ANTIBODY STUDIES WITH DNA METHYLASE

MARGARET McGARVEY

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UNIVERSITY OF GLASGOW

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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ABBREVIATIONS

BSA	-	Bovine Serum Albumin
dH_2O	-	Distilled H_2O
DMSO	-	Dimethyl Sulphoxide
DTT	-	Dithiothreitol
EDTA	-	Ethylene Diamine Tetra Acetic Acid
ELISA	-	Enzyme Linked Immunosorbent Assay
FCS	-	Foetal Calf Serum
HAT	-	Hypoxanthine, Aminopterin and Thymidine
HT	-	Hypoxanthine and Thymidine
Ig	-	Immunoglobulin
Kb	-	Kilobase
MEL Cells	-	Mouse Erythroleukaemia Cells
MEM	-	Minimal Eagles Medium
5-mC	-	5-Methyl Cytosine
O/N	-	Overnight
OPD	-	o-Phenylene Diamine
PAGE	-	Polyacrylamide Gel Electrophoresis
PBS	-	Phosphate Buffered Saline
PEG	-	Polyethyleneglycol
PMSF	-	Phenyl Methyl Sulphonyl Fluoride
RT	-	Room Temperature
RF	-	Replicative Form
S-Ado Met	-	S-Adenosyl-L-Methionine
SDS	-	Sodium Dodecyl Sulphate
TCA	-	Trichloroacetic Acid
TEMED	-	$NNN^{1}N^{1}$ - Tetramethylethylenediamine
Tween 20	-	Polyethylene Sorbitan Monolaureate
Tween 80	-	Polyethylene Sorbitan Mono oleate

CONTENTS PAGE NO.

LIST OF FIGURES

LIST OF TABLES

SUMMARY

DNA methylase catalyses the transfer of a methyl group from S-Adenosyl-L-Methionine to the 5 position on deoxycytidine residues in CpG dinucleotides of DNA. DNA methylase has been purified 2000 fold from mouse Krebs II ascites tumour cells by modification of the original purification procedure of Turnbull and Adams (1976). The purified enzyme has been used to raise polyclonal antibodies in rabbits in order to study the enzyme *in vitro*.

Partial proteolysis of DNA methylase using trypsin results in an "activated" form of the enzyme which shows an increased specific activity with both native and denatured DNA as the substrate. Western blots of trypsin-treated enzymes have shown that proteolysis results in a loss of the 190 kD and 160 kD forms of DNA methylase, with the progressive appearance of lower molecular weight, immunologically cross reacting polypeptides. *In vitro* as *in vivo*, DNA methylase acts almost exclusively at CpG dinucleotides. The increased activity of the trypsin-treated enzyme may be due to a loss of CpG specificity. Another possibility is that trypsin "activates" DNA methylase by releasing the enzyme from the constraint of acting only at hemi-methylated sites on DNA. Partial proteolysis, by leading to an increase in *de novo* methylation activity by the enzyme, may reflect changes occurring *in vivo* in embryonic cells.

Purified DNA methylase has also been used to immunize rats, for the purpose of generating monoclonal antibodies by the fusion of rat spleen lymphocytes with myeloma cells. From the positive hybridoma clones obtained, 6 were selected for further study. Purified monoclonal immunoglobulins were tested for their ability to:

(a) Inactivate DNA methylase in a standard assay.

(b) Precipitate DNA methylase from solution.

(c) Detect DNA methylase on Western blots.

Preliminary results using single clones failed to detect DNA methylase by these criteria, although the subsequent use of pooled immunoglobulin fractions has resulted in the complete inactivation of enzyme activity *in vitro*.

CHAPTER 1 : INTRODUCTION

1.1 Overview of DNA Methylation

1.1.1 The Occurrence of Methylated Bases in DNA

The existence of minor methylated bases in DNA has been recognised since 1925, when 5-methyl cytosine (5-mC) was reported as a constituent of the nucleic acid of Mycobacterium tuberculosis (Johnson and Coghill, 1925). In 1950 5-mC was identified as a minor base component in the DNA of mammals, fish, plants and some insects (Wyatt, 1950). 6-methyl adenine was identified in bacterial DNA by Dunn and Smith (1958).

The product of cytosine methylation is 5-methyl cytosine, where a methyl group from S-adenosyl-L-methionine is transferred to the 5 position on the pyrimidine ring. In some bacterial DNA, N4 methyl cytosine has also been detected.

6-methyl amino purine (6-methyladenine) is the product of adenine methylation. The methyl group, again donated by S-adenosyl-L-methionine, is attached to the amino group at the 6 position in the purine ring.

1.1.2 Distribution of Methylated Bases in DNA

The distribution of methylated bases in DNA varies greatly between species; 5-mC and 6-methyl adenine are entirely absent from the genomic DNA of some species, whilst in others only one modified base is present. In prokaryotes and lower eukaryotes 6-methyl adenine is more commonly found, while 5-mC is more prevalent in higher eukaryotes. With rare exceptions, 5-mC is the only modified base present in animal cell DNA.

1.1.3 The Effect of Methylation on DNA Structure

Methylation of cytosine residues in DNA introduces a methyl group into the major groove of the DNA helix. This modification has no effect on the base pairing with guanine residues. 5-mC is a stable component in the DNA structure although occasionally *in vivo* it may be subject to deamination to thymine (see next section). Methylation may, however, have a considerable effect on the physical structure of DNA: the substitution of a methyl group at the 5-position on cytosines results in an increase in the hydrophobic interactions involved in base stacking, thus increasing the stability of the helix. This has the effect of raising the melting temperature of the polymer and lowering its buoyant density (Gill et al, 1974; Wagner and Capesius, 1981).

In conditions of high salt, the secondary structure of DNA is known to undergo a conformational change in regions of alternating purines and pyrimidines, converting from the right-handed B form of DNA to a left-handed helix designated Z-DNA. Transition from the B form to Z-DNA is also favoured under physiological salt conditions when the DNA sequence consists of alternating 5-mC and Guanine (Behe and Felsenfeld, 1981; Pohl and Jovin, 1972). The protrusion of methyl groups into the major groove results in some destabilisation of the B-helix. In Z-DNA the major groove is filled with cytosine C_5 and guanine N_7,C_8 atoms, and the same methyl groups form a stabilising hydrophobic region. Although the presence of only four alternating 5-mCpG dinucleotides may be sufficient to effect the transition to Z-DNA, the low frequency of CpG dinucleotides in vertebrate DNA has led to some doubt about the likelihood of such

an event occurring to a significant degree *in vivo*.

The use of Z-specific antibodies has indicated the presence of Z-DNA in a variety of species, from protozoa to mammals; however, the very low incidence of 5-mC in some of these species (e.g. insects) casts some doubt on the importance of methyl cytosine in the transition to Z-DNA.

Conversion of short stretches of DNA to the Z-form may have a considerable effect on chromatin structure; the change from a right-handed helix to the left-handed form is thought to relax chromatin structure over as much as 100 Kb pairs of the immediate sequence (Adams and Burdon, 1985). In addition, Nickel et al (1982) have shown that nucleosomes do not form on Z-DNA, thus Z-DNA may constitute a sequence open to the regulatory influences of specific proteins involved in the control of transcription.

Although runs of purines and pyrimidines have been found in vertebrate DNA, they rarely occur in the promotor region of genes where they might be expected if they have a function in the control of transcription. It seems unlikely that methylation of cytosines in such sequences is sufficient to effect the transition of B-DNA to the Z configuration; more likely is the situation whereby regulatory molecules interact with one or more methyl cytosine residues to stabilise the region in the Z-form (Adams and Burdon, 1985).

1.2 DNA Methylation in Eukaryotes

1.2.1 Tissue and Species Distribution of 5-Methyl Cytosine in Eukaryotes

The genomic content of 5-methyl cytosine varies considerably between species (Vanyushin et al, 1970; Kappler et al, 1971) ranging from almost undetectable levels in yeast and insects to between 1% and 5% in mammalian cells and up to 30% 5-methyl cytosine : cytosine in plants.

The variation in 5-methyl cytosine levels in DNA also extends to different tissues of the same species (Vanyushin et al, 1970; Kappler et al, 1971). Differences in the total level of 5-methyl cytosine in the DNA from various tissues in rat, mouse, human, bull and monkey have been reported (Vanyushin et al, 1973; Gama-Sosa et al, 1983; Erlich et al, 1982; Vanyushin et al, 1970).

Studies have shown that specific genes may be under-methylated in certain somatic tissues as compared with germ line DNA (Waalwijk et al, 1978; Mandel et al, 1979). This might occur during differentiation by the replication of DNA in the absence of methylation (Razin et al, 1980). Stein et al (1983) demonstrated that several housekeeping genes have a fixed methylation pattern in all somatic and germ line DNA, characterised by a hypomethylated 5^{1} region and a methylated 3^{1} end. This pattern may direct the organisation of an active chromatin structure, allowing immediate expression of these genes, even during the early stages of development. In contrast, tissue specific genes, which are fully methylated in sperm DNA would remain in an inactive conformation.

Hypomethylation of sperm DNA has been shown in several species; sperm DNA from bulls, sheep and pigs is found to be under methylated by about one-third in comparison with DNA from other species (Vanyushin et al, 1970). This largely reflects methylation of satellite DNA sequences: most satellite DNA in the sperm of cattle and mice is hypomethylated in comparison with DNA from somatic tissues (Adams et al, 1983). Satellite DNAs are often associated with the heterochromatin present around the centromeres of chromosomes (Kurnit et al, 1973) and may have some structural function in the chromosome. Satellite DNA in germ cells may be involved in the segregation of chromosomes and in meiotic recombination events (Lewin, 1983; Kaput et al, 1979; Bostock, 1979). Methylation of these sequences may inhibit such recombination events in somatic cells (Adams et al, 1983).

In many species, under-methylation of sperm DNA is slight, perhaps indicating differences in the process of spermatogenesis in different animals (Ehrlich et al, 1982). Ageing of cells also appears to influence the level of 5-mC in salmon DNA (Berdyshev, 1967) and in cattle (Romanov and Vanyushin, 1980).

The variation observed in the 5-mC content of DNA in mammalian tissues raises questions about the role of DNA methylation, in particular, the significance of 5-mC in the control of gene expression. It is possible that tissue specific methylation patterns are a secondary consequence of changes in gene expression.

Possible functions of DNA methylation and its role in

differentiation will be discussed in Section 1.2.4.

1.2.2 Sequence Specificity of DNA Methylation

Methylation of eukaryotic DNA occurs predominantly on the cytosine residue in the dinucleotide sequence CpG (Sinsheimer, 1954; Doskocil et al, 1962; Grippo et al, 1968). Between 50% and 90% of cytosines in this sequence are methylated in eukaryotes (Gruenbaum et al, 1982).

It has been known for some time that CpG is deficient in vertebrate genomes (Josse et al, 1961) occurring at only 25% of the expected frequency predicted from base composition analysis whereby pCG = [pC x pG]. In addition, the genomic distribution of CpG is non-random: approximately 1-2% of the vertebrate genome consists of unmethylated CpG rich stretches of DNA of about 1Kb in length. These localised sequences account for approximately 50% of all unmethylated CpG dinucleotides in the genome (Bird et al, 1985). This phenomenon is discussed further in Section 1.2.3. CpG suppression is less apparent in coding DNA sequences: gene sequences contain a significantly higher level of CpG than the rest of the genome, with 5' flanking regions enriched for CpG, while 3' flanking regions are often depleted (McClelland et al, 1982; Gerber-Huber et al, 1983).

Since CpG dinucleotides are deficient only in genomes which are partially or highly methylated (i.e. in the genomes of vertebrates and some invertebrates) (Salser, 1977) the phenomenon of CpG suppression may result from selection against 5-mC, possibly due to steric hindrance at the DNA helix level. Methylation of cytosine residues introduces a methyl group into the major groove

of the DNA helix which may result in structural changes in both DNA and chromatin (Nussiner, 1984). Mutation of 5-mC to thymine could also account for the loss of CpG dinucleotides from the genome: Deamination of 5-mC to thymine *in vitro* occurs only in single stranded regions of DNA, where the NH_2 group is not involved in hydrogen bonding (Adams and Eason, 1984). If this situation reflects events *in vivo* then deamination may occur less frequently in regions of high G+C content where "breathing" of DNA is restricted due to increased helical stablity.

If the CpG deficiency observed in vertebrates is due to the progressive mutation of 5-mC to thymine throughout evolution, the remaining CpG dinucleotide residues are presumably of importance in the DNA function. They may have been preserved as a result of their failure to be methylated (see below). The reason as to why specific genes retain very highly localised levels of unmethylated CpG dinucleotides, and the relationship between the methylation status and the activation state of a gene remains unclear.

