Determination of Inheritance of Methylated DNA

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ABSTRACT

I have studied the maintenance of methyl groups at the HpaII sites of autonomously functioning DNA upon transformation into mammalian cells. DNA molecules, containing the SV40 origin, were methylated in vitro with the bacterial modification enzyme HpaII methylase, which methylates the internal cytosine of the 5'-CCGG-3' sequence. The DNAs were then transfected into a SV40 monkey cell line (COS) and extracted 48 hours later to determine methylation. The question of whether the DNAs were replicated was also asked. Restriction enzymes and Southern blotting were used to determine both methylation and replication. It was found that the DNAs replicated by COS cells lost their methylation, while the DNAs not replicated maintained their methylation. As a result, it is suggested that COS cells not be used in experiments requiring a specific methylation pattern to be maintained in transfected DNA.

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I thank the University Honors Program for giving me the opportunity to pursue an independent research project. I would also like to thank Dr. D. Peterson for this opportunity. In addition, I thank Dr. Peterson for his unending patience as I stumbled through the first few months of this project, and I appreciate and commend him on his ability to "stand back and watch" as I made my mistakes --- allowing me to actually perform an "independent" project. I also thank Dr. Peterson for sacrificing his time to meet with me regularly, attend the Honors Program functions, and for all the time spent helping me prepare for presentations and this paper.

Much of the thanks for all the things I learned during the past nine months goes to both Kim and Mark Toohey. Their patience and understanding never ended --- no matter how many times I asked a particular question or how uneducated the question was. I truly appreciate the time, materials, and lab space they sacrificed on my behalf.

Finally, I'd like to thank Kathy Reifuss, Jim Marich, Wen-Liang Kuo, Joel Steelman, and Kelly Marcom for making this such an enjoyable lab experience. Special thanks goes to Stuart Wright for preparing this paper in such an excellent manner and on such short notice (esentially none!). The generosity of his time is greatly appreciated.

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SUMMARY

I have studied the maintenance of methyl groups at the HpaII sites of autonomously functioning DNA upon transformation into mammalian cells. DNA molecules, containing the SV40 origin, were methylated in vitro with the bacterial modification enzyme HpaII methylase, which methylates the internal cytosine of the 5'-CCGG-3' sequence. The DNAs were then transfected into a SV40 monkey cell line (COS) and extracted 48 hours later to determine methylation. The question of whether the DNAs were replicated was also asked. Restriction enzymes and Southern blotting were used to determine both methylation and replication. It was found that the DNAs replicated by COS cells lost their methylation, while the DNAs not replicated maintained their methylation. As a result, it is suggested that COS cells not be used in experiments requiring a specific methylation pattern to be maintained in transfected DNA.

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INTRODUCTION

The regulation of gene expression in higher eucaryotes is a highly complex phenemenon which is at present poorly understood. Regulation is likely to occur in several steps, one of which is the initiation of transcription. To complicate matters, the regulation of initiation is likely to occur in a variety of ways. Naturally, the genetic code plays an important role in regulation, and as such elucidation of more complex coding principles is a major challenge of today's molecular biologists. However, it may be too simplistic to look for control of transcription to reside solely in specific DNA sequences; transcriptional control may also be found in complex structures of DNA. It has become increasingly apparent that the double stranded DNA can assume many different structures that differ strikingly from its classical B form (Dickerson et al., 1982; Zimmerman, 1982). Since certain DNA sequences can cause specific alterations (Dickerston et al., 1982; Zimmerman, 1982), it is important to determine under which conditions these DNA structural transitions can occur and which modifications might stabilize certain transitions. DNA methylation at highly specific sites is a modification that could have this effect (Behe and Felsenfeld, 1981; Behe and Zimmerman, 1981; Klysik et al., 1983; Moller et al., 1981). In addition, a methylated base with a methyl group protruding into the major groove of B DNA can efficiently provide a signal in its own right. It is this role of methylation, as a molecular signal potentially important in the cell-specific determination of steroid-responsive genes, that this work concentrates on.

Background Information

To fully appreciate the significance of this work, a general understanding of the mechanism of steroid hormone action is necessary. Steroid hormones appear to function via a common pathway (for review see Gorski and Gannun, 1976; Yamamoto and Alberts, 1976). The hormone itself is able to enter the cell. Upon entering the cell the hormone binds with high affinity to a receptor protein found in the soluble fraction of the cell forming a hormone-receptor complex. The hormone-receptor complex becomes localized in the nucleus, binds to specific genes characteristic to the hormone, and alters transcription of the gene. Immediately a question can be raised in regards to this mechanism. What are the signals that determine whether or not a particular gene is recognized by the hormone-receptor complex? The basis of this work is to determine whether DNA methylation, acting as a molecular signal, may provide at least part of the answer to this question.

The molecular signals that determine steroid responsiveness could be of two (and not mutually exclusive) types. One type, the "trans" mechanism, is one in which the signal is provided by a specific entity generally capable of acting at various sites within the cell. For example, the trans signal could take the form of a receptor or a specific repressor protein. The other type of molecular signal is termed the "cis" mechanism. This type of signal is the result of structural or conformational aspects of the genes themselves. DNA methylation provides an example of cis control.

