpression of oncogenic Ras leads to induction of DNA MeTase activity and hypermethylation of DNA and induction of cellular transformation.

5) Modulation of the Ras pathway alters the nature and the level of AP-1 binding activities in Y1 cells.

The demonstration here that DNA MeTase activity and DNA methylation patterns can be altered by extracellular and central cellular signals raises the possibility that methylation patterns exhibit more plasticity in developed somatic tissues than previously thought. If this is true, we should revise our thinking of the biological role of methylation patterns from the static maintainers of epigenetic information to dynamic regulators of the genome which are constantly responsive to extracellular and intracellular signals. As mentioned in the previous section detailed deletion analysis of the DNA MeTase promoter is being performed, this work should yield clues into other potential signaling systems regulating this gene. However, an essential component of such a study would be to test the functionality of the pathway, that is, can modulation of the pathway lead to stable changes in the methylation status of the genome. Making use of dominant negative mutant proteins

such as those of the PKC family, the CREB family and others to downregulate pathways could help answer these questions. Also of interest is the potential cross talk of such signaling systems and how they might integrate to influence the final DNA Methylation pattern of the genome.

As suggested earlier, hypermethylation in cancer cells is a common feature and appears to demonstrate some site specificity, that is targeting specific group of sequences, while other sequences are protected from methylation. CpG rich island sequences which are usually found in the 5' region of housekeeping genes and are generally hypomethylated in most normal somatic tissues (Bird et al., 1985) but are often hypermethylated in cancers (Antequera et al., 1990). Jones et al have shown that hypermethylation of the MyoD CpG island occurs in transformed cell lines (Jones et al.,1990) and methylation proceeds progressively along with cellular transformation (Rideout et al., 1994). Consistent with this we show here that down regulation of the Ras pathway leads to demethylation of specific sites within the MyoD gene supporting the idea that aberrant methylation of this CpG island is a marker of the transformed state. Hypermethylation of CpG islands is also associated with oncogene-induced transformation of human bronchial epithelial cells (Vertino et al., 1993). The CpG islands of the estrogen receptor gene is hypermethylated in human breast cancer cells the loss of this receptor is associated with hormone resistance (Ottaviano et al., 1994) making traditional chemotherapy that targets this receptor ineffective. The other class of genes known to undergo specific hypermethylation is the tissue specific genes that characterize the differentiated state of the normal parental cell and are inactivated in the dedifferentiated cancer cell type. A good example of this and the one used in this study is the adrenal specific 21-hydroxylase gene (C21) which is hypomethylated and expressed in the adrenal cortex and is hypermethylated and inactivated in the adrenocortical carcinoma cell line Y1 (Szyf et al., 1989, 1990). When an exogenous C21gene is introduced into Y1 cells it is specifically de novo methylated, suggesting that the cancer cell maintains an elevated capacity to specifically recognise this gene and target it for de novo methylation (Szyf et al., 1989). As mentioned above, Szyf 1990 proposed the existence of DNA cis-acting elements that regulate the methylation of neighbouring sequences. Recent studies by Turker's group have revealed a cis acting "de novo

methylation center" in the upstream region of the mouse APRT gene (Turker et al., 1991; Mummaneni et al., 1993) this sequence can lead to inactivation of the APRT gene supporting the hypothesis that centers of methylation also function as centers of inactivation (Mummaneni et al., 1995). Additional cis-acting methylation inducing sequences have since been identified in exon 7 of the p53 gene (Magewu and Jones, 1994) and the α-fetoprotein control region (Hasse and Schulz, 1994). Playing an opposing role are cis acting signals that protect juxtaposed sequences from de novo methylation, to date these "hypomethylation signals", include those found in the CpG island of the thy-1 gene (Szyf et al., 1990) and HPPRT (Brandeis et al., 1994), and recently the DNA binding site for the transcription factor Sp1 has been shown to possess such activity (Brandeis et al., 1994).