Selective methylation of cytosines may influence the interaction of DNA sequences with regulatory molecules. The presence or absence of a methyl group protruding into the major groove of the helix is known to have a decisive effect on the binding of the lac repressor in E coli. The A:T nucleotide pair at position 13 of the binding site sequence is crucial: Mutation of T to U or C at this site substantially reduces the affinity of the lac repressor for its target sequence. When 5-mC was substituted for C at position 13, the stability of the repressor-operator complex was equivalent to that of

the wild-type, indicating that the presence of a methyl group at this position is essential for recognition of the operator sequence by the lac repressor protein (Fisher and Carruthers, 1979). Changes in the major groove are also known to affect the binding of restriction methylases, histones and hormone receptors to DNA (Lin et al, 1976; Yuan et al, 1970; Kallos et al, 1978).

Further, direct evidence that methylation can alter the binding of proteins to DNA comes from the footprinting studies by Becker et al (1987) which demonstrated that *in vitro* methylation of cloned DNA from the tyrosine aminotransferase gene of rat hepatoma cells completely abolishes the binding of at least one protein factor.

1.2.3 The Role of DNA Methylation in Gene Expression

Cell specific methylation patterns represent an additional level of information to the genetic message encoded in DNA. In eukaryotes, the function of this additional information remains unclear, although various hypotheses for the role of DNA methylation have been proposed. These include:

a) Methylation as a marker for the repair of mismatches in DNA (Meselson, 1975);

b) Methylation as a mechanism for developmental regulation (Holliday and Pugh, 1975);

c) Methylation as a regulator of chromosomal replication (Taylor, 1977).

In recent years, however, the most widely studied hypothesis has been the proposed role of methylation in the regulation of gene expression (Scarano, 1971; Riggs, 1975; Holliday and Pugh, 1975; Sager et al, 1975).

Methylation of cytosine residues introduces a methyl group into the major groove of the DNA helix (Razin and Riggs, 1980). This structural alteration may affect the protein-DNA interactions necessary for gene transcription. Keshet et al (1986) demonstrated that the presence of methyl groups in DNA can affect the chromatin structure adopted by genes following their introduction into cells by DNA mediated gene transfer. Thus methylation of DNA can result in a stable alteration in the local configuration of a gene.

Naveh-Many and Cedar (1981) have demonstrated that all active genes (as measured by DNase 1 sensitivity) from calf thymus, chicken erythrocyte and mouse L-cell DNA are undermethylated in comparison to the rest of the genome, suggesting that hypomethylation is a general phenomenon in actively transcribed genes. In addition, Razin and Riggs (1980) noted that certain tissue-specific genes are undermethylated only in the tissue in which the gene is expressed.

Tissue specific genes are nearly fully methylated in both male and female germ lines, and remain in this state during early development and in all non-expressing adult somatic tissues. Only in the specific tissue of expression is the gene found to be demethylated. In contrast, housekeeping genes which are constitutively expressed in all tissues generally contain unmethylated "islands" of CpG both in the germ line and in all somatic tissues (Bird, 1985). Many vertebrate genes (with the exception of some tissue specific genes) have been shown to contain HTF-like sequences in regions of transcriptional control.

The original function of methylation in invertebrates may have been to inactivate non-coding regions of the genome as no transcriptionally active sequences have been found in the methylated fraction of invertebrate DNA (Bird et al, 1980). The phenomenon of HTF islands in vertebrates may have arisen as the extent of DNA methylation and subsequent deamination of 5-mC to thymine spread throughout the vertebrate genome. It is possible that selective pressure to maintain the promotor region of housekeeping genes free from the repressive effects of methylation resulted in the

constitutive binding of specific factors to DNA, thus sterically excluding these regions from the action of DNA methylase. For housekeeping genes in particular, it is essential that initiation of transcription is not impeded. Holler et al (1988) proposed that the constitutive binding of the transcription factor Sp1 to its target sites prevents methylation of these regions. Binding sites for this transcription factor are commonly found in HTF islands, specifically in the promotor region of genes.

It may be necessary for HTF islands to remain free of methylation to allow expression of their associated genes; transcription of genes with HTF islands is inhibited when the island is methylated. For example, on the inactive X-chromosome, methylated HTF sequences have been found in the region of both the hypoxanthine phosphoribosyl transferase (HPRT) and glucose-6-phosphate dehydrogenase (G6PD) genes (Toniolo et al, 1984; Yen at al, 1984; Wolf et al, 1984).

The presence of unmethylated CpG dinucleotides, the common feature of HTF sequences, is thought to determine whether DNA sequences remain accessible to factors involved in the regulation of transcription. DNA-protein interactions may interfere with the process of methylation and lead to hypomethylation of certain gene sequences. Inactivation of a gene may lead to the displacement of bound factors (which normally prohibit methylation) from the associated island, thereby allowing access to DNA methylase. Methylation may therefore serve as a means of ensuring the continued inactivation of certain genes from one cell generation to the next.

The question remains as to whether demethylation of tissue specific genes occurs prior to transcriptional activation. Yisraeli et al (1986) demonstrated that methylation inhibits expression of the α-actin gene when it is introduced into fibroblasts, but when introduced into myoblasts the gene is expressed despite the presence of 5-methyl cytosine. Activation of tissue specific genes presumably involves recognition of these genes in the methylated state by specific transcription factors, and the simultaneous demethylation and transcription of these genes. Alternatively, the observation that many tissue-specific genes are unmethylated in their "target" tissue but methylated in non-expressing cells (Doerfler, 1983) may be due to the loss of methyl cytosine at a limited number of 5-mCpG dinucleotides *following* transcriptional activation. Thus under-methylation of these genes may be an effect, rather than a cause, of transcriptional activation.

The transcription of several genes can be inhibited by methylation (Felsenfeld, 1982). Busslinger et al (1983) demonstrated that *in vitro* methylation of the 5' end of a γ-globin gene rendered the gene inactive, following transformation into mouse cells, while transformation of a gene unmethylated at the 5' but methylated in the transcribed region resulted in expression of the γ-globin gene. Similarly, both the hamster adenine phosphoribosyl transferase (aprt) gene and the mouse dihydrofolate reductase (dhfr) gene are consistently found to be hypomethylated at the 5' end, although heavily methylated elsewhere in the gene. Both genes encode essential metabolic enzymes, and are therefore expected to be expressed in all

cells (Stein et al, 1983). *In vitro* methylation of the hamster aprt gene at all CCGG sites by Hpa II methylase inhibits the expression of this gene in mouse L cells (Stein et al, 1982). These results suggest that modification of the 5' end of genes by methylation may be a factor in the regulation of gene expression.

In contrast, Keshet et al (1985) showed that methylation of the coding sequences of the Herpes thymidine kinase gene can inhibit transcription of the gene in mouse fibroblasts, despite the fact that the promotor itself was not modified. This implies that methylation at regions outwith the promotor may also impede RNA synthesis, presumably by disrupting the binding or activity of essential trans-acting factors.

5 azacytidine (5-azaC), an analogue of cytosine, is a potent inhibitor of DNA methylase (Jones, 1984). Cells treated with 5-azaC have a greatly reduced level of 5-m Cytosine in their DNA, and treatment of certain cells with 5-aza C often results in the reactivation of hitherto transcriptionally inactive genes (Compere et al, 1981; Harris, 1982). Genes on the inactive X-chromosome have also been successfully reactivated by treatment with 5-azaC (Mohandas et al, 1981).

There is also evidence that genes which have been activated in cell lines can be subsequently inactivated by restoration of DNA methylation (Hickey et al, 1986; Clough et al, 1986; Flatau et al, 1984).

It is likely, however, that 5-azaC interferes with a

variety of general metabolic processes in the cell, and may exert effects on gene expression not only by interfering with the action of DNA methylase, but also by blocking the interaction of other controlling factors with DNA. It is possible that the level of methylation is adjusted as a consequence of this latter event.

While there is evidence to suggest that some 5-methyl cytosines are involved in the regulation of gene expression, it is clear from other studies that a change in the methylation status of a gene is not the sole factor involved. In some instances methyl groups may generate a local chromatin configuration rendering genes inaccessible to transcription factors, but the absence of methylation is not sufficient for transcription to occur.

It remains to be demonstrated whether DNA methylation is itself a causative factor in the regulation of gene expression or if it is merely the result of variations in gene activity. If methylation is shown to be actively involved in the regulation of transcription, this role is limited to certain organisms and possibly even to certain genes. It appears unlikely that 5-methyl cytosine was evolutionarily selected and retained in the genome in this capacity. The level of cytosine methylation is considerably lower in invertebrates than in vertebrates and no definitive example of a methylated gene in invertebrates has been found. Drosophila melanogastor DNA is not detectably methylated, yet cellular determination and differentiation are known to occur. Clearly factors other than methylation are involved in the regulation of these processes in invertebrates.

It seems likely that a series of factors will govern the control of gene expression. The level of this control may vary according to the degree of sophistication of gene regulation required by particular organisms. Methylation may be one of a number of factors involved in the regulation of certain vertebrate genes, perhaps acting to maintain the activation status of particular DNA sequences in differentiated cells. While regulation of gene expression by selective transcription is one potential point of control, it is possible that further regulation of gene expression occurs at a later stage, notably in the processing of precursor RNA to mature messenger RNA.

1.2.4 DNA Methylation and Differentiation

The differentiation of cells deriving from a single zygote into the highly specialised arrangement of cells and tissues of higher eukaryotes is thought to involve a process of determination or commitment, when the cell is set on a particular developmental pathway. The controls which govern the differentiation of cells are likely to involve changes at the level of the genome. There is some evidence to suggest that changes in the methylation pattern of DNA occur during the process of cellular differentiation, demonstrated by tissue specific differences in the 5-methyl Cytosine content of DNA (Gama-Sosa et al, 1983; Sano et al, 1982) and the demethylation observed at specific sites around active genes (Bird, 1978).

Razin et al (1984) investigated the relationship between variations in the levels of DNA methylation and mouse cell differentiation. These workers found that mouse teratocarcinoma cells induced to differentiate *in vitro* undergo substantial demethylation, losing 30% of methyl moieties following treatment with a variety of inducers. More recently (Razin et al, 1988) it was shown that *in vitro* induction of differentiation in Murine erythroleukaemia cells (MEL cells) results in a transient genome-wide demethylation, followed by remethylation of the DNA. As much as 60% of the 5-mC content of the DNA was replaced by Cytosine 10-18 hours after the induction of differentiation, with remethylation completed by 24 hours after induction. It was proposed that changes in the methylation pattern may be involved in the commitment of cells to differentiate and that erasure of the pre-existing methylation

pattern would enable the establishment of a new methylation pattern, characteristic of the next stage of differentiation. The transient replacement of 5-mC by cytosine in the DNA of both MEL cells and F9 teratocarcinoma cells (Razin et al, 1984; Bestor et al, 1984) strongly implies that the methylation pattern of DNA in induced cells is an important factor in the differentiation process.

Chemical inhibition of methylation by 3-deaza-adenosyl homocysteine (DZA Hcy) was found to inhibit differentiation (Sherman et al, 1985; Razin et al 1988) and also completely inhibited the hypomethylation of MEL cells.

Events during early mouse development also indicate that variations in the pattern of methylation occur during the early stages of development. Razin et al (1984) observed a decrease in the level of methylation in the yolk sac and placenta of embryonic mice, while the DNA of the embryo itself was found to become *de novo* methylated. Such changes in the methylation pattern of DNA may be linked to the differentiation mechanism of cells.

Hypomethylation may be a necessary step in the commitment of these cells to differentiate, but it is unlikely that hypomethylation of DNA is itself sufficient for terminal differentiation to occur. A series of events, such as the binding of various tissue specific trans-acting factors to DNA are likely to control the expression of genes in cells undergoing differentiation.

1.3 Eukaryotic DNA Methylases

1.3.1 Properties of Eukaryotic Methylases

The enzymatic transfer of methyl groups from S-adenosyl-L methionine to residues in eukaryotic DNA was first demonstrated by Burdon et al (1967) using a crude preparation from Mouse Krebs II ascites cells. Since then mammalian DNA methyltransferases have been purified from a number of tissues including rat spleen (Kalonsek and Morris, 1969), rat liver (Morris and Pih, 1971; Simon et al, 1978), Hela cells (Roy and Weissbach, 1975), mouse ascites cells (Turnbull and Adams, 1976), Bovine Thymus (Sano et al, 1985), mouse erythroleukaemic cells (Bestor and Ingram, 1983) and human placenta (Pfeifer et al, 1983). The molecular wt of the native enzyme is reported in the range 120,000 - 280,000 and may vary in different vertebrate tissues. The enzyme isolated from mouse ascites cells has a molecular wt in the region of 180,000. With the exception of the bovine thymus enzyme, mammalian methylase activity is found in the cell nucleus. Adams and Davis (1985) have reported the presence of two methylase fractions in a cell homogenate. One fraction consists of "soluble" enzyme which is only loosely associated with the nucleus and can be extracted with dilute salt solutions. This fraction accounts for 70-80% of the cell's methylase activity, and the isolated enzyme is capable of both maintenance and *de novo* methylation. The remaining enzyme activity stays firmly bound in the nucleus, even under 2M NaCl extraction conditions. The bound form of the enzyme may be associated with a nuclear matrix-like structure near the replication fork of DNA (Burdon et al, 1985). Bound enzyme has been shown to act predominantly on cytosine residues

in the nascent strand of a DNA duplex following replication, and thus appears to function mainly as a maintenance enzyme.