Modified bases are found in the genomic DNAs of most organisms. In vertebrates, 5-methylcytosine (^mC) is the only modified base and is

usually found in the dinucletide CpG (Browne and Burdon, 1977) though other variations have been observed (Sneider, 1980). Methylated cytosine results from enzymatic transfer of the methyl group of 5-adenosylmethionine, and evidence indicates that methylation occurs as an early post-replicative step (Burdon and Adams, 1969; Kappler, 1970).

As mentioned above, DNA methylation is an example of cis control; as such, it may act as a signal in steroid responsive gene regulation. In fact, evidence is increasing linking DNA methylation patterns to patterns of gene expression (for review see Razin and Riggs, 1980). Detailed study of the species- and tissue-specific variations in the extent of methylation have been noted, and it has been shown that the extent of methylation differs between active and inactive genes. Actively transcribed genes are undermethylated, while inactive genes were usually, but not always, methylated (McGhee and Ginder, 1979; Mandel and Chambon, 1979; van der Ploeg and Falvell, 1980). Other correlations between methylation and gene expression have been made using DNaseI sensitivity as a measure of gene activity. Studies show that moderate sensitivity to nuclease usually extends throughout an entire active transcription unit (Weisbord, 1982). In animal cell DNA, an average of 70% of all CpG dinucleotides are methylated; however, in active nuclear regions in which increased sensitivity to DNaseI is observed (Weintraub, 1976; Garel, 1977) only 30-40% of the CpG dinucleotides are methylated (Naveh-Many, 1981). Further evidence of the importance of DNA methylation in regulating gene expression has been obtained with the ability to introduce foreign genes into a variety of cells and with the subsequent ability of these genes to be expressed. Through these types of

experiments a direct test of the causal relationship between DNA methylation and the inhibition of gene expression is made possible. A number of experiments have tested whether a gene, methylated in vitro, is expressed either in vivo or in suitable in vitro transcription systems. DNA methylation proved to have no effect on expression in in vitro systems (Busslinger et al., 1983), while methylated genes are not expressed in micro-injected Xenopus oocytes (Vardimon et al., 1982; Fradin et al., 1982) or in transformed mouse L-cells (Stein et al., 1982b).

Before proposed experiments to determine the effects of methylation on hormone-induced gene transcription can be performed, two requirements must be met. First, we must obtain a hormone inducible gene system. And second, we must determine the state of methylation of the DNA being investigated. Mouse mammary tumor virus (MMTV) satisfies the first requirement. MMTV is a retrovirus, and as such the RNA genome of MMTV is encoded by the viral DNA forms of these viruses (proviruses) covalently integrated into the nuclear DNA of infected cells. The integrated forms of these proviruses contain long terminal repeats (LTRs), approximately 1.3 kb in size, at both ends of the 10 kb provirus (Weiss et al., 1982). MMTV is unique among retroviruses in that MMTV transcription is inducible by glucocorticoid hormones such as dexamethasone (Ringold et al., 1975; Ringold et al., 1977; Ringold et al., 1975b). Glucocorticoid receptor protein has been shown to bind to a specific 340 bp fragment within the LTR. The binding region, termed the glucocorticoid response element (GRE), has many properties similar to those of enhancer elements (Chandler et al., 1983; Khoury and Gruss, 1983). Through the use of the

GRE it is possible to confer hormone inducibility on heterologous promoters to which it is linked in cis.

Under normal conditions, endogenous copies of MMTV are not expressed (Varmus et al., 1973) though proviral copies of the MMTV genome are present in all inbred mouse strains and in most wild mice (Cohen and Varmus, 1979). For example, even though some mouse strains contain as many as three genomic regions with sequence homology to MMTV (Cohen et al., 1979), there is no detectable MMTV RNA in the spleen, liver, or mammary gland of these mice (Marcus et al., 1981; Maxam and Gilbert, 1977). It has been suggested that methylation may be one paramater that affects the potential for endogenous gene expression. Experiments show that transcriptionally inactive endogenous MMTV genes in liver cells are highly methylated at CpG, while transcriptionally active, exogenously acquired MMTV genes in mammary tumors are not extensively methylated (Cohen, 1980).

The second requirement, determining the methylation state of the tranfected DNA, must also be satisfied before a conclusion concerning the effects of methylation can be drawn. In other words, to conclude whether or not methylation of a test gene affects hormone induction, we must first conclude whether or not the test gene is methylated. To do this, we cannot simply transfect cells with DNA methylated in vitro. In vitro methylation will only insure that the pre-transfected DNA is methylated. During the time course of a typical transfection experiment (48-72 hours) the DNA may lose its methylation pattern; perhaps as a result of non-functional methylation enzymes or as a result of demethylation activities (Gjerset and Martin, 1982). In order to insure methylation

has been maintined, the DNA should be investigated after existing in the host cell system for a period of time corresponding to that of a typical experiment. In this way only can validation of methylation be achieved.

In this paper, I address the issue of whether the methylation pattern of a genome functioning autonomously in a particular line of simian cells (COS) is faithfully maintained. In addition, I seek to determine the extent of replication of this autonomously functioning genome.

RESULTS

Experimental Design

These experiments were performed with transiently replicating DNA in COS cells. COS cells were developed from a simian cell line (CV-1) known to be permissive for lytic growth of simian virus (SV40). In order for viral replication of SV40 to occur, T antigen, a protein encoded by SV40, must be present. Establishment of COS was achieved by transforming CV-1 cells with an origin-defective mutant of SV40 which codes for wild-type T antigen. The transformed cells were found to contain T antigen, retain complete permissiveness for lytic growth of SV40, and support the replication of pure populations of SV40 mutants with deletions in the early region (for review see Gluzman, 1981).