What are the mechanism responsible for regional site-selective de novo methylation or protection from de novo methylation? A model proposed by Szyf suggest that DNA methylation like transcription and other genome functions involves transacting factors that interact with cis acting DNA elements such as thy-1 and SP1 elements and target juxtaposed sequences for methylation or protection from methylation. These factors may be ubiquitous like some of the histone proteins such as H1 (Higurashi and Cole, 1991; Johnson et al., 1995) or nonhistone proteins such as HMGs (Paranjape et al., 1994). Another class of "modifying proteins" has to be cell and sequence specific to explain the cell specific methylation of certain sites (Szyf, 1989). The cell specific proteins could induce a general repressing mechanism such as histone binding and nucleosomal condensation. For example, a cell specific repressor can cause the precipitation of an inactive chromatin structure and this in turn can trigger DNA methylation by a general mechanism that attracts the DNA MeTase to inactive chromatin structure sites. The identification and characterisation of such putative factors will be a major advance in the understanding of the generation and function of DNA methylation patterns. In addition to the regulation of DNA MeTase by the Ras oncogene it will be of interest to determine whether other transforming stimuli can induce the DNA MeTase. Recent data showing that stabilisation of DNA MeTase levels and CpG island hypermethylation precedes SV40

induced immortalisation of human fibroblasts suggests that this might be the case (Vertino et al., 1994). The SV40 large T antigen appears to inhibit the function of the retinoblastoma tumor suppressor protein which is a regulator of cell cycle progression (Riley et al., 1994). It is tempting to speculate that tumor suppressors such as the Rb protein play an inhibitory role in controlling DNA MeTase activity.

An interesting consequence of downregulating the Ras pathway in Y1 cells was the change in composition of the AP-1 DNA binding complexes observed. Future experiments will be aim at determining the components of these different complexes using various antibodies and supershift assays, and how modulating the pathway alters the formation of these complexes.

The all important question remains, are the changes in DNA methylation observed following the attenuation of Ras signaling critical for the reversal of the transformation process in Y1 cells or is this merely a misleading coincidence? Addressing this question necessitates inhibition down stream of the Ras pathway at the level of the DNA MeTase itself. The following chapter discusses our strategy to gain specific inhibition of the DNA MeTase and its biological consequences.

Chapter 3: Expression of Antisense to the DNA Methyltransferase mRNA induces DNA Demethylation and Inhibits Tumorigenesis. A.R. MacLeod and M. Szyf. J. Biol. Chem. **270**. 8037-8043 (1995).

In the previous chapter we showed that a common oncogenic signalling pathway can activate the DNA MeTase as a downstream target, leading to the stable epigenetic modification of the genome. This suggests a mechanistic link between known oncogenic signals, DNA hypermethylation and the transformed state. The critical question that remained to be answered was whether indeed the level of expression of the endogenous DNA MeTase plays a causal role in tumors that are induced by naturally occurring

oncogenic signal transduction pathways. To address this question, we again used the adrenocortical carcinoma cell line Y1 as a model system. As mentioned above, Y1 is a cell line that is derived from a naturally occurring adrenocortical tumor in an LAF1 mouse (Yasumura et al., 1966), therefore oncogenic transformations were initiated *in vivo*. We hypothesised that if the level of expression of DNA MeTase activity is critical for the oncogenic state, then the transformed state of a cell should be reversed by partial inhibition of DNA methylation. Previous attempts to modulate the DNA MeTase relied on the drug 5-azaC, this nucleoside analog has many cellular targets other than the DNA MeTase itself, therefore its usefulness in elucidating the specific role of DNA methylation in cancer is limited. To circumvent this problem we designed an antisense strategy to inhibit the DNA MeTase. The lab has previously demonstrated that forced expression of an "antisense" mRNA to the most 5' 600 bp of the DNA MeTase message (pZαM) can induce limited DNA demethylation in 10T1/2 cells (Szyf et al., 1992). To directly test the hypothesis that the tumorigenicity of Y1 cells is controlled by the DNA MeTase, we transfected either pZαM or a pZEM control into Y1 cells.

In this work we have demonstrated that:

1) Expression of the antisense construct leads to a stable reduction in DNA MeTase mRNA, protein activity and to a limited reduction in DNA methylation content of the genome and hypomethylation of specific sites aberrantly methylated in Y1 cells.

2) Y1 cells with reduced DNA MeTase have slowed growth rates and an increased dependence on serum for survival.

3) Serum deprivation of the antisense expressing cells causes them to initiate an active cell death program (apoptosis).

4) Expression of the antisense construct results in the appearance of a lower molecular

weight DNA MeTase mRNA species, possibly by inducing alternative splicing.