When solubilised, the properties of the bound form of DNA methylase are similar to those of the soluble enzyme fraction, with regard to substrate requirement, ionic requirement and pH optima (Burdon et al, 1985) and it is likely that the two fractions represent the same catalytic molecule. *In vivo*, the soluble enzyme is thought to become firmly, though transiently, bound with DNA in the nuclear matrix structure, being released again only after methylation has taken place. The soluble fraction may simply represent a circulating pool of enzyme available for attachment at sites within the nucleus, or it may have a more individual role in the cell, possibly in the methylation of repaired stretches of DNA (Kastan et al, 1982).

1.3.2 Substrate Specificity and Requirements

DNA methylases isolated from eukaryotes have no co-factor requirement, and are in fact inhibited by most divalent cations with the exception of Mg^{2+} and Ca^{2+} (Adams and Burdon, 1983). Enzyme activity is enhanced in the presence of chelating agents. The preferred substrate for known eukaryotic DNA methylases is the unmethylated strand in a hemi-methylated duplex, where the methylation rate is 10-30 X greater than that of *de novo* methylation. In low salt, enzyme molecules are known to aggregate. This condition also allows the DNA duplex to "breathe", that is, single stranded regions become available. It is thought that only under these conditions can DNA methylase bind and interact with

DNA (Adams, 1979). This is supported by EM data of Pfeifer et al (1985) which indicates that mouse DNA methylase preferentially binds to single stranded regions of DNA. *In vivo*, such regions will occur at the sites of DNA replication and excision repair.

Proteins associated with DNA *in vivo* may interfere with the ability of the enzyme to modify CpG sites. In isolated nuclei, methyl groups are preferentially added to DNA which is susceptible to digestion with micrococcal nuclease (Razin and Cedar, 1977) implying that DNA binding proteins may inhibit the action of DNA methylase. This was confirmed by Kautiainen and Jones (1985). Studying the effects of DNA binding proteins on methylation they demonstrated that hemi-methylated CpG sites which are readily methylated in purified DNA may remain unmethylated when DNA is associated with chromatin proteins. Histones became associated with DNA shortly after synthesis, forming nucleosomal structures: Core histones are deposited within ten minutes and linker histones ten to twenty minutes after replication, an association known to inhibit methylation (Davis et al 1986). This effect is probably due to a close association of protein with the methyl acceptor site in DNA.

1.3.3 Methylation and DNA Synthesis

Although initiation of methylation occurs shortly after DNA synthesis, a period of several hours may elapse before methylation of the nascent DNA is complete (Burdon et al, 1969; Evans et al, 1973; Adams, 1971; Adams, 1974; Geraci et al, 1974; Theiss et al, 1978; Kiryanov et al, 1980).

Woodcock et al (1983) demonstrated that in several mammalian

cell lines this "delayed" methylation is completed prior to the next S-phase and initiation of a new round of DNA replication - presumably essential to avoid loss of methylation patterns.

A short lag of about one minute between synthesis and methylation (reported by Kappler, 1970 and Gruenbaum et al, 1983) may be due to the ligation of Okazaki fragments in the nascent DNA strand prior to methylation (Adams, 1974; Hotta and Hecht, 1971; Drahorsky and Wacker, 1975). One cause of the longer delay in the completion of methylation *in vivo* is almost certainly due to the association of proteins, such as histones, with DNA. Such interactions interfere with the action of DNA methylase, resulting in the persistence of hemi-methylated sites in DNA for several hours after replication. Woodcock et al (1984) suggested that two different enzyme activities are responsible for immediate and delayed methylation, citing different sensitivities of "immediate" and "delayed" methylase to inhibitors.

Since no distinct differences have been found between DNA sequences methylated immediately or after a delay (Woodcock et al, 1984) it seems likely that the second enzyme would act as an accessory to the first, restoring symmetry to remaining hemi-methylated sites following passage of the replication fork and the binding of chromatin proteins; Some time may elapse before methylase has access to all hemi-methylated sites in nucleosomal DNA.

Davis et al (1985) showed that nuclear matrix-associated DNA methylase (see Section 1.3.1) was responsible for delayed

methylation in mammalian nuclei. This enzyme fraction may also be responsible for delayed methylation *in vivo*.

1.3.4 Establishment and Maintenance of Methylation Patterns

The methylation patterns in mammalian cell DNA are heritable in a tissue and species specific manner (Bird, 1984; Doerfler, 1983; Jaenisch et al, 1984; Razin et al, 1984). The methylation patterns within cellular DNA are believed to be established during embryogenesis, and are subsequently maintained in a stable state throughout somatic cell division.

As described in Section 1.3.2 the preferred substrate for known eukaryotic methylases is the unmethylated strand in a hemi-methylated duplex, where the rate of methylation is 10-30 X that of *de novo* methylation (Gruenbaum et al, 1982; Bolden et al, 1984).

De novo and Maintenance Methylation

De novo methylation - methylation of symmetrical unmethylated CpG dinucleotides has been shown to occur in early mammalian embryos (Jahner et al, 1982; Brinster et al, 1983). As described in Section 1.2.1, it is highly unlikely that methylation of CpG sites is random - it appears that the enzyme preferentially methylates certain CpG sites during *de novo* methylation (Pfeifer, 1985). The function of this modification in undifferentiated cells may be to selectively control gene expression (Riggs and Jones, 1983). Alternatively, *de novo* methylation may be a secondary consequence of gene inactivation (by some other means) and may function to imprint the inactivity of these sequences throughout

successive cell generations (Bird, 1986). Inhibition of *de novo* methylase activity is observed upon prolonged incubation of the enzyme with high concentrations (200-300 ug/ml) of unmethylated double stranded DNA (Palitti et al, 1987). The inactivation process appears to be the result of a progressive interaction of the enzyme with DNA, independent of the presence of S-adenosyl-L-methionine, suggesting that *de novo* methylating activity could be regulated by DNA itself.

Maintenance methylation, the continuance of pre-existing methylation patterns in DNA following DNA synthesis, is probably essential for maintaining phenotypic stability of differentiated cells (Doerfler, 1983; Razin and Riggs, 1980). Holliday and Pugh (1975) and Riggs (1975) suggested that methyl groups on cytosine residues are inherited in a semi-conservative fashion following DNA synthesis. The combination of the symmetrical placement of methylated residues on the DNA duplex coupled with an enzyme acting preferentially at hemi-methylated sites (confirmed by Adams et al, 1979) would facilitate the stable inheritance of the methylation pattern following DNA replication. The totality of this theory has been challenged more recently by Bolden et al (1987) whose results suggest that the placement of methyl moieties on cytosines in the daughter strand of the duplex following DNA synthesis is at least to some extent determined by the primary sequence of this target strand, and is not solely determined by the methylation pattern in the complementary parent strand.

Most somatic cell methylation is believed to be carried

out by a maintenance enzyme (Gruenbaum et al, 1982). However, there is no direct evidence for the existence of distinct *de novo* and maintenance methylase activities, since all DNA methylases isolated to date exhibit both activities, and it is believed that one enzyme species performs both reactions (Pfeifer et al, 1983; Grunwald et al, 1984). Bestor and Ingram (1983) demonstrated that the ratio of maintenance : *de novo* enzyme remains unchanged during purification, while both enzyme activities were shown to be inhibitied by the same monoclonal antibody (Drahovsky, 1984).

The dual functions presumably result from modification of enzyme activity, either indirectly by the influence of cellular factors, or by a change in the nature of the enzyme itself, possibly a structural modification, influencing its activity on different DNA substrates. Such modulations may be essential to enable site specific *de novo* methylation to occur during the establishment of methylation patterns in differentiating cells, and prevent inappropriate *de novo* methylating events which would interfere with the existing tissue specific methylation pattern in the cell, whilst allowing the stable inheritance of methylation patterns from one somatic cell generation to the next.

1.4 Structure and Mode of Action of DNA Methylase

1.4.1 Preliminary Studies on DNA Methylase

Total purification of DNA methylase has proved difficult due to the low levels of enzyme found in cells, and the instability of the enzyme *in vitro*: DNA methylase immunoprecipitated from two human cell lines indicated that the enzyme represents approximately 0.002% of the total protein (Pfeifer et al, 1986), while Adams et al (1986) found that enzyme incubated in the absence of substrate for six hours at 37°C subsequently loses 62% of its activity on single stranded DNA and 39% of its activity on a native DNA substrate. Pfeifer et al (1986) demonstrated that affinity purified DNA methylase from mouse P815 mastocytoma cells was liable to proteolytic degradation when incubated at 20°C for 16 hours: following SDS polyacrylamide gel electrophoresis the 190 kD native enzyme was found to be converted to smaller polypeptides of 175 kD, 150 kD, 130 kD and 115 kD. It is not clear whether this decomposition is due to the presence of endogenous proteinases in the enzyme preparation or if the enzyme itself is capable of autocatalysis. The same authors have shown that the composition of the purified protein is to some extent dependent on the purification procedure employed. The lower molecular mass polypeptides may still be enzymatically active as no substantial loss of enzyme activity is reported. Another possibility is that the "cleavage" event merely involves the introduction of nicks at various sites within the enzyme, which do not cause dissociation of the enzyme *in vitro*, but which are sufficient to cause it to dissociate upon SDS gel electrophoresis.

Some form of enzyme cleavage, either by autocatalysis

or by the action of a specific proteinase may occur *in vivo* as a means of modulating the activity of DNA methylase on different substrates. For example, while maintenance and *de novo* methylation are believed to be carried out by the same enzyme, some structural modification may be necessary to facilitate methylation at CpG dinucleotides without recognition of 5-mC on the opposite strand.

1.4.2 Factors Influencing the Binding of DNA Methylase to DNA and the Rate of the Methylation Reaction

While the initial binding of DNA methylase to a DNA polymer does not require the presence of methylatable cytosines (Drahovsky et al, 1972; Bolden et al, 1984) it is likely that flanking sequences will influence the binding and catalytic action of the enzyme. The number and spacing of CpG sites are likely to be important factors, as is the length of the DNA polymer itself. Pfeifer et al (1985) found that a minimum length of 90 base pairs of DNA was required for the binding reaction of affinity purified human DNA methylase, although binding was apparently independent of the nucleotide composition of the DNA and the presence of S-adenosyl-L-methionine.

The rate of methylation on native DNA is likely to be influenced by the number of CpG sites available for methylation. In higher eukaryotes, DNA is deficient for CpG and a large proportion of the CpG sites present are already methylated. Km values of 30 to 70 mg/ml have been found for the native DNA of higher eukaryotes in comparison with the much lower Km values (0.5 to 2.5 ug/ml) found for native unmethylated bacterial DNA

and insect DNA.

In higher eukaryotes, even under conditions of a vast excess of enzyme to DNA (one enzyme molecule for twelve base pairs of DNA) over forty hours elapses before completion of the methylation reaction, using M. luteus DNA as a substrate. This lag may be due to an initial failure to methylate at certain sites by the enzyme, perhaps as a result of the proximity of CpG sites and the inability of the enzyme to recognize or gain access to all available sites.

1.4.3 Models for DNA Methylase Structure and the Enzymatic Methylation of DNA

Due to the difficulties of isolating sufficient quantities of purified DNA methylase, details of the structure and mode of catalysis of the enzyme are still speculative. It is presumed that DNA methylase will have domains for the recognition of paired unmethylated CpG dinucleotides as well as domains for the binding of S-adenosyl-L-methionine and DNA.

An early model for the enzymatic methylation of DNA by Drahovsky and Morris (1971) envisaged the enzyme binding to a DNA molecule at or near one end and "walking" along the molecule, methylating sites as it encountered them. The results of Turnbull and Adams (1976) disputed this as a plausable mechanism, finding that mouse ascites DNA methylase binds loosely and reversibly to DNA molecules, methylating only when it binds to CpG sites. More recently Burdon and Adams (1983) surmised that DNA methylase can

travel along a DNA polymer, dissociating when it reaches the end of the molecule and also perhaps at certain randomly positioned sequences such as fully methylated CpG dinucleotides.

Speiss et al (1988) analysed the structure of DNA methylase purified from mouse P815 mastocytoma cells by electron microscopy. The resulting schematic model depicted a hemi-elliptical globular structure bearing a small projection. It has been proposed that the globular mass carries the methylation centre of the enzyme with the appendix perhaps representing the CpG recognition site, although further work is required before definitive functional roles can be assigned to the morphological domains identified so far.

1.4.4 Cloning of Mammalian DNA Methylase

Bestor et al (1988) have recently cloned and sequenced a cDNA coding for DNA methylase from Friend erythroleukaemia cells.