Since COS cells produce SV40 T antigen and are permissive for SV40 viral replication, transfecting COS cells with plasmids containing the SV40 viral replication orgin but lacking regions necessary for viral transcription (SV-ORI vectors) results in high copy number of the plasmid. Using DNA blotting experiments, it is estimated that COS cells

tranfected with SV-ORI DNA contain between 200,000 and 400,000 copies of DNA after 48 hours (Mellon et al., 1981). This efficiency of replication is comparable to that of wild type SV40 in normal monkey cells (Tooze, 1980). Thus, the COS cell system provides a means of amplifying cloned eucaryotic genes in mammalian cells.

The plasmids used in this experiment were specifically designed to assess the effect of methylation on hormonally induced transcription and as such contain specific regions required for this purpose. I will briefly identify these regions in the next paragraph. In addition, I have included an explanation for their use in hormone induction experiments in the Discussion section of this paper.

The vector synthesized in our lab for use in DNA methylation experiments is pKM1 (see Figure 1). The parent plasmid for pKM1 construction is pSVOd (Mellon et al., 1981). pKM1 consists of a portion of pBR322 DNA for replication in E.coli. A region of pBR322 termed the "poison" sequence has been deleted. The poison sequence has been shown to inhibit SV40 replication in simian cells (Lusky and Botchan, 1981). pKM1 is further characterized by the gene coding for the bacterial enzyme (Gorman et al., 1982) chloramphenicol acetyl transferase (CAT). Just upstream from the CAT gene is the hormone-responsive promoter supplied by the MMTV LTR. A polyadenylation signal is provided by SV40 along with the SV40 replication origin. In addition, pKM1 contains a region from the replication origin of the filamentous bacteriophage M13. The importance of each of these regions is discussed in greater detail in the Discussion. An additional plasmid, pKM2, has also been constructed. pKM2 is essentially identical to pKM1 with the addition of the SV40

enhancer element in the SV40 origin region. It should be noted that the results shown in this paper are obtained from experiments using pKM1 only, though pKM2 showed identical results.

In vitro Methylation of pKM1

pKM1 was methylated in vitro with the bacterial modification enzyme HpaII methylase. HpaII methylase recognizes the tetranucleotide sequence 5'-CCGG-3' and methylates the internal cytosine by transfer of a methyl group from 5-adenosylmethionine to the 5-position the base. The methylation efficiency was determined utilizing the restriction enzymes HpaII and MspI. These enzymes recognize the same tetranucleotide sequence as HpaII methylase. However, HpaII will not cleave the DNA if the internal cytosine is methylated; MspI will cleave both methylated and unmethylated DNA.

Efficiency of the HpaII methylase reaction (methylation of pKM1) was determined by extensive digestion of the DNA with HpaII and MspI followed by fractionization of the digests by electrophoresis on 1.5% agarose gels. Methylated pKM1 treated with HpaII was identical to undigested pKM1. That is, HpaII was unable to digest methylated pKM1. Digests of methylated pKM1 with MspI showed fragmentation identical to that seen in digestion of unmethylated pKM1 (see Figure 2).

Replication of pKM1 in COS cells

The ability to determine whether transfected DNA has been replicated relies on determining susceptibility of the DNA to digestion by the restriction enzyme MboI. This restriciton enzyme recognizes the sequence

5'-GATC-3' and will cleave this sequence if the internal adenine is not methylated. Cellular genes and viral DNA molecules cloned in dam[+] E. coli acquire methylation of the adenine in the sequence 5'-GATC-3', the same sequence recognized by MboI. pKM1 was propagated in dam[+] E. coli and as a result contains this methylation pattern. Following transfection of animal cells, these adenine methylations are progressively lost upon replication of the DNA (Peden, et al., 1980). Thus if pKM1 transfected into COS cells is replicated, it will become sensitive to MboI as a direct result of loss of adenine methylation. Unreplicated pKM1 will remain insensitive to MboI.

The plasmid pKM1 was assayed for DNA replication in COS cells. The DNAs were introduced into COS cells utilizing DEAE-dextran (Experimental Procedure). Low molecular weight DNA was isolated 48 hr after transfection by the procedure of Hirt (1967) and was digested with MboI and SauIIIA. SauIIIA recognizes the same sequence as MboI but is insensitive to methylation and, thus, will cut DNA whether it has been replicated in mammalian cells or not. The DNA was then fractionated by electrophoresis on a 1.5% agarose gel, transferred to a nylon filter (Southern, 1975) and hybridized with ^{32}P-labelled pKM2 DNA. The filter was exposed to film for 24 hr.

The replication of in vitro COS cells appears to be rather inefficient. Plasmid DNA that has been replicated in mammalian cells should show identical fractionation after digestion with MboI as that seen after SauIIIA digestion. pKM1 does not show identical fractionation with these two enzymes (see Figure 3). In all cases, high molecular weight DNA, representing undigested pKM1, is present in the MboI lane.

These bands are absent in he SauIIIA lane. However, faint bands in the MboI lane corresponding to those of the SauIIIA digestion are apparent, suggesting that some of the DNA assayed is replicated by the COS cells. Using relative band intensity as a measure of the amount of DNA in each digestion band, the percentage replicated appears to be low.