5) Demethylation of Y1 DNA by DNA MeTase antisense expression results in reversal of the tumorigenic phenotype suggesting that DNA MeTase plays a critical role in tumorigenesis.

Whereas there is no question that methylation patterns are changed in cancer cells, the true meaning of these changes has baffled the field for the last two decades. Since the acceptable dogma was that demethylation induces gene expression and that cancer involves activation of oncogenes, it was logical to propose that hypomethylation is involved in carcinogenesis, and in fact many cancer cells exhibit genome wide hypomethylation (Feinberg and Vogelstein, 1983). The pharmacological implication of this model was that inhibiting the DNA MeTase with agents such as 5-azaCytidine , 5-deoxyazacytidine or an antisense approach would be carcinogenic and therefore should not be used in therapy. Since the mechanism responsible for hypomethylation in cancer was unknown and since most of the field focused on correlative studies, the pharmacological potential of DNA methylation modifiers was left untouched for more than two decades. The main activity in the field was to provide direct evidence that hypomethylation agents such as 5-azaCytidine would lead to cancer. Whereas chronic injection of 5-azaCytidine led to increased cancer in male Fischer rats (Carr et al., 1984), injection of the deoxy azacytidine analog which is a more specific and more effective inhibitor of DNA methylation led to reduction of tumorigenicity well below control levels (Carr et al., 1988). These latter results were overlooked because of the prevailing hypothesis that hypomethylation is involved in the carcinogenic process. Since our demonstration here that inhibition of DNA MeTase can be antitumorigenic it has been shown that 5azadC treatment can prevent the initiation of neoplasia in a mouse model of APC (adenomatosis polopus coli) supporting our hypothesis that hypermethylation plays a role in neoplasia and nullifying the hypothesis that hypomethylation can lead to neoplasia (Laird et al., 1995). Whereas the experiments by Laird and Jaenisch show that initiation of neoplasia could be inhibited by 5-azadC, our results suggest that the transformed state of the Y1 adrenocortical cell could be reversed by

inhibiting the DNA MeTase by antisense RNA or by 5azadC at concentrations that are not cytotoxic. The experiments by Laird and Jaenisch also show that chronic and systemic administration of a hypomethylation drug is not toxic, these results have very important therapeutic implications for future use of methylation inhibitors as anticancer agents. Furthermore, our experiments suggest that inhibition of the DNA MeTase by only 2-fold is sufficient of alter the transformed state, this is consistent with previous reports showing such an induction in the DNA MeTase could transform NIH 3T3 fibroblasts (Wu, J., et al 1990).

Because of the "differentiating" potential of 5-azaCytidine (Jones, 1985; Taylor, 1993) it was considered as a potential therapy for leukemias that were blocked in their differentiation program (Taylor, 1993). A recent supplement of the journal Leukemia has been dedicated to some of the preclinical and clinical results obtained with 5-aza-2'-deoxycytosine (5-azaCdR) in leukemias and the results were encouraging (Leukemia, 1993 supplement). However, in some of these treatments the classic side effects of nucleoside therapy were observed such as inhibition of myelogenesis and resistance. Experimental evidence and clinical trials with 5-azaCdR provided some indication that demethylation might be of advantage in anticancer therapy and are consistent with the hypothesis that hypermethylation plays a causal role in cancer. One should be very careful in ascribing all the effects of 5-azaCdR to the inhibition of the DNA MeTase because as previously mentioned it is known to have many other cellular functions. A detailed account of these side effect is warranted. First, 5-azaCdR is incorporated into the genome, the effects of the presence of nucleoside analogues such as 5-azaCdR on genomic functions is still unknown. For example, can it have an impact on genomic stability and the ability of different DNA binding proteins to interact with a DNA containing 5azaCdR rather than C in the recognition sequence. Second, Jutterman et al have recently shown that incorporation of 5-azaCdR into DNA causes trapping of bulky DNA MeTase molecules onto the DNA which might be responsible for the toxicity of the drug (Jutterman et al., 1994). Third, 5-azaCdR is a nucleoside analogue that might have a potential inhibitory effect on enzymes utilising cytidine and its different phosphorylated metabolites such as cytidine kinases and DNA polymerase. Fourth, 5-azaCdR could be converted to the riboazaC form and inhibit

RNA methylation in addition to DNA methylation. Inhibitors of DNA MeTase that do not require additional metabolism in the cell and are not incorporated into DNA are necessary. Different strategies could be utilised to develop such inhibitors. In the attempt to inhibit the DNA MeTase in the most specific means possible we employed the antisense expression system previously shown to lead to limited demethylation in 10T1/2 cells (Szyf, 1992).