The protein was found to consist of a 1000 amino acid N-terminal region and a 570 amino acid C-terminal region. The latter was shown to bear significant homology to bacterial type II restriction methyltransferases, suggesting a common ancestry of mammalian and bacterial methyltransferases; this region is proposed to contain the catalytic centre of the enzyme, represented by the sequence motif Pro Cys X_6 Gly.

No enzymatic function has yet been assigned to the N-terminal region. This domain contains a cysteine-rich region characteristic of the consensus sequence for metal binding "fingers" found in other nucleic acid binding proteins (Evans

et al, 1988), although it remains to be established whether the N-terminal region does bind metal ions. Proof of a metal binding capacity may indicate a role for the N-terminal domain in the regulation of enzymatic methylation by the C-terminal domain.

1.5 Methods and Approaches to Studying DNA Methylase

DNA methylase activity is predominantly present in rapidly dividing cells, with negligible activity detected in stationary cells. The enzyme, however, represents only a very small proportion (approximately 0.002%) of the total cell protein (Pfeifer et al, 1986) and is in addition extremely heat labile and prone to degradation during purification. Thus considerable difficulties have been encountered in attempts to purify DNA methylase to homogeneity, and in the study of the enzyme *in vivo*. Simple extraction and assay of DNA methylase from cells often provides little information as synthetic DNA substrates may prove ineffective in reflecting the *in vivo* activity of the enzyme.

In order to study DNA methylase itself and the effect of methylation on DNA, both purified enzyme and a means of precise detection of enzyme *in vitro* and *in vivo* are required. The use of monoclonal and polyclonal antibodies directed against epitodes specific to DNA methylase used in conjunction with immunodetection techniques should provide a means of identifying DNA methylase activity in cells, from enzyme synthesis to degradation.

To maximise enzyme yields, the purification strategy requires the use of selected purification procedures, chosen for rapid and efficient recovery of enzyme with minimal loss of enzyme activity. The recovery of purified, active DNA methylase is essential for *in vitro* analysis of size, reactivity and stability of the enzyme.

The application of these techniques should provide us with a greater understanding of both DNA methylase and the methylation reaction, and of the effect of DNA methylation on cellular development.

CHAPTER 2 : MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

General chemicals used were of analytical grade and were supplied by The British Drug House, Dorset, UK. Chemicals obtained from other sources are listed below:

Sigma Chemical Co Ltd, Dorset, UK

Aminopterin, Bovine serum albumin, Coomassie Blue (G), Ethidium bromide, Hypoxanthine, α_2-macroglobulin, o-Phenylene diamine, Phosphorylase B, Phenyl methyl sulphonyl fluoride, Thymidine, Trypsin, Trypsin inhibitor, Tween 20 (Polyoxyethylene sorbitan monolaureate), Tween 80 (Polyoxyethylene sorbitan mono oleate).

Koch-Light Laboratories, Bucks, England, UK

m-Cresol, Dimethyl sulphoxide, Triton X-100.

FSA Supplies, Loughborough, England UK

Acrylamide, N,N^{1}methylene bisacrylamide, Sodium dodecyl sulphate.

Boehringer-Mannheim, East Sussex, UK

Dithiothreitol, Polyethylene glycol 1500, Tris (hydroxymethyl) methylamine.

Amersham International PLC, England, UK

Biotin-Streptavidin detection system, Hybond-C hybridisation transfer membrane.

Miscellaneous

Ammonium sulphate was supplied by Fisons PLC, Loughborough, UK; Ecoscint scintillation fluid was supplied by National Diagnostics, New Jersey, USA; Salicylic acid was supplied by Aldrich Chemical Company Ltd, Dorset, UK; Heat inactivated horse serum was from Gibco, Paisley, Scotland; Pansorbin was supplied by Behring Diagnostics, La Jolla, California, USA; Freund's adjuvant was from Difco Laboratories, Detroit, USA; Fuji Rx x-ray film was from Fuji Photo Film Co Ltd, Tokyo, Japan; Millex-GS filter units were supplied by Millipore SA, France; Whatman filter paper was from H. Reeve-Angel & Co Ltd, London, UK.

2.1.2 Nucleic Acids

Micrococcus luteus DNA

ΦX174 RF DNA

Salmon testes DNA

E. coli transfer RNA

were supplied by Sigma Chemical Co, Poole, Dorset, UK.

2.1.3 Restriction Enzymes

Restriction enzymes and REact buffers were obtained from Bethesda Research Laboratories, Cambridge, UK.

2.1.4 Radiochemicals

S-adenosyl-L-[Methyl ^{3}H] methionine (^{3}H Ado Met) was obtained from the Radiochemical Centre, Amersham, England. ^{125}I-NaI was supplied by the Western Infirmary, Glasgow University, Scotland.

2.1.5 Chromatography Media

CN Br Sepharose

Heparin Sepharose

Sephacryl S300

Sephadex G50

QAE Sephadex A50

were supplied by Pharmacia Ltd, Milton Keynes, UK.

Cellulose Phosphate P11

was supplied by Whatman Biosystems Ltd, Maidstone, Kent, UK.

2.1.6 Cell Culture Materials

RPMI 1640, Glasgow MEM, MEM without L-methionine, Foetal calf serum, Newborn calf serum, L-Glutamine, Non-essential amino acids, Sodium bicarbonate were supplied by Gibco Ltd, Paisley, Scotland.

2.1.7 Cell Lines

The rat myeloma cell line Y3.Ag.1.2.3 was obtained from the MRC Laboratory of Molecular Biology, Cambridge, UK. (Galfre et al, 1979)

2.1.8 Animals

DA rats were propagated in the animal house of Glasgow University, Biochemistry Department.

2.1.9 Disposable Plasticware

96 well microtitre ELISA plates were supplied by Dynatech

Laboratories Ltd, Sussex, UK.

Tissue culture plates (96 well and 24 well) and Biofreeze vials were supplied by Costar, Northumbria Biologicals Ltd, Northumbria, UK.

Tissue culture flasks (25 cm^2, 75 cm^2 and 125 cm^2) were supplied by Corning Glass Works, New York, USA.

Petridishes and sterile syringes were supplied by Becton Dickinson and Co Dublin, Eire.

Centrifuge tubes were supplied by Elkay products Inc, MA, USA.

2.1.10 Solutions

2.1.10.1 SDS Mix

1% (w/v) SDS

2 mM EDTA

5% (v/v) Butanol

2.1.10.2 Stopper

3 g p-amino salicylic acid

0.125 M Nacl

25 mg salmon testes DNA

50 ml SDS mix (see section 2.1.10.1)

Adjust volume to 100 ml with dH_2O

2.1.10.3 Phenol Mix

88% (v/v) phenol

12% (v/v) m-Cresol

0.1% (w/v) 8-hydroxyquinoline.

2.1.10.4 Bradford's Reagent

100 mg Coomassie Brilliant Blue G was dissolved in a 50 ml solution of 95% ethanol in dH_2O. 100 ml of 85% (w/v) phosphoric acid was added, and the solution made to one litre with dH_2O. The solution was filtered on Whatman No 1 filter paper and stored in the dark.

2.1.10.5 Ado [^{3}H] Met Stock Solution

25µCi TRA (0.5µCi/µl)

300µCi TRK (1 µCi/µl)

2650µl dH_2O

2.1.10.6 Preparation of HT and HAT Medium

100 x HT Stock solution containing 10 mM hypoxanthine and 1.6 mM thymidine, was prepared by dissolving 1.36 mg hypoxanthine plus 39 mg thymidine in dH_2O to a final volume of 100 ml. The solution was heated to 60°C to dissolve the hypoxanthine and filter sterilised.

100 X Aminopterin stock (4×10^{-5}M) was prepared separately to a final concentration of 1.8 mg aminopterin/100 ml dH_2O. The solution was filter sterilised and stored in the dark at -20°C.

50 X HAT stock was prepared by mixing equal volumes of stock HT and Aminopterin. The solution was aliquoted in small volumes and stored at -20°C in the dark.

HT and HAT medium were diluted in complete medium before dispensing to cells on microtitre plates.

2.1.10.7 Cell Culture Solutions

2.1.10.7.1 Complete Medium

RPMl 1640

2 mM L-Glutamine

1% (v/v) non-essential amino acids

Supplemented with 10% (v/v) or 20% (v/v) foetal calf serum.

2.1.10.7.2 Serum-Free Medium

RPMl 1640

2 mM L-Glutamine

1% (v/v) non-essential amino acids

The composition of RPMl 1640 is shown in Table 1.

The composition of non-essential amino acids is shown in Table 2.

2.1.10.7.3 EC 10 Medium

450 ml dH_2O

50 ml Glasgow (MEM) 10 X conc

10% (v/v) newborn calf serum

2 mM L-Glutamine

25% (v/v) sodium bicarbonate

2.1.10.7.4 Minus Methionine Medium

MEM minus L-methionine medium

10% (v/v) newborn calf serum

2.1.11 Buffers

2.1.11.1 Buffer M

50 mM tris HCl pH 7.8

1mM EDTA

1 mM DTT

0.01% (w/v) NaN_3

10% (v/v) glycerol

6 mg/ml PMSF in ethanol was added to buffer M at 1% (v/v) immediately before use.

2.1.11.2 Phosphate Buffered Saline

PBS A : 10 g/l NaCl

0.25 g/l KCl

1.44 g/l Na_2HPO_4

0.25 g/l KH_2PO_4

PBS B : 1 g/l $CaCl_2$

PBS C : 1 g/l $MgCl_2dH_2O$

Phosphate buffered saline was prepared as three separate solutions (A, B and C) and mixed in the ratio 8:1:1 before use.

2.1.11.3 Immunoblotting Buffer

20 mM tris HCl pH 7.2

0.15 M NaCl

0.5% (v/v) Tween 20

0.5 mg/ml NaN_3

2.1.11.4 ELISA Buffers

PBS	)	Antibody Diluent
0.5 mg/ml BSA	)	
PBS	)	Blocking Buffer
10 mg/ml BSA	)	
PBS	)	Wash Buffer
0.1% (v/v) Tween 20	)	
PBS	)	Conjugate Diluent
1% (w/v) BSA	)	

Citrate Phosphate Buffer (McILVAINE'S BUFFER)

Solution A : 0.1 M citric acid

Solution B : 0.2 M Na_2HPO_4 12 H_2O

17.9 ml of solution A was added to 32.1 ml of solution B and the pH adjusted to 6.0 with HCl. The solution was made to a final volume of 100 ml with dH_2O.

OPD Solution (Developer)

4 mg OPD

0.01% H_2O_2

9 ml citrate phosphate buffer

4N H_2SO_4 to stop the reaction

2.1.11.5 Immunoprecipitation Buffers

TKM Buffer

100 mM tris

100 mM KCl

5 mM $MgCl_2$

5N HCl to pH 8.2

TKM/Triton X-100 Buffer

100 mM tris

5N HCl to pH 8.2

100 mM KCl

5 mM $MgCl_2$

1% (v/v) Triton X-100

1% (w/v) sodium deoxycholate

0.5% (w/v) SDS

2.1.12 Electrophoresis Solutions and Buffers

2.1.12.1 Polyacrylamide Gel Buffers

Resolving Gel Buffer

1.5 M tris

0.4% SDS

5N HCl to pH 8.8

Stacking Gel Buffer

500 mM tris

0.4% SDS

5N HCl to pH 6.8

Acrylamide Stock Solution

30% (w/v) acrylamide

0.8% (w/v) N,N' - methylene bisacrylamide

Ammonium Persulphate Stock Solution

10% (w/v) ammonium persulphate in dH_2O

SDS Sample Buffer

0.1875 M tris HCl pH 6.8

6% (w/v) SDS

30% (v/v) glycerol

15% (v/v) 2-mercaptoethanol

0.003% (w/v) bromophenol blue

Reservoir Buffer

25 mM tris

0.19 M glycine

0.1% (w/v) SDS

2.1.12.2 Agarose Gel Buffers

Reservoir Buffer

50 mM tris pH 8.3

400 mM boric acid

5 mM EDTA

Sample Buffer

25% (v/v) glycerol

2% (w/v) SDS

0.025% (w/v) bromophenol blue

2.1.12.3 Immunoblot Transfer Buffer

25 mM tris pH 8.3

0.192 M glycine

20% (v/v) methanol

0.02% (w/v) SDS

2.1.13 Stains and Destains

Coomassie Stain

0.1% (w/v) Coomassie Blue G

50% (v/v) methanol

10% (v/v) acetic acid

Destain

10% methanol

10% acetic acid.