Additional digestions were performed in order to insure that the MboI digestion was cutting to completion. Since lack of cutting by MboI is interpreted as DNA that has been unreplicated, it is essential to insure that MboI activity is not inhibited by extraneous fators. To do this, small aliquots were taken from the MboI digestion of the transfected pKM1 and added to .25 ug SV40 DNA. The SV40 digestion was incubated, along with pKM1 digestion, for 1 hr at 37°C, and the sample was electrophoretically fractioned on 1.5% agarose and stained with ethidium bromide. Photographs taken in the presence of ultraviolet light showed digestion of the SV40 DNA, indicating active MboI is present in the pKM1 digestion. This test substantiates the earlier finding that pKM1 is not efficiently replicated by COS cells.

Maintenance of methylation of pKM1 in COS cells

Experiments have shown that exogenous DNA molecules methylated in vitro maintain their methyl groups, upon integration into the cell DNA, for over 25 generations (Pollack et al., 1980; Wigler et al., 1981). In order to determine if this holds true for autonomously replicating DNA, I transfected COS cells with pKM1 methylated in vitro with HpaII methylase. I then isolated the low molecular weight DNA (Hirt, 1969), treated the DNA with the isoschizomers HpaII and MspI, electrophoretically

fractioned the DNA on 1.5% agarose, and transferred the DNA to a nylon filter. After probing the DNA with ^{32}P-labelled pKM2, I exposed the filter to film for 24 hr.

As discussed earlier, the restriction enzymes HpaII and MspI allow determination of internal cytosine methylation at the tetranucleotide 5'-CCGG-3'. DNA methylated at this cytosine is resistant to digestion by HpaII, whereas all DNA (methylated or unmethylated) is sensitive to MspI digestion. Thus, if COS cells are capable of maintaining the methylation of the transfected plasmid DNA, the DNA obtained from the Hirt extraction will be resistant to HpaII, as is the pre-transfected DNA (see Figure 2). My experiments show that this is not the case. The methylation pattern is not faithfully maintained by COS cells. Though some plasmid NDA remains intact upon digestion with HpaII, it appears that a large quantity of DNA is digested (see Figure 3), demonstrating conclusively that the methyl groups introduced at CpG sites in vitro are lost.

To investigate further the methylation state of the transfected DNA, HpaII and MspI double digests were performed on the MboI digested sample. By comparing the bands produced by the 3 digests (MboI; MboI/HpaII; MboI/MspI) we can determine whether or not the small amount of plasmid DNA that was replicated by the COS cells maintained its methylations pattern.

The replicated DNA is that which is digested with MboI. This DNA is represented by the bands in the "Digested" region of the MboI lane (see Figure 3). Subsequent incubation of this replicated DNA with HpaII and MspI results in the disappearance of two bands previously present. Apparently, the replicated DNA is sensitive to HpaII. We can also see

that the unreplicated plasmid DNA, represented by the two bands in the "Undigested" region of the MboI lane (see Figure 3), is resistant to HpaII digestion. Two conclusions can be drawn from these results. First, the small amount of DNA that is replicated by COS cells has lost its methylation pattern. Second, the DNA that is not replicated by COS cells maintained it methylation pattern.

DISCUSSION

The results presented in this paper clearly show that COS cells are unable to stably maintain methylation of DNA functioning autonomously within the cell; the reason for their inability is not so clear. Animal cells are believed to contain essentially two methylases, one know as a de novo methylase and the other as a maintenance methylase (Razin and Friedman, 1981; Razin and Riggs, 1980). De novo methylase is not a primary concern here; it imposes a specific methylation pattern on DNA not previously methylated. On the other hand, maintenance methylase recognizes hemimethylated DNA as its specific substrate (Gruenbaum et al., 1982). All biochemical evidence indicates that DNA is methylated in an early post-replicative step (Bardon and Adams, 1969). Only the newly synthesized strand becomes methylated (Brid, 1978). Since the CpG dinucleotide, which bears most of the vertebrate methylation, is a simple palindrome, methylation on one strand is able to direct the methylation on the newly synthesized strand via maintenance methylase action. In this way, specific methylation patterns of DNA can be perpetuated in the cell population.

The results presented in this study show an absence of maintenance methylation. Some possible reasons for the lack of methylation in autonomously replicating DNA were suggested by Subramanian (1982) and include: (1) The maintenance methylase can not keep pace with the rapidly replicating DNA (2) Preliminary evidence indicates that in animal cells, DNA methylation, like DNA replication, starts from unique origins and from there proceeds to other sites in the cell DNA. Such origins for DNA methylation may be absent in autonomously replicating DNAs (3) Cellular DNA and automously replicating DNA may replicate in separate compartments within the nucleus (4) The chromatin structure for cellular DNA might be so arranged that certain CpG sites are exposed for methylation, where as autonomously replicating DNA may not have this arrangement. Whatever the reason, this experiment shows that maintenance methylase action is absent on the autonomously replicating plasmid in COS cells.

As mentioned earlier, it was hoped that COS cells would provide a suitable system for determining the effect of methylation on glucocorticoid-regulated gene expression. The COS cell system was initially attractive for a number of reasons described in detail by Mellon et al. (1981). First, it has been shown that plasmids that contain the SV40 viral origin but lack regions necessary for transcription can be efficiently replicated and transcribed after transfection into this cell system. Second, since COS cells provide the large T antigen no helper virus is necessary. Third, replication in COS cells does not require actively transcribed viral genes, thus avoiding interference from viral promoters, splicing junctions, and termination sites which could interfere with analysis of cloned gene transcripts.