The implication of the present study is that increased DNA methylation capacity is in fact required to maintain the transformed state of Y1 cells and that by modulating the level of the DNA MeTase the state can be reversed to a less lethal one. What are the mechanisms by which DNA hypermethylation exerts its oncogenic effect? Baylin has previously suggested that an imbalance of DNA methylation involving regional hypermethylation result in abnormalities of chromatin condensation which in turn lead to inactivation of genes, predisposition to mutations and allelic deletions which are associated with the progression of tumorigenesis (Baylin, 1991). The inactivation or loss of critical loci involved in tumor suppressor functions could mediate transformation. An alternative hypothesis that has been suggested by Jones and his colleagues is that methylated cytosine are mutation hot spots since methylated cytosines can be transformed by spontaneous or enzyme catalysed deamination into thymidine, which following replication will result in mutation of the original C-G base pair into a T-A pair (Jones et al., 1992). It has been suggested that 30 to 40% of all human germline point mutation occur at methylated CpG sequences. Jones and his colleagues have shown using direct genomic sequencing that three sites which are known to be hotspots for mutations in the p53 gene were found to be methylated in target human tissue examined (Rideout et al., 1990). Hypermethylation can result in an increase in the number of deaminated CpGs which might strain the mismatch repair machinery that is normally responsible for removing G T mismatches. Another exciting and surprising result by Jones's group which provides a biochemical basis for the proposed increase in deamination of methylated cytosines in cancer shows that the DNA MeTase enzyme itself might catalyse the deamination of methylated cytosines (Shen et al., 1992). If the DNA MeTase can catalyse the deamination of 5-methyl Cytosine in addition to methylation of cytosines, the observed increase in DNA MeTase activity in cancer cells (Kautien and Jones 1986: El-Deiry et al., 1991) can result in an increase in the rate of C to T transitions (Shen

et al., 1992; Laird and Jaenisch 1994).

Another provocative finding was that DNA demethylation led to the initiation of an apoptotic death program when Y1 cells were deprived of serum. Apoptosis is known to involve the induction of endonucleases that degrade the DNA into the characteristic 180 bp internucleosomal ladder (Wyllie, et al., 1981). It is tempting to speculate that demethylation of critical sites allows access of these death-program related endonucleases to the genome of the targeted cell.

If the critical transforming function of hypermethylation is an increase in the rate of mutagenesis, then then damage incurred by hypermethylation should not be reversible. Our results however support the hypothesis that DNA MeTase inhibition can reverse the transformed phenotype. This is of obvious importance should DNA MeTase inhibitors be developed as cancer therapeutics.

One questions that remains to be answered is why cancer cells overexpressing the DNA MeTase often exhibit genome wide hypomethylation in addition to regional hypermethylation. Recently, Szyf identified a general demethylase activity and demonstrated its induction by Ras (Szyf et al, 1995). If both DNA MeTase and demethylase are induced by oncogenic signaling pathways, demethylation and hypermethylation of different sites cold occur at the same time provided the two enzymes show different substrate specificity. One possible example of two distinct subsets of methylation sites that show opposite propensities to be methylated are the CpG rich islands (Bird et al., 1985) (regions of DNA usually at the 5' of housekeeping genes that are nonmethylated in most tissues) versus CpG sites positioned around tissue specific genes (Yisraeli and Szyf, 1984; Razin and Kafri, 1994). In early embryonic stem cells, CpG islands are demethylated (Frank et al., 1991) and protected from de novo methylation whereas other sites are de novo methylated (Szyf et al., 1990). In cancer cells on the other hand CpG islands are de novo methylated (Jones et al., 1990; Rideout et al., 1994; Baylin et al., 1986) whereas other sites are hypomethylated (Feinberg and Vogelstein, 1983). Two alternative hypotheses can explain this switch in the methylation state of these classes of CpG sites. One possible hypothesis is that different MeTases or demethylases are induced in early embryogenesis versus cancer. Alternatively, the switch in methylation