Amido Black Stain

0.1% (w/v) Amido Black

45% (v/v) methanol

10% (v/v) acetic acid

Destain

45% (v/v) methanol

10% (v/v) acetic acid

TABLE 1 : COMPOSITION OF RPMI 1640

	mg/litre
$Ca(NO_3)_2$	69.49
KCl	400.0
$MgSO_4$ $7H_2O$	100.0
NaCl	6000
$NaHCO_3$	2000
Na_2HPO_4	800.7
Glucose	2000
Sodium phenol red	5.00
L-arginine Hcl	200.0
L-asparagine H_2O	56.82
L-aspartic acid	20.00
L-cysteine disodium salt	59.15
L-glutamic acid	20.00
L-glutamine	300.0
Glutathione	1.00
Glycine	10.00
L-histidine HCl H_2O	15.00
L-hydroxyproline	20.00
L-isoleucine	50.00
L-leucine	50.00
L-lysine HCl	40.00
L-methionine	15.00
L-phenylalanine	15.00
L-proline	20.00
L-serine	30.00
L-threonine	20.00
L-tryptophan	5.00
L-tyrosine	20.00
L-valine	20.00
Biotin	0.20
D-Ca pantothenate	0.25
Choline chloride	3.00
Folic acid	1.00
I-Inositol	35.00
Nicotinamide	1.00
p-aminobenzoic acid	1.00
Pyridoxal HCl	1.00
Riboflavin	0.20
Thiamin HCl	1.00
Vitamin B12	0.005

TABLE 2 : COMPOSITION OF NON-ESSENTIAL AMINO ACIDS

	mg/litre
L-alanine	8.9
L-asparagine H_2O	15.0
L-aspartic acid	13.3
Glycine	7.5
L-glutamic acid	14.7
L-proline	11.5
L-serine	10.5

2.2 Methods

2.2.1 Purification of DNA Methylase from Mouse Krebs II Ascites Tumour Cells

2.2.1.1 Propagation of Krebs II Ascites Cells in Mice

Krebs II ascites cells were propagated and maintained in Porton mice by the method of Martin et al (1961). The cells were harvested after seven days of growth as described by Turnbull and Adams (1976).

2.2.1.2 Preparation of Nuclei from Krebs II Ascites Cells

Nuclei were prepared from mouse Krebs II ascites cells by the method of Turnbull and Adams (1976) and stored at -70°C before use. All purification procedures were carried out at 4°C.

2.2.1.3 Extraction of DNA Methylase from Nuclei

Frozen nuclei were thawed quickly at 37°C and removed to 4°C. The nuclei were stirred with an equal volume of buffer M until clumps had dispersed, followed by two volumes of buffer M containing 0.4 M NaCl. The nuclei were stirred for ten minutes and centrifuged at 1000 g, for 15 min. The supernatant was removed and the pellet stirred with a further 1 volume of buffer M and 1 volume of buffer M containing 0.4 M NaCl. The nuclei were centrifuged as above, and the supernatants from the two spins pooled (Fraction II).

2.2.1.4 Preparation of Phosphocellulose

Phosphocellulose P11 cation exchange matrix was swollen according to manufacturer's instructions, then stirred with buffer M containing 1 mg/ml BSA. The matrix was washed in buffer M, then in buffer M containing 0.6 M NaCl until the E280 value was $\leq$ 0.01. The phosphocellulose was re-suspended in an equal volume of buffer M containing 0.2 M NaCl to give a 50% (w/v) phosphocellulose suspension and stored at 4°C.

2.2.1.5 Phosphocellulose Chromatography

Fraction II was absorbed onto phosphocellulose in buffer M containing 0.2 M NaCl. 12 ml of 50% (w/v) phosphocellulose suspension was used per 100 ml fraction II. The mixture was stirred for 20 min at 4°C and centrifuged at 1000 g for 10 min. The pellet was washed twice in buffer M containing 0.2 M NaCl, and the enzyme eluted batchwise with buffer M containing 0.5 M NaCl (Fraction III).

2.2.1.6 Ammonium Sulphate Precipitation

Fraction III was made 50% saturated with respect to ammonium sulphate. The solution was stirred for a further 20 min and centrifuged at 14000 g for 20 min. The supernatant was discarded.

2.2.1.7 Gel Filtration on Sephacryl S300

The pellet was re-dissolved in 10 ml of buffer M and applied to a column of Sephacryl S300 equilibrated with the same buffer. The enzyme was eluted with buffer M containing 0.2 M NaCl. Fractions eluting at 1.15 - 1.6 X void volume were pooled (Fraction

IV) and made to 70% saturation with ammonium sulphate. The solution was stirred and centrifuged as described above.

2.2.1.8 Preparation of tRNA Sepharose

3g of CN-Br Sepharose was swollen and washed by a method recommended by the manufacturer. 10 mg of E. coli tRNA was dissolved in a buffer containing 0.1 M $NaHCO_3$/0.5 M NaCl pH 8.3, and mixed with the gel for 12 h at 4°C. Excess ligand was removed by washing with the same buffer, and remaining active groups were blocked using 0.1 M tris HCl pH8. The gel was then washed with three cycles of alternating pH using:

(a) 0.1 M sodium acetate
0.5 M NaCl pH4

(b) 0.1 M tris
0.5 M NaCl pH8

2.2.1.9 Affinity Chromatography on tRNA Sepharose

The pellet obtained in Section 2.2.1.7 was redissolved in 1 ml of buffer M and desalted on a 19 ml Sephadex G50 column. The non-retarded fraction was bound directly onto a 10 ml tRNA Sepharose column, and the enzyme eluted with buffer M containing 0.2 M NaCl (Fraction V).

2.2.1.10 Assay of DNA Methylase Activity

2.2.1.10.1 Preparation of Denatured M. luteus DNA

M. luteus DNA was dissolved in 50 mM KCl to a final concentration of 0.5 mg/ml, and heated to 100°C for 10 min. The solution was cooled on ice and stored at -20°C.

2.2.1.10.2 DNA Methylase Assay

DNA methylase activity was assayed in a final volume of 70 ml containing 5 mg M. luteus DNA, 3.3 µM S-adenosyl L-[Me-H^3] methionine, 50 mg BSA and enzyme in buffer M. When denatured M. luteus DNA was used as the substrate, 100 mM NaCl was also included. The reaction mixture was incubated at 37°C for 1 h and the reaction stopped by addition of 0.5 ml of stopper solution (2.1.10.2). Protein was removed by treatment with phenol containing 8-hydroxyquinoline and m-cresol, and the DNA was isolated by precipitation with two volumes of ethanol. RNA was removed by incubation with 50µl 0.3 M NaOH, at 37°C for 1 h. The DNA was precipitated onto 3 mm Whatman filter squares using 5% (v/v) trichloroacetic acid in which the filters were washed four times. The filters were then washed in methylated spirits, dried in ether, placed in scintillation vials and incubated with 0.5 ml of 0.5 N perchloric acid at 60°C for 30 min. 5 ml of Ecoscint scintillation fluid was added and the samples counted on a Beckman scintillation counter.

One unit of activity is defined as the amount required to catalyse the incorporation of one pmol of methyl groups into

M. luteus DNA in 1 h at 37°C under standard assay conditions.

2.2.1.10.3 Measurement of Protein

The protein content of samples was assayed by the method of Bradford (1976) using bovine serum albumin as a standard.

2.2.1.11 Polyacrylamide Gel Electrophoresis

8.75% (w/v) Resolving Gel

10 ml stock buffer;

11.7 ml acrylamide stock;

18 ml dH_2O;

50µl TEMED;

200µl ammonium persulphate stock.

6% Stacking Gel

5.5 ml dH_2O;

2.5 ml stock buffer;

2 ml acrylamide stock;

75µl TEMED;

150µl ammonium persulphate stock.

Preparation of Samples for PAGE

20 ml of gel sample buffer was added to each of the protein samples which were then heated to 100°C for 10 min, before applying to a polyacrylamide slab gel. Gels were subjected to electrophoresis for 5 h at 40 mA.

2.2.1.12 Preparation of Polyclonal Antiserum Against DNA Methylase

Preparation of polyclonal antiserum in rabbits against mouse DNA methylase was described by Adams et al (1986). Rabbits were subcutaneously injected with purified DNA methylase in Freund's complete adjuvant and given booster injections after

3 weeks and 6 weeks. After a further 2 weeks the rabbits were bled and serum recovered. The antibody titre by ELISA was 1/12800 (see Figure 6).

2.2.1.13 Immunoblotting

Following gel electrophoresis, proteins were transferred onto a nitrocellulose membrane by the method of Towbin (1979). The membrane was washed in immunoblotting buffer (Section 2.1.11.3) and incubated in the same buffer containing 1% (v/v) heat-inactivated horse serum and 0.03% (v/v) polyclonal antiserum (Section 2.2.1.12) for 2 h at RT. The membrane was washed as above and incubated with 1μCi I^{125}-labelled protein A in blotting buffer for 2 h at RT. Unbound I^{125}-protein A was removed by washing in blotting buffer. The membrane was dried overnight and autoradiographed.

2.2.1.14 Immunoprecipitation of Mouse DNA Methylase

2.2.1.14.1 Radiolabelling of Mouse L929 Cells

5 x 10^6 mouse L929 cells were grown in 75 cm^2 flasks containing 80% (v/v) MEM minus methionine medium plus 10% (v/v) EC10 for one hour at 37°C. 500μCi of ^{35}S-methionine was then added to the medium and the cells were incubated at 37°C. The cells were harvested 18 hours and 48 hours after addition of the radioactive marker, and nuclei were prepared.

2.2.1.14.2 Preparation of Nuclei from Mouse L929 Cells

The cell medium was discarded and the flasks rinsed twice in PBS. The cells were scraped into PBS and pelleted by centrifugation at 1000 g for 5 min. The pellet was re-suspended

in 1 ml PBS containing 1% (v/v) Tween 80 and 1% (v/v) PMSF solution (6 mg/ml PMSF in ethanol). The cell suspension was homogenised at 600 rpm and the homogenate centrifuged at 1000 g for 10 min. The nuclei were re-suspended in two volumes of buffer M containing 0.4 M NaCl, and vortexed for 10 min at 4°C. Following centrifugation at 1000 g for 5 min, the supernatant obtained was used for immunoprecipitation of DNA methylase.

2.2.1.14.3 Pansorbin Preparation

100 µl of Pansorbin (standardised *S. aureus* cells) was centrifuged at 13000 rpm for 5 min and the supernatant discarded. The pellet was washed in TKM/Triton X-100 buffer, and centrifuged as above. The pellet was re-suspended in 1 ml of the same buffer. 50µl of the washed Pansorbin suspension was added to each sample undergoing immunoprecipitation.

2.2.1.14.4 Immunoprecipitation

Nuclear extracts were incubated with $^1/_{20}$ vol of polyclonal antiserum (Section 2.2.1.12) for 10 min at 37°C, then at RT for 1 h. The volume was adjusted to 100 µl with TKM buffer and incubated at RT for 15 min. 50µl of Pansorbin suspension was then added to each sample. Incubation was at RT for 2 h. The samples were centrifuged at 13000 rpm for 5 min and the supernatant discarded. The pellets were washed X 3 in TKM buffer and X 1 in TKM/Triton X-100 buffer. The preparations were centrifuged as above and the pellets were redissolved in 20 µl SDS gel sample buffer. The samples were heated to 100°C for 10 min, then centrifuged at 13000 rpm for 10 min. The supernatants were applied to 8.75%

acrylamide gels and electrophoresed as described in Section 2.2.1.11. Following electrophoresis, the gel was subjected to fluorography (Section 2.2.2.5), dried and autoradiographed.

2.2.2 Limited Proteolysis of DNA Methylase Using Trypsin

2.2.2.1 Partial Proteolysis of DNA Methylase

Purified samples of DNA methylase (tRNA Sepharose fraction) were incubated at 37°C with a solution of trypsin prepared at 100µg/ml in H_2O, to a final enzyme protein:trypsin ratio of 200:1. The reaction was stopped at the required time by the addition of equimolar concentrations of trypsin inhibitor and the samples stored on ice. Samples were assayed as described in Section 2.2.1.10.2.

2.2.2.2 Methylation of ΦX174RF DNA Using Proteolysed Enzyme

1µg of ΦX174RF DNA was incubated in a final volume of 100µl containing 50µg BSA, 4.95 µCi S-adenosyl-L-[Methyl ^3H] methionine, and proteolysed DNA methylase in buffer M. The mixture was incubated at 37°C for 4 h. A 10 µl aliquot was then removed into 490 µl stopper and assayed by the normal method. The remainder was added to 400 µl of a solution containing 3% p-amino salicylate, 0.125 M NaCl, 1% SDS, 2 mM EDTA, 5% (v/v) n-butanol. Protein was removed by extraction with 88% (v/v) phenol, 12% (v/v) m-cresol, 0.1% (v/v) 8 hydroxyquinoline and DNA was precipitated by addition of 1/10 vol of 3M sodium acetate pH 5.2 and 2 1/2 volumes of cold ethanol for 12h at -20°C. The DNA was pelleted by centrifugation at 13000 rpm for 10 min at 4°C, and washed with

1 ml of cold ethanol. The DNA was repelleted as above and the ethanol drained. The pellet was allowed to dry at RT.