Finally, transient experiments performed in the COS cell system allow transcription assays to be performed in much less time than those performed in stable cotransformation procedures (Wigler et al., 1979). However, as this experiment indicates, there are some limitations to the COS cell system. Besides not being able to stably maintain a methylation pattern, it has been suggested that the high gene copy number observed in COS cells may result in transcriptional properties that do not reflect those of normal cells. For instance, in cell processes that require regulatory molecules (such as steroid receptor proteins) it is possible that the large gene number may titrate out such necessary molecules. In addition, some studies show that mRNA of several mammalian genes are not efficiently translated in COS cells (Mellon et al., 1981). In any case, with COS cells unable to stably maintain methylation in autonomously functioning DNA, we have sufficient reason to search for a new cell system that meets our requirement.

Because future methylation experiments in this lab have awaited the outcome of this work, a brief discussion of the proposed work will be presented here. In addition, the reason for constructing the plasmid pKM1 (see Figure 1) will become apparent. As mentioned throughout this paper we are primarily interested in whether a specific methylation pattern will have an effect on glucocorticoid responsiveness. Thus, while searching for a cell system that will maintain methylation we are concurrently working to construct defined methylation patterns in vitro, using a strategy developed by Stein et al. (1982) and Busslinger et al. (1983). The procedure requires the presence of the M13 origin on pKM1. With the M13 origin and appropriate male strains of E. coli containing

the plasmid, we can obtain single stranded pKM1 (Zagursky and Berman, 1984). Once single stranded pKM1 is obtained, specific methylation patterns can be constructed by in vitro second strand synthesis. Using primers hybridized to the single stranded pKM1, second strand synthesis will be performed in the presence of 5-methylcytosine by Klenow polymerase. The result is double stranded pKM1 with a defined methylation pattern. Methylated cytosine residues will be present on only one strand and only in the region not occupied by the DNA primer (see Figure 4).

Once defined methylation patterns are achieved, transfection into a suitable cell system will follow with subsequent determination of glucocorticoid responsiveness. Glucocorticoid responsiveness is characterized by increased gene expression. The bacterial enzyme chloramphenicol acetyl transferase (CAT) provides a convenient assay for gene expression, thus accounting for its presence and location (adjacent to the MMTV LTR) in pKM1. Transcription of the CAT gene can be easily quantitated. Crude extracts of cells transfected with pKM1 will be assayed by providing the substrates, cholramphenicol and acetyl CoA, for the CAT reaction. By transfecting cells with pKM1 methylated in various specific regions, treating the cells with glucocorticoid, and comparing CAT activity of each of these preparations, the effects of various methylation patterns on glucocorticoid induced transcription can be achieved.

EXPERIMENTAL PROCEDURES

Cells and Enzymes

COS cells (Gluzman, 1981) were grown as a monolayer culture in Dulbecco's modified Eagle's medium (Gibco) supplemenmted with 10% fetal calf serum. The cells were propagated in 100 mm petri dishes and were split at approximately 90% confluency. To split the cells, the medium was removed and the cells were washed twice with 1X phosphate buffered saline calcium and magnesium free (1X PBS(CMF) = 200 mg KCl, 200 mg $KH_2 PO_4$, 8.0 g NaCl, 2.16 g Na_2HPO_4 made up to 1 liter with distilled water). The cells were then trypsinized with a 1X trypsin: EDTA (Gibco) solution for 1 minute at room temperature and scraped from the plate with a rubber policeman. The cells were re-plated after approximately 1:6 dilution.

Restriction enzymes HpaII, MspI, MboI, and SauIIIA were purchased from either Bethesda Research Laboratories or New England Biolabs and were used according to the manufacturer's recommendations. The modification enzyme HpaII methylase was purchased from New England Biolabs. The nucleotides ^{32}P-dATP, ^{32}P-dGTP, dTTP and dCTP were purchased from New England Nuclear.

In vitro methylation

DNA methylation reactions were carried out in the buffer recommended by New England Biolabs. The reactions were performed at various (methylase per µg DNA) ratios, with the length of incubation varying accordingly. For example, if one unit of HpaII methylase, defined as the amount of enzyme required to protect by <90% 1.0 µg λ DNA against cleavage by HpaII

endonuclease, was used to methylate 1.0 µg DNA, the reaction was incubated for 1 hour at 37°C. If one unit was used to methylate 3 µg DNA, the reaction was incubated for 3 hours at 37°C. After methylations, the methylated DNA samples were precipitated with ethanol before restrictions or transformations were performed. The efficiency of the methylation reaction was determined by restriction with HpaII and MspI. Samples of methylated DNA, 2 µg, were incubated with HpaII and MspI. The fragments were then fractionated on a 1.5% agarose gel, stained with ethidium bromide and visualized under U.V. light. DNA undigested by HpaII but digested by MspI was assumed to be adequately mehtylated and appropriate for transfection.