specificity is determined by a change in transacting factors interacting with cis-acting signals that determine the accessibility of these sites to the methylation or demethylation machinery. One such example is the cis-acting signal located in the CpG island of the thy-1 promoter and HPRT gene (Szyf et al., 1990; Brandeis et al., 1994) which can protect CpG sites from de novo methylation in early embryogenesis. Differential expression of the factor(s) interacting with these sequences can explain the differences in CpG island methylation observed in cancer cells versus early embryonic cells. The model that demethylation is modulated by local signals and proteins interacting with them could also explain the discrete site specific demethylation of tissue specific genes during development.

Chapter 4: Expression of antisense mRNA to the DNA Methyltransferase inhibits v-Ha-Ras initiated transformation of C2C12 cells. A.R. MacLeod, V. Bozovic, J. Rouleau and M.Szyf. (Submitted manuscript).

The picture that emerges from our studies is that DNA methylation patterns are an important element controlling genomic programs. Cancer is one example of the loss of cellular control over such programs. If the level of DNA MeTase activity is one critical control of the pattern of methylation, partial inhibition of DNA MeTase activity should result in a switch in genomic programs. The nature of the newly adopted program will be dictated by all the other components currently present in the cell. Therefore, demethylation will activate only functions that were preprogrammed to be expressed by existing transcription factors but were inhibited by aberrant or programmed methylation. This can explain why partial inhibition of methylation in predifferentiated cells does not result in a chaotic change in phenotype but rather a programmed move to the next stage in differentiation (Jones, 1985; Szyf et al., 1992). Moreover, since the pattern of methylation is determined as suggested by signals in the DNA and proteins that interact with these signals (Szyf, 1991), the pattern of methylation would probably be restored to the original state in most sequences. It is therefore expected that systemic demethylation will have

minor effect on programmed somatic cells. However, it should be effective in reactivating latent programs or bringing a cell to a natural differentiation state that it is otherwise blocked from attaining. In such cases, the necessary transcription machinery is present in the cell. The concept that the level of DNA MeTase expression can control a genomic program is especially critical in cancer where terminal differentiation is often repressed in favor of the oncogenic state. This idea is exemplified in the study of myogenic differentiation and transformation. In muscle cells, terminal differentiation requires the activation of several of muscle-specific genes (including α-actin, myosin heavy and light chains, and acetylcholine receptors). The transcription of some of these muscle-specific genes is activated by products of the MyoD gene family (including MyoD,MyoH and myogenin) (Weintraub et al., 1991). These proteins are called master regulatory proteins and are thought to be responsible for initiating the direction of the cellular program. However, expression of the activated ras oncogene in these cells inhibits their differentiation, most probably by transcriptional silencing of regulatory control loci such as MyoD1, myf 5 (Lasser, et al., 1989). In light of our observations that Ras can induce the DNA MeTase and that this activity has potential gene repressing function, we hypothesised that inhibition of differentiation and transformation of C2C12 myoblasts by Ras may be mediated by induction of the DNA MeTase.

In this paper we show that:

1) Expression of v-Ha-Ras in C2C12 cells increases DNA MeTase steady state mRNA levels and protein activity levels.

2) Expression of v-Ha-Ras in C2C12 cells leads to gene specific hypermethylation of the MyoD and p53 loci.

3) Minimal expression of v-Ha-Ras causes cellular transformation and loss of the differentiated phenotype of C2C12 cells.

4) Coexpression of antisense mRNA to the DNA MeTase along with v-Ha-Ras inhibits Ras initiated increase in DNA MeTase, transformation and DNA hypermethylation.

The results presented in this work complements our previous findings and demonstrate the DNA MeTase is not only involved in maintaining the transformed state but is actively involved in the initiation of transformation by v-Ha-Ras. Here, we actively transformed the myoblast cell line C2C12 with v-Ha-Ras whereas the Y1 cells used in the previous studies were isolated from a naturally occurring mouse tumor and are thus already transformed.