2.2.2.3 Restriction Enzyme Digest of Methylated ΦX174 RF DNA

Methylated ΦX174 RF DNA (isolated as described in Section 2.2.2.2) was re-suspended in 8 µl dH_2O. 10 units of REact buffer was added to each sample, followed by 10 units of the appropriate restriction enzyme. The solution was mixed briefly and incubated at 37°C for 3 h.

2.2.2.4 Agarose Gel Electrophoresis

Samples were prepared for electrophoresis by incubation with 5 µl of sample buffer at 68°C for 10 min. Samples were then applied directly to 1% (w/v) agarose slab gels and electrophoresed at 50 mA, for 3 h.

2.2.2.5 Fluorographic Detection of Radioactivity

1M Sodium salicylate was prepared by the solution of salicylic acid in the presence of equimolar sodium hydroxide. The pH was adjusted to 6.0 and the gels were soaked in this solution for 1-2 h at RT. The gels were washed in dH_2O, dried and autoradiographed.

2.2.3 Production of Rat Monoclonal Antibodies

2.2.3.1 Immunisation

DA rats were injected subcutaneously with 100-200µg of purified DNA methylase (Fraction V). For the primary immunisation the enzyme was added to an equal volume of Freund's complete adjuvant and mixed thoroughly before injection. A booster immunisation of 100µg of DNA methylase mixed with an equal volume of Freund's incomplete adjuvant was administered intraperitoneally three to four weeks later. A final boost containing 150µg of DNA methylase in PBS was given intravenously three days prior to the fusion.

2.2.3.2 Preparation of Spleen Lymphocytes

Rats were killed in ether and swabbed with 70% (v/v) ethanol. The spleen was dissected from the rat using sterile instruments and placed in a petri dish containing 5 ml of serum free medium. Subsequent procedures were carried out in a sterile hood. The spleen was gently scraped with two 21-G needles to release lymphocytes. The spleen capsule was then removed. Spleen cells were passed through sterile syringes fitted with 21-G needles (twice) and twice through 25-G needles. The cells were harvested by centrifugation at 800 g for 10 min, and the pellet washed in serum free medium. The cells were pelleted by centrifugation as above. A typical rat spleen yielded 10^8 cells.

2.2.3.3 Growth and Harvesting of Myeloma Cells

The Y3 cell line was grown in complete medium (Section 2.1.10.7.1) containing 10% (v/v) foetal calf serum. Y3 cells were

maintained in a state of rapid growth for two to three days before the fusion. Cells were harvested from the medium by centrifugation at 800 g for 10 min. The cells were washed in serum-free medium (Section 2.1.10.7.2) pelleted as above, and a sample count taken. Approximately 2-5 x 10^7 myeloma cells were required for fusing with 10^8 spleen cells.

2.2.3.4 Fusion of Myeloma and Spleen Cells

Approximately 10^8 rat spleen cells were mixed with 4 x 10^7 myeloma cells in a conical tube and centrifuged at 800 g for 5 min. The medium was removed and the cell pellet loosened by gentle tapping. 1 ml of 50% PEG 1500 plus 5% dimethyl sulphoxide was added over a period of 30 seconds, with continuous gentle agitation of the mixture for a further 60 seconds. 5 ml of serum-free medium was then added over a period of 1-2 min, followed by a further 5 ml of the same medium. The cell suspension was centrifuged at 800 g for 10 min, washed with 10 ml of serum-free medium and centrifuged as above. The pellet was re-suspended in 5 ml of complete medium containing 20% (v/v) foetal calf serum. Aliquots of 0.125 ml were dispensed to 36 x 1 ml tissue culture wells containing 0.4 ml of the same medium supplemented with 1 X HAT medium. The plating density was approximately 3 x 10^6 cells/well. The plates were sealed on two sides with adhesive tape and incubated at 37°C in a 4% CO_2 incubator.

Controls included fused cells grown in medium without HAT, Y3 cells grown in HAT medium and spleen cells grown in HAT medium.

2.2.3.5 Feeding Hybridoma Cultures

Feeding the fused cells was carried out weekly, by removal of half the culture medium and replacement with complete medium containing 20% (v/v) foetal calf serum and 1 X HAT. When clones were established and growing rapidly the serum supplement of the medium was reduced to 10% (v/v).

2.2.3.6 Selection of Hybridomas in HAT Medium

Fused cells were maintained in HAT medium for four to five weeks. The hybrids were then cultured in HT medium for one week prior to growth in normal cell medium.

2.2.3.7 Detection of Positive Clones by ELISA

Three weeks after fusion, the supernatants of wells supporting cell growth were removed for assay, to identify clones secreting specific antibody.

96 well Dynatech microtitre plates were coated with a solution containing 6.25µg/ml purified DNA methylase (tRNA Sepharose fraction) in PBS, and incubated overnight at 4°C. The antigen was then shaken from the wells and the plates were immersed in PBS, then blotted dry on absorbent paper. The wells were blocked with a solution containing 10 mg/ml BSA in PBS for 1h at RT. The plates were then washed X 3 in PBS containing 0.1% (v/v) Tween 20. 100 µl aliquots of antiserum diluted in PBS containing 0.5 mg/ml BSA, or hybridoma supernatants were added to each well and the plate incubated for 2h at RT. The plate was washed as described above, and treated with 100µl/well of a solution containing biotinylated sheep anti-rat

whole Ig, diluted $^{1}/_{100}$ in PBS/1% (w/v) BSA. Incubation was for 1h at RT. After washing, each well was incubated with 100 µl of streptavidin biotinylated horse-radish peroxidase, diluted $^{1}/_{400}$ in PBS/1% (w/v) BSA for 30 min at RT. The plates were washed as above and 100 µl of OPD solution (Section 2.1.11.4) was added to each well. Incubation was at RT for 30 min in the dark. The reaction was stopped by the addition of 50 µl 4N H_2SO_4/well and the absorbance at 492 nm measured on a Titertek multiscanning spectrophotometer.

Controls included spleen cell supernatants and myeloma cell supernatants in place of hybridoma supernatants. Other controls involved the use of PBS in place of antigen, and minus antiserum (PBS supplemented with 0.5 mg/ml BSA).

2.2.3.8 Cloning Hybridomas

Hybridomas were cloned by limiting dilution. Cells were plated at theoretical densities of 60, 14.5, 3.5, 0.85, 0.21 cells/well on 96 well tissue culture plates. Clones growing at estimated densities of 0.8 cells/well were recloned several times before assuming monoclonality.

2.2.3.9 Freezing Hybridomas

Aliquots of cells were added to 1 ml of a solution containing 90% (v/v) foetal calf serum and 10% (v/v) dimethylsulphoxide. Ampoules were frozen in a cryospeed freezing chamber and transferred to gaseous phase nitrogen (-120°C).

2.2.3.10 Expansion of Clones

Hybridomas were expanded *in vitro* by transfer of cells to 24 well tissue culture plates, followed by growth in 25 cm^2, 80 cm^2 and 125 cm^2 tissue culture flasks.

2.2.3.11 Purification of Immunoglobulin from Hybridoma Supernatants

2.2.3.11.1 Ammonium Sulphate Precipitation

50 ml of cell supernatant was harvested from culture flasks of hybridoma clones. The medium was centrifuged at 1000 g for 10 min to pellet cells. The supernatant was removed and made to 48% saturation with respect of ammonium sulphate. The solution was stirred on ice for a further 20 min and centrifuged at 14000 g for 20 min. The supernatant was drained and the pellets stored at -20°C.

2.2.3.11.2 Fractionation on QAE Sephadex A-50

QAE Sephadex A-50 was swollen in PBS pH 6.5 for 36 h and degassed under vacuum before pouring into a 20 ml column. The column was washed with PBS pH 6.5 until the E280 value was $\leq$ 0.01. The ammonium sulphate pellet obtained in Section 2.2.3.11.1 was re-suspended in 1 ml of PBS pH 6.5 and applied to the column. The sample was eluted with the same buffer at a flow rate of 1 ml/ minute, and 1 ml fractions were collected. Fractions were assayed for protein content by the method of Bradford (1976), and tested by ELISA to identify the peak immunoglobulin fractions.

2.2.3.12 Titration of Immunoglobulin

Peak Immunoglobulin fractions (determined by ELISA assay) were titrated to quantitate the activity of the purified Ig. Serial dilutions of immunoglobulin were prepared in PBS containing 0.5 mg/ml BSA. Dilutions ranged from $^1/_2$ - $^1/_{256}$. The samples were tested by ELISA (Section 2.2.3.7).

2.2.3.13 Neutralisation of DNA Methylase Activity Using Purified Ig

3µg samples of purified DNA methylase (tRNA sepharose fraction) were incubated with various amounts of monoclonal immunoglobulin in PBS, for 12 h at 4°C. The mixture was then incubated with the standard DNA methylase assay reagents for 1 h at 37°C, and assayed as described in Section 2.2.1.10.2. Controls included Ig fractions isolated from unused culture medium.

2.2.3.14 Precipitation of DNA Methylase Using Purified Ig

3µg of purified DNA methylase (tRNA Sepharose fraction) were incubated with various amounts of monoclonal Ig in PBS, for 12h at 4°C. The mixture was then centrifuged at 1000 g for 10 min to pellet immune complexes, and the supernatant assayed for methylase activity as described in Section 2.2.1.10.2. Controls included Ig fractions isolated from unused culture medium.

2.2.3.15 Immunoblotting Using Monoclonal Antibodies and I^{125}-Labelled Protein A

Samples of purified DNA methylase (tRNA Sepharose fraction) were subjected to gel electrophoresis and transferred

to a nitrocellulose membrane by the method of Towbin (1979). The membrane was cut to separate single lanes of enzyme, and the strips placed in stoppered tubes. Non-specific sites were blocked by incubation with 3 ml of blotting buffer (Section 2.1.11.4) containing 1% (v/v) heat inactivated horse serum, for 5 min at RT. 100µl of monoclonal antibody (peak QAE Sephadex-A50 fraction) was added to the mixture and the membrane incubated for a further 2h at RT with constant gentle shaking. After washing to remove non-bound material, bound immunoglobulin was probed by incubation with I^{125} labelled protein A, for 2h at RT. The nitrocellulose strips were washed with blotting buffer, dried on 3 mm filter paper and autoradiographed.

CHAPTER 3 : PURIFICATION OF DNA METHYLASE

3.1 Purification of DNA Methylase from Mouse Krebs II Ascites Cells

3.1.1 Introduction

For the purposes of further characterisation of DNA methylase using polyclonal antiserum, and to obtain a supply of purified enzyme for the production of monoclonal antibodies, an enzyme purification strategy was devised. This was based on the method of Turnbull and Adams (1976) but with several significant modifications. In this section a detailed description and rationale of the purification described in Section 2.2.1 will be presented. Results of a typical purification are shown in Table 3.

3.1.2 The Choice of Mouse Ascites Tumour Cells as a Source of DNA Methylase

Nuclei isolated from rapidly dividing cells are known to show a greater level of incorporation of methyl groups into DNA than nuclei from resting cells (Adams et al, 1973; Tosi et al, 1972; Adams et al, 1974). Szyf et al (1985) have demonstrated that DNA methylase activity coincides with the activation of the cell's replication machinery.

Mouse Krebs II ascites tumour cells were chosen as the enzyme source because of the high levels of DNA methylase present and the large number of cells obtainable, prohibitively expensive by tissue culture resources.

3.1.3 Extraction of DNA Methylase from Ascites Nuclei

Stirring nuclei in buffer M containing NaCl to a final concentration of 0.2 M was found to be the optimum initial extraction method. Lower salt concentrations failed to release the bulk of enzyme activity from the nuclei and no significant increase in recovery was observed on extraction with a final concentration of 0.3 M NaCl. Concentrations of greater than 0.3 M NaCl reduced recovery of enzyme by promoting aggregation of the nuclei.

Two consecutive 0.2 M NaCl extractions of the nuclei were carried out, releasing 88% of the enzyme activity into solution. The enzyme was recovered in the supernatant, following centrifugation of the nuclear suspension at 1200g for 10 minutes (Fraction II).

3.1.4 Phosphocellulose Ion Exchange Chromatography

3.1.4.1 Preparation of the Matrix

Phosphocellulose was prepared as a 50% (wet wt/vol) suspension in buffer M containing 0.2 M NaCl. Non-specific binding sites were blocked by treatment with 1% (w/v) BSA. The matrix was thoroughly washed and equilibrated before use.

3.1.4.2 Titration of Phosphocellulose

To determine the optimum amount of phosphocellulose required to bind DNA methylase recovered in Fraction II, varying

amounts of phosphocellulose suspension, ranging from 8 ml to 36 ml, were mixed with aliquots of Fraction II. The samples were subsequently treated as described in Section 2.2.1.5. Following elution with buffer M containing 0.5 M NaCl, the samples were assayed for DNA methylase activity as described in Section 2.2.1.10.2.

Figure 1 shows the results of phosphocellulose titration. Treatment with 12 ml of phosphocellulose suspension per 100 ml of Fraction II results in optimum recovery of enzyme activity.