Transformation of COS cells

COS cells were subcultured at a dilution of approximately 1:6, 1 day prior to transfection, and fresh media was added to the cells 4 hours prior to transfection. DNA was introduced into COS cells utilizing a DEAE-dextran method (McCutchan and Pagano, 1968). Methylated DNA was resuspended in a solution of DME containing no serum (10 µg DNA/plate, 4 ml DME/plate), DEAE-dextran (100 µg/µg DNA, and penicillin-streptomycin). Before exposing the cells to this solution, the fresh media was removed from the cells, and they were washed 2 times with serum-free madia. After washing, 4 ml of the above solution was added to each plate and the plates were maintained at 37°C in a water saturated atmosphere containng 5% CO_2. The cells were exposed to the DNA-DEAE-dextran preparation for 6 hours. After aspiration of the transformation

solution, the cells were grown in DME supplemented with 10% fetal calf serum for 48 hours. The low molecular weight DNA ws then harvested.

DNA Extraction

DNA from transformed cells was isolated as decribed by Hirt (1967). The DNA harvest began 48 hours post transfection. The cell medium was removed and the cells were washed 2 times with 37°C PBS(CMF). The cells were lysed by treatment with 4 ml Hirt lysing solution (0.6% SDS, 10 mM EDTA, pH 7.5) per plate. The plates were tipped gently until the viscous lysate was mobilized (approximately 3-5 minutes) and lysates were pooled in 250 ml polypropylene centrifuge bottles (2 plates/bottle). A second aliqout of lysing solution was added to the plates, the plates were tipped gently for 2-3 minutes and the lysate produced was pooled with the first lysates. A .25 volume 5 M NaCl was added to the lysates, and the solution was mixed until appearing homogenous. The centrifuge bottles were the stored >12 hours at 4°C.

To remove cellular DNA and protein the lysates were centrifuged for 30 mintes (4°C, JA-20, 17K RPM). The cleared lysate was carefully trans- ferred to another 250 ml polypropylene centrifuge bottle and subjected to RNaseA (500 µg/bottle) for 15 minutes at 37°C. One lysate volume of freshly prepared phenol (pH 7.0) was added to the lysate and mixed for 10 minutes at moderate speed with a magnetic stirrer, followed by addition of 1 lysate volume chloroform: isoamyl alcohol (24:1) mixed as before. The phases were separated by centrifugation as before. The acqueous phase was transferred to another bottle. If a white precipitate was

observed at the interface, the acqueous phase was re-extraced with chloroform: isoamyl alcohol until the precipitate was no longer visible. Two volumes of absolute ethanol was added to the acqueous solution followed by mixing by hand and chilling the solutino to -20°C for >12 hours. The precipitated nucleic acids were collected by centrifugatino as before and resuspended in TE.

Southern Blot Analysis

A fraction (1/30 - 1/15) of the Hirt (1967) extracted DNA was digested with restriction enzymes according to the manufacturer's recommendations and fractionated by eletrophoresis through a 1.5% agarose gel. The gel was soaked 30 minutes in a 0.2 N NaOH, 0.5 M NaCl solution and washed 3 times in a solution made of 40 mM Tris/HCl (pH 7.6), 20 mM NaAcetate, and 1 mM EDTA, 10 minutes per wash. The DNA was blotted onto a nylon membrane (Bio-Rad). Filters were then air dried and baked for 2-3 hours at 80°C in a vacuum oven.

Filter Hybridization, Probing, and Exposure to Film

Filters were prehybridized overnight at 41°C in sealed plastic bags containing 7 ml/100 cm^2 of a solution consisting of 3 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate), 50% deionized formamide, 50 µg/ml salmon sperm DNA, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone and 0.1% ficoll. After removal of the prehybridization solution, DNA sequences were detected by hybridization with ^{32}P-labeled nick-translated probes (Rigby et al., 1977) consisting of either pKM1 or pKM2

$(0.5 \times 10^6$ cpm/blot) in prehybridization solution containing 10% (W:V) dextran sulfate. After incubation at 41°C for 12-24 hours, filters were washed 4 times in 400 ml of 2 × SSC, 0.1% SDS, with shaking, for 5 minutes. The filters were then washed twice in 400 ml 0.1 × SSC, 0.1% SDS at 50°C for 30 minutes, followed by a rinse in 200 ml of 0.1 × SSC. After drying, filters were exposed for 12-36 hours to Kodak XAR-5 X-ray film with Dupont Cronex Lightning-Plus intensifying screens.

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Much of the thanks for all the things I learned during the past nine months goes to both Kim and Mark Toohey. Their patience and understanding never ended --- no matter how many times I asked a particular question or how uneducated the question was. I truly appreciate the time, materials, and lab space they sacrificed on my behalf.