A caveat when using transfected vectors to express the desired antisense mRNA is that the transfection and selection procedure may select against the clones of interest. For example if the desired phenotype is a nontransformed cell then isolating relatively fast growing clonal populations obviously undermines this attempt. In addition, there may be regulated expression or progressive inactivation of the transfected gene, in this case the steady state expression in the cell line may not reflect the level of expression at the time of transfection. This is of primary importance when you are modulating an activity with lasting effects such as the covalent modification of the genome by the DNA MeTase. With this and its potential as a therapeutic in mind we have developed antisense oligonucleotides to inhibit the DNA MeTase. These drugs can then be administered to cells in culture to monitor the immediate and long term effects of DNA MeTase inhibition. Future experiments will include treating v-Ha-Ras transformed C2C12 cells with such inhibitors and monitoring the changes in methylation patterns as well as gene expression profiles. Reestablishment of the proper myogenic program to v-Ha-ras transformed C2C12 myoblast should involve transcriptional activation of genes in the MyoD family. Preliminary evidence suggests that MyoD expression lost in the v-Ha-Ras C2C12 transfectants is induced in the antisense expressing cell lines, however more experimentation is required for a definitive result.

Chapter 5: Antisense oligonucleotides to the DNA Methyltransferase inhibit tumor growth *in vivo*. A.R. MacLeod, V. Bozovic, E. Van Hoffe and M Szyf. (submitted manuscript).

It was the discovery of simple DNA sequences elements in the promoter of the DNA MeTase gene that inspired questions about the complex cellular regulation of the gene and then to investigating its potential deregulation in cancer. Understanding the biochemical mechanisms involved in the regulation of DNA methylation would be of great interest to biologist, along with this however, is the ultimate goal of developing effective anticancer therapeutics. If inhibition of the DNA MeTase by antisense technology is to be a cancer therapy it is clear that some means other than expression from a transfected vector will be necessary. As described in the introduction, modified DNA oligonucleotides that can penetrate cells are gaining support as effective agents used to modulate gene expression. Therefore, to test the therapeutic potential of specific inhibition of the DNA MeTase, a series of DNA antisense phosphorothioate oligonucleotides complementary to the sequence flanking or near the translation initiation codon of the DNA MeTase mRNA were synthesised. Because we have extensively characterized the Y1 adrenocortical cell line in terms of transformation state, morphological phenotype and DNA methylation profiles we tested the ability of the various oligos to inhibit the endogenous DNA MeTase in Y1 cells and the resulting effect on cellular transformation in *ex vivo*. The oligo with the best antitumorigenic potential in the *ex vivo* studies was then used for the *in vivo* experiments on tumor bearing mice.

In this study we show that:

1) Antisense treatment of Y1 cells reduced the steady state level of DNA MeTase mRNA and protein activity and lead to DNA hypomethylation whereas control oligos with scrambled sequence had no effect.

2) Y1 cells treated with DNA MeTase antisense oligonucleotides in culture show

morphological characteristics consistent with non transformed, growth arrested cell. Control oligonucleotides do not induce such change in Y1 cells.

3) Electron microscopic analysis revealed the accumulation of lipid vacuoles in Y1 cells treated with antisense oligonucleotides and not in those treated with control oligonucleotides.

4) Cell cycle analysis demonstrated that cells treated with antisense oligonucleotides accumulated in the G_0 and G_2/M compartments (data not shown).

5) Cells treated with antisense oligonucleotides lost their ability to grow in an anchorage independent fashion.

6) Antisense oligonucleotides to the DNA MeTase administered subcutaneously inhibited the growth of Y1 cells into tumors when injected into the syngeneic mouse strain LAF-1.

This study demonstrates that antisense phosphorothioate oligonucleotides to the DNA MeTase can inhibit the DNA MeTase and have potential as an antitumorigenic therapy. As discussed previously an increase in DNA methylation activity may lead towards cancer by a programmed methylation of a large number of tumor suppressor loci and that inhibition of methylation by a means such as antisense may reverse this process. The finding that inhibition of DNA MeTase by antisense oligonucleotides leads to an increase in the population of Y1 cells residing in the G_0 and G_2/M compartments (data not shown) suggests that hyperactivation of DNA MeTase might also be directly involved in control of the cell cycle. In support of such hypothesis are results by Tasheva and Roufa showing hypermethylation of an origin of replication in growing cells and hypomethylation of the same sequences in arrested cells (Tasheva and Roufa, 1994). This might suggest a direct role for hyperactivation of DNA MeTase in the loss of growth control in cancer cells. Szyf has proposed a model implicating the DNA MeTase in the control of origins of replication