3.1.4.3 Phosphocellulose Chromatography

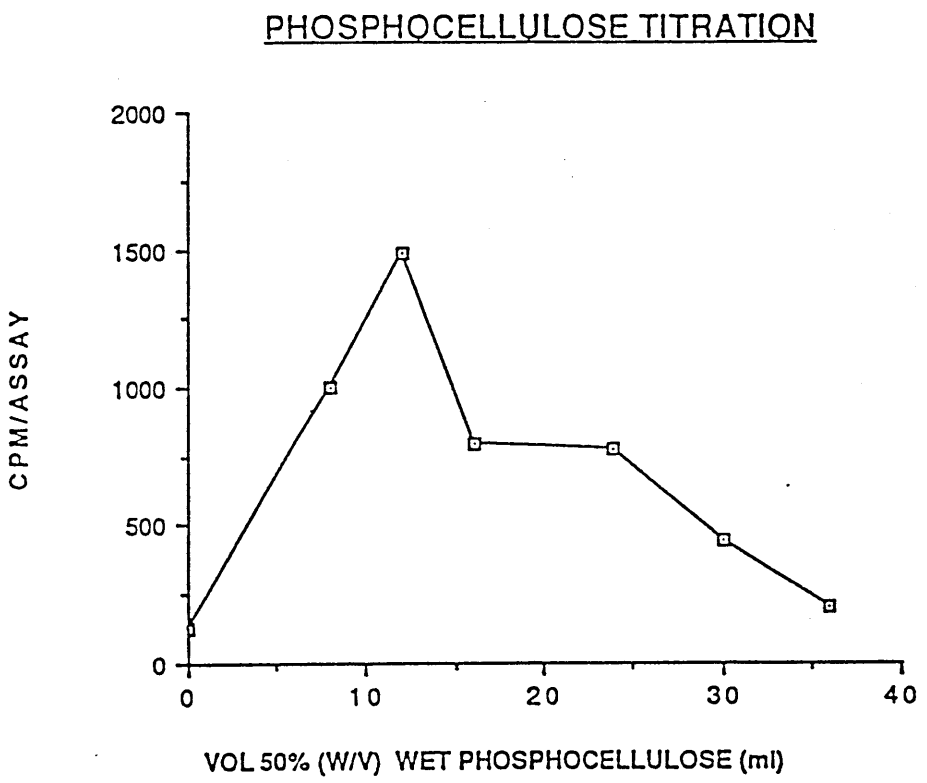
Fraction II was treated with 12 ml phosphocellulose suspension/100 ml as described in Section 2.2.1.5. Greater than 90% of the total activity was routinely recovered from the pooled batch elutions (Fraction III) with a 13-fold increase in specific activity over Fraction II.

3.1.5 Ammonium Sulphate Precipitation of DNA Methylase

Previous results (Turnbull and Adams, 1976) have shown that optimum precipitation of DNA methylase occurs at concentrations of 30% - 60% saturation with ammonium sulphate, with negligible amounts of enzyme being precipitated at concentrations outwith these limits.

50% saturation with respect to ammonium sulphate was used to precipitate DNA methylase from Fraction III, prior to gel filtration on Sephacryl S300. The precipitate obtained was drained

Figure 1



Varying amounts of phosphocellulose suspension were mixed with aliquots of Fraction II to give final values of 8 ml, 12 ml, 24 ml, 30 ml and 36 ml of 50% phosphocellulose suspension added per 100 ml of Fraction II. Following washing and subsequent elution with buffer M containing 0.5 M NaCl, optimum enzyme activity was recovered from the use of 12 ml of 50% phosphocellulose suspension/100 ml of Fraction II.

thoroughly to ensure complete removal of the supernatant and stored at -20°C until use.

3.1.6 Gel Filtration of DNA Methylase on Sephacryl S300

Sephacryl S300 was used as the gel filtration matrix. Particles of size 600,000 M.W. are excluded from the beads. Figure 2 shows the elution profile obtained following application of an ammonium sulphate precipitate redissolved in buffer M containing 0.2 M NaCl on a 380 ml Sephacryl S300 column, using the same buffer as eluant. The void volume of the column was 108 ml. Fractions eluting at 1.2 - 1.6 X void volume (130 - 164 ml) were pooled (Fraction IV). DNA methylase elutes as a single peak, after the major protein peak of the sample. Purification of 1527 fold over the nuclear suspension was achieved at this stage. However, only 42% of the activity applied to the column was recovered, although it is possible that some loss of activity occurred during the preceding ammonium sulphate precipitation.

3.1.7 Ammonium Sulphate Precipitation of Fraction IV

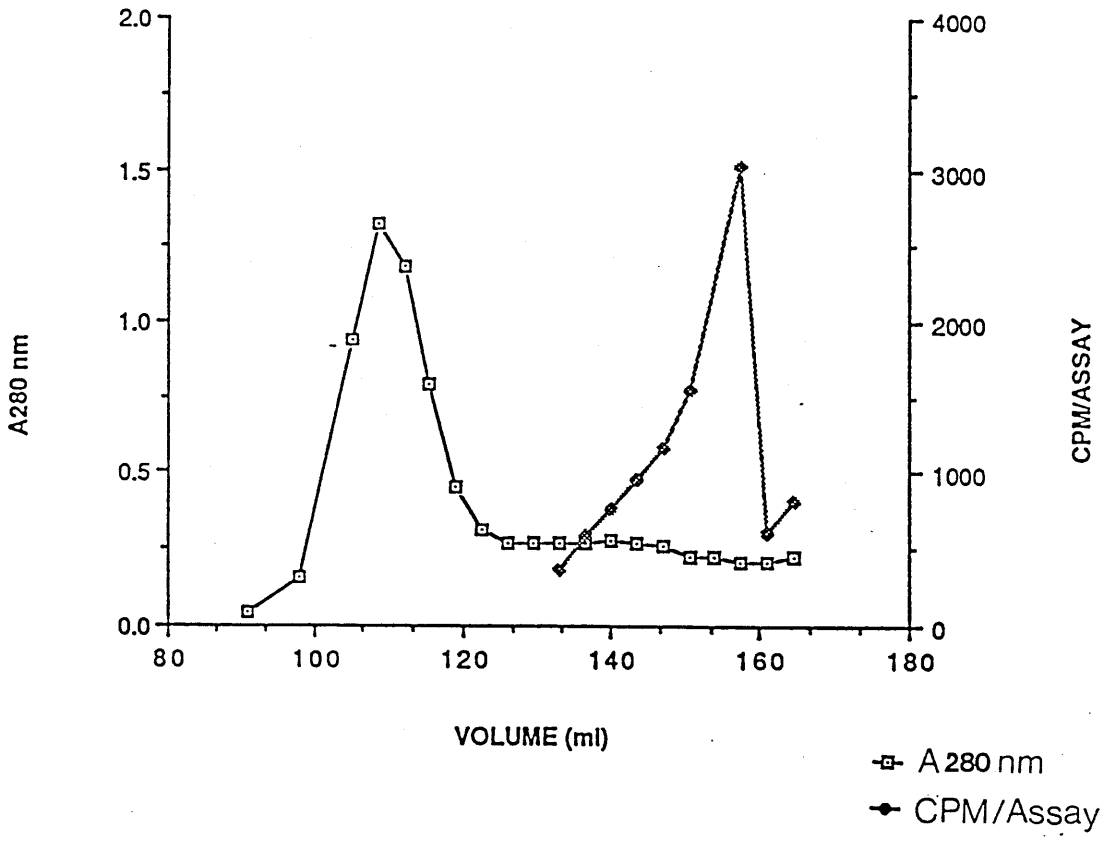
To reduce the volume of Fraction IV prior to affinity chromatography, the sample was made to 70% saturation with respect to ammonium sulphate. This level of saturation was chosen to ensure precipitation of all the DNA methylase activity from the sample.

3.1.8 Affinity Purification of DNA Methylase

Affinity purification of DNA methylase was undertaken to remove contaminating proteins of similar size and charge from the

FIGURE 2

S300 GEL FILTRATION ELUTION PROFILE



DNA methylase (Fraction III, ammonium sulphate precipitated) was subjected to gel filtration on Sephacryl S300 and eluted with buffer M containing 0.2 M NaCl. 4 ml fractions were collected and the absorbance at 280 nm monitored. DNA methylase activity was assayed for samples eluting between 1.2 - 1.6 X void volume, i.e. 130 - 164 ml.

preparation which had failed to be removed by the preceding steps. Several affinity matrices were considered: Heparin Sepharose readily bound the enzyme in low salt conditions (0.2 M NaCl). However, purification on this matrix resulted in a loss of over 80% of the enzyme activity initially applied, presumably due to irreversible binding of the enzyme on the column.

An affinity column of anti-methylase polyclonal immunoglobulin cross-linked to CNBr Sepharose was prepared. DNA methylase bound to this matrix under conditions of 0.2 M NaCl. Again, finding a suitable eluant proved difficult. Buffer M containing 0.25% (v/v) Triton X-100 plus 3.5 M $MgCl_2$ failed to release enzyme activity. It seems likely that DNA methylase was either irreversibly cross-linked to the immunoglobulin matrix or the elution conditions may have adversely affected enzyme activity.

E. coli transfer RNA cross-linked to Sepharose provided a suitable affinity ligand for the binding and elution of DNA methylase.

3.1.9 Affinity Chromatography on tRNA Sepharose

1 ml of redissolved ammonium sulphate precipitate (Section 3.1.7) was de-salted on a 19 ml column of Sephadex G50. The non-retarded fraction containing the enzyme was pumped directly onto a 10 ml column of tRNA Sepharose at a flow rate of 10 ml/hr. The A280 of the sample eluting from G50 Sephadex was monitored using a Uvicord detector and chart recorder. Following application of the protein peak eluting from G50 Sephadex onto the tRNA column, this

column was washed with 1 volume of buffer M. The enzyme was then eluted in buffer M containing 0.2 M NaCl and 1 ml fractions were collected and assayed. Figure 3 shows the A280 elution profile of the sample from tRNA Sepharose (Fraction V).

3.1.10 Overall Purification of DNA Methylase from Mouse Ascites Nuclei

Table 3 shows a summary of data obtained from a typical DNA methylase purification. A final purification of 2080-fold over the nuclear suspension was achieved.

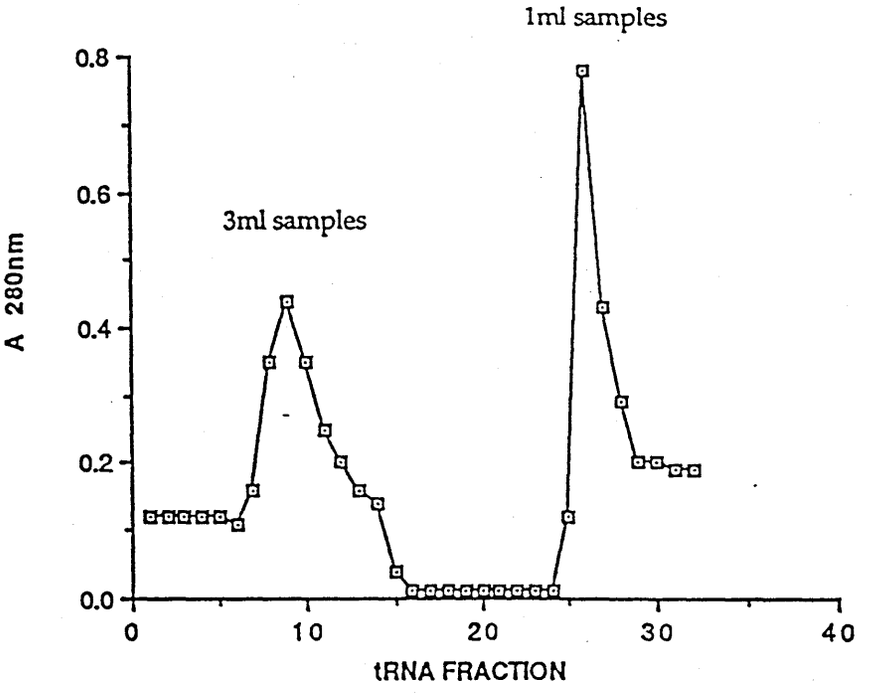
Figure 4 shows a Coomassie blue-stained gel of Fractions II-V. Adams et al (1979) reported the molecular weight of mouse ascites DNA methylase between 160 kD and 185 kD. The major bands present in tracks 5, 6, and 7 in Figure 4 correspond to 160 kD, 175 kD and 190 kD, although a number of other protein bands are also visible.

Figure 5 shows the result of a Western blot probed with polyclonal antiserum (see Section 2.2.1.12) and illuminated with ^{125}I-labelled protein A. The antiserum selectively interacts with two protein bands corresponding to 190 kD and 160 kD.

The purified enzyme fraction visualised in track 1, Figure 5 showing approximately equal amounts of the 190 kD and 160 kD forms of DNA methylase, showed considerably greater activity ($>$9X) than the purified enzyme shown in track 2, Figure 5, in which the 160 kD form is predominant.