Finally, I'd like to thank Kathy Beifuss, Jim Marich, Wen-Liang Kuo, Joel Steelman, and Kelly Marcom for making this such an enjoyable lab experience. Special thanks goes to Stuart Wright for preparing this paper in such an excellent manner and on such short notice (esentially none!). The generosity of his time is greatly appreciated.
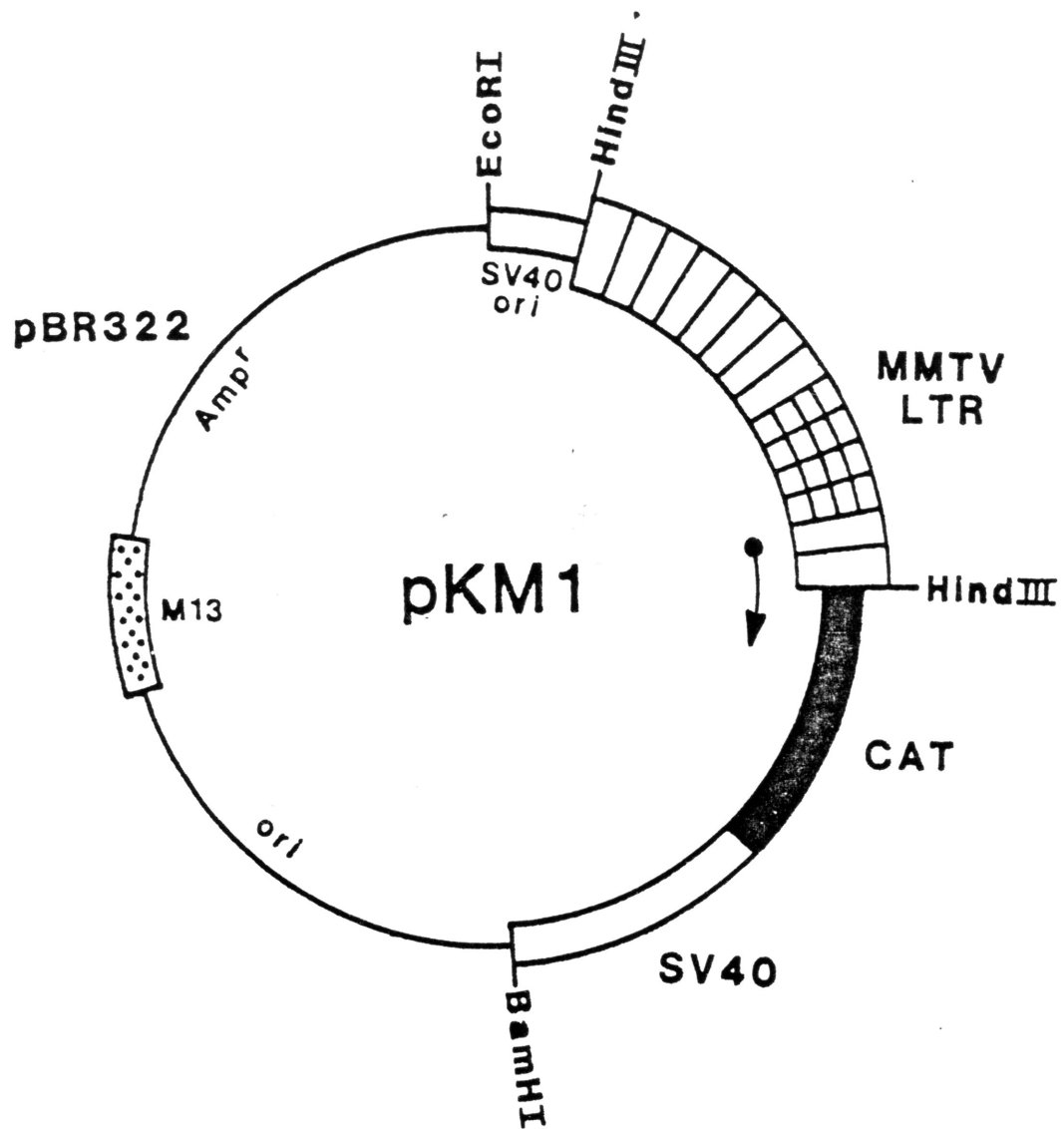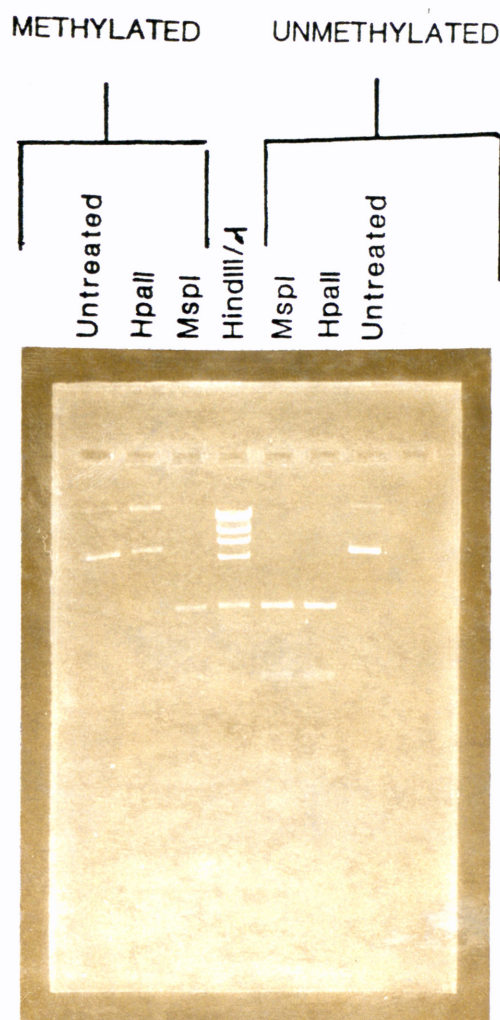
Figure 1.   Restriction Map of pKM1

Figure 2.   Differential Sensitivity to HpaII and MspI of In Vitro
Methylated and Unmethylated pKM1