(see Fig.7): Methylated origins initiate replication (Tasheva and Roufa, 1994). In non replicating cells DNA MeTase activity is low as has been shown before (Szyf et al., 1991) and origins are therefore maintained in a hypomethylated state (Tasheva and Roufa, 1994). Induction of DNA MeTase activity at the G1-S boundary (Szyf et al., 1991) initiates the methylation of origins of replication and the origin fires. Once replication is initiated, the origin becomes hemimethylated, that is the nascent strand is not methylated. The origin remains inaccessible to the DNA MeTase throughout S phase possibly by its association to the putative fixed "replication factories" (Coverely and Laskey, 1994) as is the case in E.coli (Campbell and Kleckner, 1990). Once one round of replication has been completed, the origin is again accessible for methylation and a new round of DNA replication can be accomplished. Tasheva and Roufa suggested that when a cell retreats from the cell cycle, a demethylase removes the methyl groups from the origin (Tasheva and Roufa, 1994). Aberrant induction of the DNA MeTase by activation of the Ras signaling pathway might result in methylation of origins and the aberrant initiation of DNA replication. Such overexpression of DNA MeTase in cancer cells may induce the premature methylation and firing of normally silent origins leading to the chromosomal abnormalities observed in cancer cells.

The methylation of origins observed by Tasheva and Roufa is interesting because it is not limited to the dinucleotide sequence CG and in fact methylation of cytosine in dinucleotides CA,CT,CC and CG is found. The question that begs experimentation is whether the CpG DNA MeTase is also responsible the methylation of the other dinucleotides in the origin of replication or as has been suggested before, other DNA methyltransferases exist. Recent purification and expression in a baculovirus system of the murine DNA MeTase from erythroleukemia cells (Glickman and Reich, 1994; Xu et al., 1995) demonstrated the presence of a single protein encoding the murine DNA MeTase in these cells suggesting that previous reports of more than one form of MeTase in murine erythroleukemia cells (Bestor et al., 1983) could be explained as proteolytic digestion products of the 190kDa protein (Xu et al., 1995). Homologous knockouts of the DNA MeTase resulted in dramatic but not complete inhibition of the DNA MeTase activity in

homozygote embryos supporting the hypothesis that other DNA MeTases exist (Li et al., 1992). The cloning and characterisation of the 5'genomic sequences of the DNA MeTase revealed some putative alternative splice sites as well as alternative initiation site that might encode different proteins from this one gene. If these putative splice variants of the DNA MeTase exhibit different substrate specificities, this could explain the methylation of DNA other than that restricted to the CpG dinucleotide. This is an interesting possibility however repeated efforts to clone more mammalian DNA MeTases have failed.

If antisense oligonucleotides directed against the DNA MeTase are ever to reach the clinic, progress will be needed in the area of efficacy. It is my opinion that concentrations used in my *in vivo* experiments are too high to be of clinical use mainly due to cost ineffectiveness. Chemical modifications increasing delivery, enhancing stability and perhaps increasing degradation of the target will help increase efficacy. Antisense may not turn out to be the drug of choice to inhibit the DNA MeTase in cancer therapy, however, it has provided the biological understanding required to design any effective drug.

Therefore any approach that can inhibit DNA methylation with minimal side effects has potential as an antitumorigenic treatment. Among other approaches are the use of analogues of the substrates of the DNA MeTase; (S-adenosylmethionine) SAM and CpG containing DNA. The availability of the crystal structure of the bacterial HhaI CpG MeTase (Cheng, et al., 1993) and the striking homology between mammalian and bacterial CpG methylases (Bestor et al., 1988) should enable the rational design of such inhibitors. SAH (s-adenosyl homocysteine) an analogue of SAM and one of the products of the methylation reaction is an inhibitor of DNA methylation (Mixon and Dev, 1983). However, SAH will inhibit a large number of different methylation reactions in the cell and must have nonspecific effects (Vedel et al., 1978). An additional approach that has been shown to be effective in inhibiting DNA methylation is the use of inhibitors of SAH hydrolysis such as periodate-oxidized adenosin (liteplo and Kerbel, 1986). Again, nonspecific effects are expected because of the predicted effect on all cellular methylation reactions. Another potential inhibitor is a double stranded oligonucleotide bearing 5-fluorocytidine replacing cytidine in the CG recognition sequence which is a well characterised mechanism driven inhibitor of the DNA MeTase (Osterman et al., 1988). This oligo has yet to be tested in

vivo and it stands to reason that the presence of the fluoride molecule also might have deleterious effects. It is therefore clear that while the study of the regulation of DNA MeTase provides us with general direction as to the potential candidates for DNA MeTase inhibitors, future work will be required to develop this family of drugs. These drugs would probably have a wide range of therapeutic application in cancer therapy and other genetic diseases.

OTHER TARGET DISEASES

Arguments presented above suggest that a general inhibition of DNA MeTase can result in specific changes in methylation patterns and modulation of gene expression profiles. Potentially any disease involving aberrant gene expression is a target for treatment with DNA Methylation modulators. Other examples of such diseases include thalassemia and sickle cell anemia. In these situations the adult mature β-globin protein is rendered defective by a point mutation resulting in defective red blood cells (Ingram, 1952; Orkin et al. 1982). Mammals bear a set of fetal globin genes that are switched off at birth and methylation is possibly one mechanism of stabilising the repressed state (Shen and Maniatis, 1980; Busslinger et al., 1983). Demethylation could be used to reactivate the fetal γ-globin which is structurally normal in these patients. Reactivation of fetal globin genes will result in a fraction of red blood cells that express non mutated biologically functional hemoglobin chains. 5-azaCytidine and 5-deoxyazacytidine were successfully used in the past to activate the fetal globin genes in primates (Desimone et al., 1982) and sickle cell anemia patients (ley et al., 1982). These clinical trials were discontinued because of the toxic effects of 5-deoxyazacytidine. Recently hydroxyurea (an inhibitor of ribonucleoside diphosphate reductase) has been effectively used in sickle cell anemia patients which is suggested to activate fetal globin by a different mechanism, possibly by induction of reprogramming of red blood cell maturation. Since hydroxyurea is a cytotoxic agent with a wide range of side effects it will be of advantage to have at hand agents that

can specifically inhibit DNA methylation with limited cytotoxicity. Another clinically relevant situation where gene reactivation is of interest is genetic diseases involving parentally imprinted genes. Parentally imprinted genes are different from most of our genome since only one allele is expressed. Mutation of the active allele will result in complete loss of function even though the other allele is genetically intact but repressed by methylation. It stands to reason that in many cases of parentally imprinted genetic diseases demethylation could activate the parentally silenced allele.

Exogenously introduced genes, for example viruses, are targets for inactivation that involves de novo methylation. Doerfler has suggested that de novo methylation might be an immune response at the cellular level aimed at protecting cells from introduction of foreign genetic material (Doerfler, 1991). Adenoviruses and retroviruses (Chalitta and Kohn, 1994) have been shown to undergo *de novo* methylation *in vivo*. However, the repression of retroviral vectors is problematic when retroviral vectors are used for in vivo delivery of genes in gene therapy protocols (Chalitta and Kohn, 1994). Challita and Kohn have recently shown that such an inhibition is associated with methylation *in vivo* of the retroviral vector (Challita and Kohn, 1994). As discussed previously, cis acting sequences directing de novo methylation and those imparting protection from such methylation exist, these signals must interact with factors that mediate methylation or protection. Therefore, two different strategies can be envisioned to overcome this problem. Cis-signals that protect adjacent sequences from methylation (Szyf et al., 1990) could be engineered into the delivery vectors in addition to deletion of negative control regions (Flanagan et al., 1989). Such an approach has been recently shown to increase expression and decrease DNA methylation of MoMulv LTR driven neomycin resistance gene in F9 embryonic carcinoma cells (Challita et al., 1995). Similarly, future developments of gene therapy mediated therapeutics might also involve generating modified vectors that had deleted the de novo methylation signals and containing a number of cis-hypomethylating signals. Targeting the specific transacting factors mediating the methylation or protection of specific sites provides another potential strategy. The latter is a long term goal as the these "methylation modulating factors" have yet to be discovered.

In summary, it seem clear that DNA methylation plays important roles in many biological

processes and that its deregulation is involved in oncogenesis. Future work should be aimed at improving compounds used to inhibit or modulate this activity to realise their potential as therapeutics.

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