The methylations and restrictions were performed as described in
Experimental Procedures. The restriction digests were fractionated by
electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.
Pay particular attention to the difference in sensitivity to HpaII of
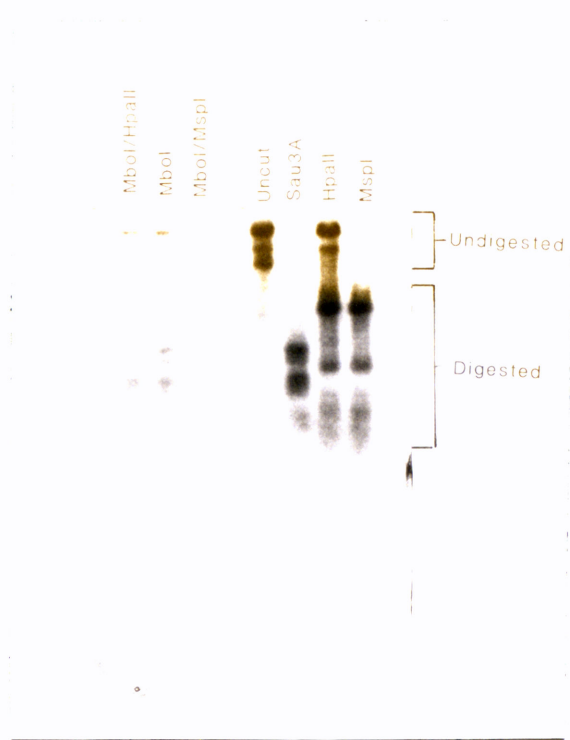methylated and unmethylated DNA.

Figure 3   Determination of Methylation and Replication of DNA
Transfected into COS Cells.

COS cells were transfected with in vitro methylated pKM1. Forty-eight

hours later, plasmid DNA was collected by the Hirt procedure, purified,

treated with various restriction enzymes, separated by electrophoresis on

a 1.5% agarose gel, and transfered to a nylon filter. pKM1 DNA was

visualized by hybridization with pKM1 ^{32}P-DNA and exposure to Kodak XR

film. Notice that the MboI lane contains some undigested DNA, and the

HpaII lane contains bands in the Digested region. MboI double digest

with HpaII results in the loss of two bands that were present in the

digested region of the MboI lane; however, the DNA in the Undigested

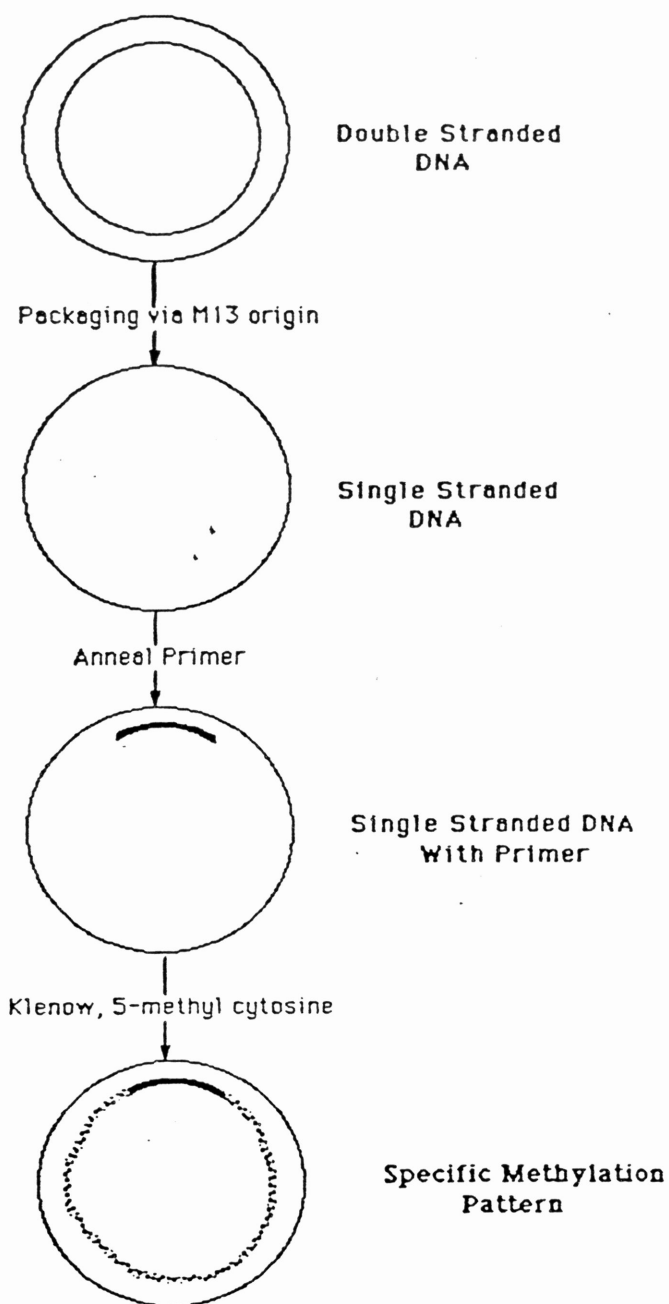region of the MboI lane appears unaffected by HpaII digestion.

Synthesis of Specific Methylation Patterns

Double Stranded
DNA

Packaging via M13 origin

Single Stranded
DNA

Anneal Primer

Single Stranded DNA
With Primer

Klenow, 5-methyl cytosine

Specific Methylation
Pattern

Figure 4   Schematic Representation of the Procedure to Synthesize
Specific Methylation Petterns In Vitro. p.

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