Figure 3

ELUTION PROFILE OF DNA METHYLASE FROM tRNA SEPHAROSE



DNA methylase (ammonium sulphate precipitate) was de-salted on a Sephadex G50 column with the non-bound fraction applied directly to a tRNA Sephadex column for affinity purification.

Samples	1 - 13	G50/tRNA Run through
	14 - 22	tRNA Wash (Buffer M)
	23 - 32	tRNA Elution (Buffer M + 0.2 M NaCl)

Fraction	Activity (U/mg)
9	413
10	212
25	1089
26	2081
27	2461
28	2238

TABLE 3

PURIFICATION OF DNA METHYLASE FROM MOUSE KREBS II ASCITES TUMOUR CELLS.

FRACTION		TOTAL PROTEIN (mg)	TOTAL ACTIVITY (Units)	SPECIFIC ACTIVITY (Units/mg)
NUCLEAR SUSPENSION	I	45170	45120	1
0.2M NaCl NUCLEAR EXTRACT	II	1155	39600	34
PHOSPHO-CELLULOSE	III	79	35640	451
SEPHACRYL S300	IV	9.7	14820	1527
tRNA SEPHAROSE	V	0.49	1020	2082

FIGURE 4

Coomassie Blue-stained SDS polyacrylamide gel electrophoresis patterns of fractions from various stages of DNA methylase purification.

Track	Sample
1	Markers: 200 kD, 165 kD, 155 kD
2	Fraction II
3	Fraction III
4	Fraction IV
5)	Individual 1 ml samples eluted from tRNA Sepharose
6)	Track 5 - Fraction 25
	6 - Fraction 26
7)	7 - Fraction 27

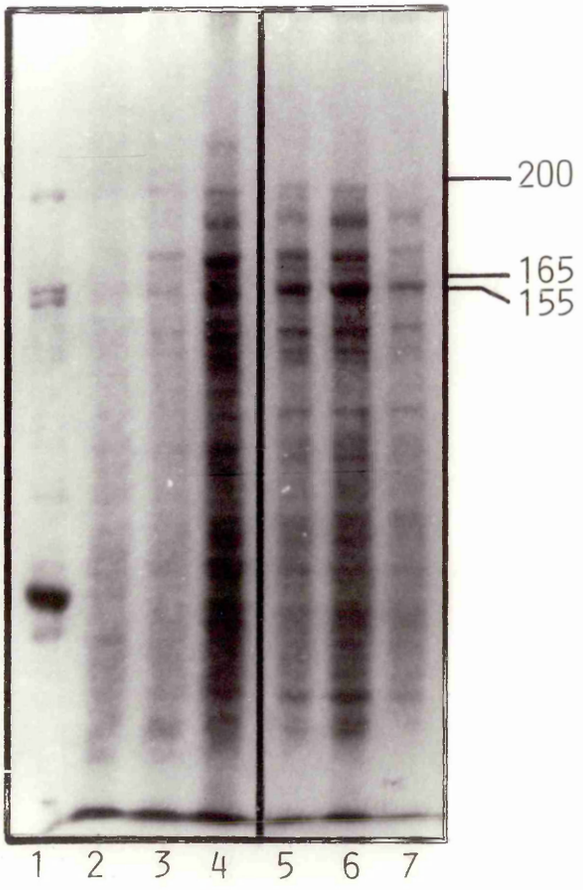

200
165
155
1 2 3 4 5 6 7

FIGURE 5

Western blot of purified DNA methylase (peak fractions eluting from tRNA Sepharose) probed with polyclonal antiserum and I^{125}-labelled protein A.

Track	Sample
1	DNA methylase (Fraction V) eluting from tRNA Sepharose (Fraction 25)
2	DNA methylase (Fraction V) eluting from tRNA Sepharose (Fraction 26)

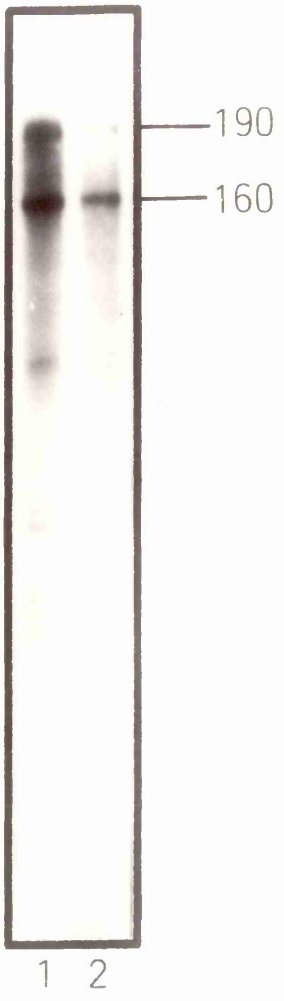
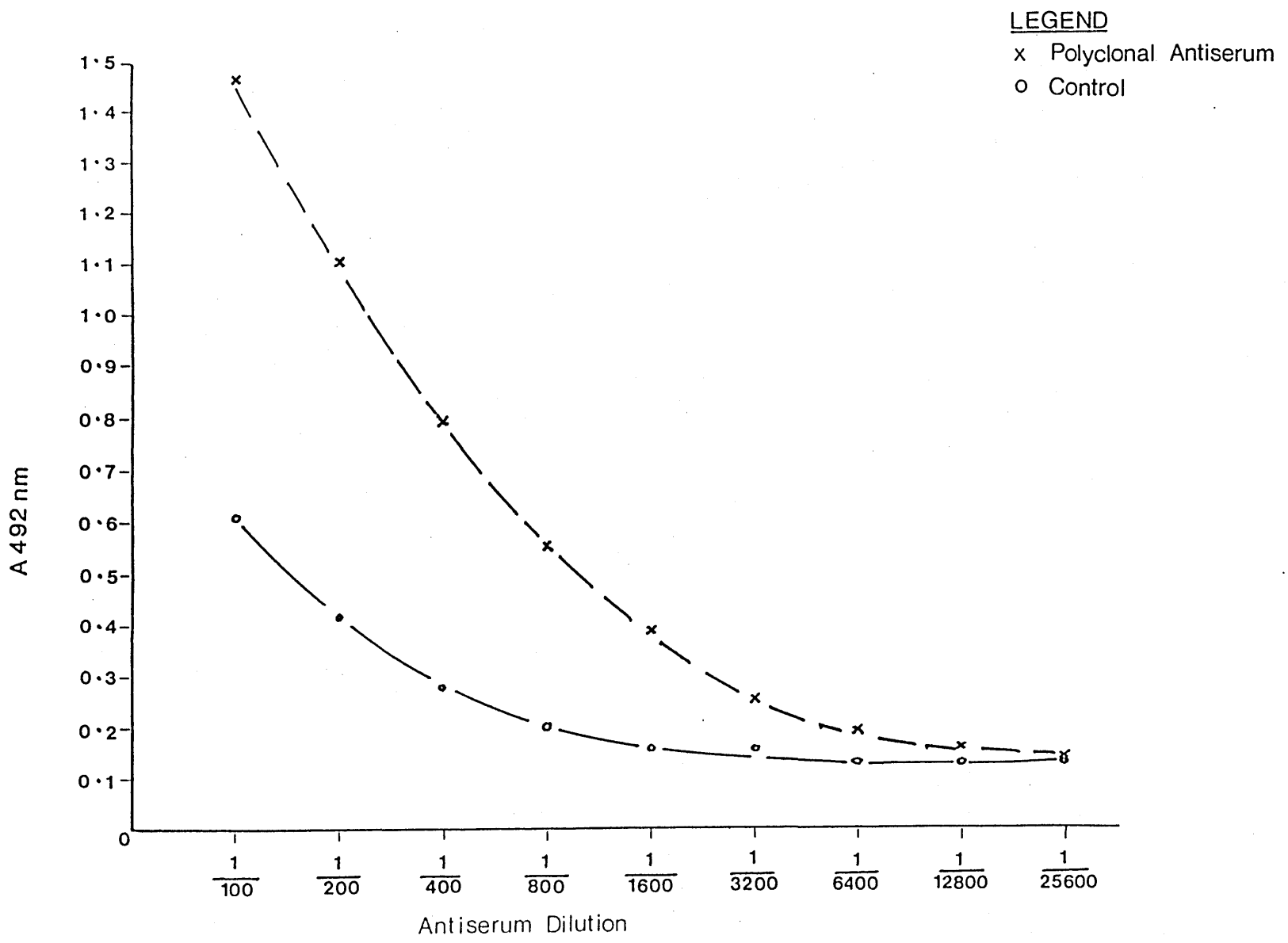

190
160
1 2

Figure 6

Figure 6 shows the titration by ELISA of polyclonal antiserum raised against DNA methylase (see Section 2.2.1.12). Serial dilutions of antiserum ranging from 1/100 - 1/25600 were prepared in PBS containing 0.5 mg/ml BSA. The control shows ELISA readings obtained using pre-immune serum.

FIGURE 6 TITRATION OF POLYCLONAL ANTISERUM



3.2 Immunoprecipitation of DNA Methylase from Mouse L929 Cells

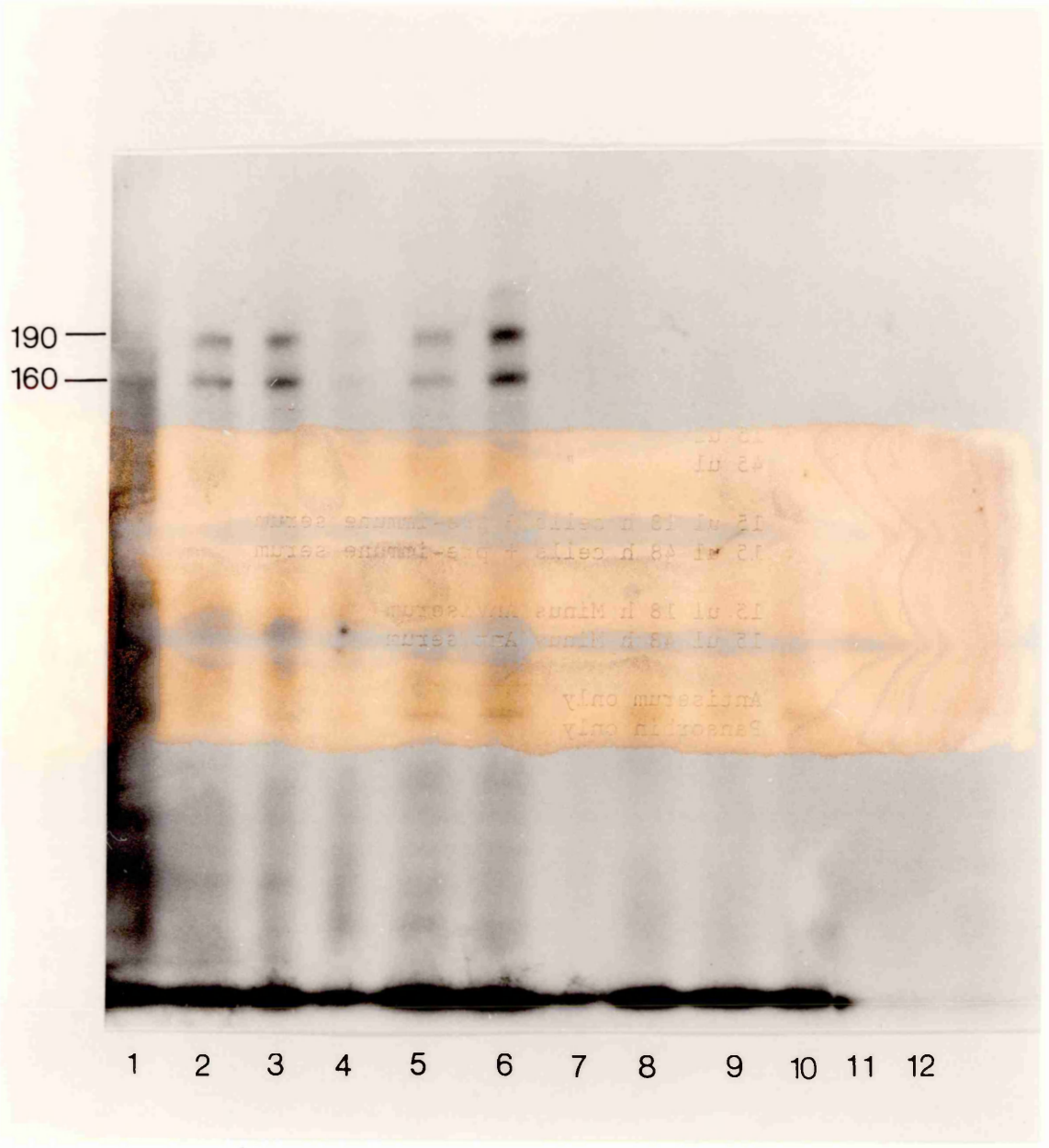
To investigate the size of DNA methylase at different stages of cell growth, the enzyme was immunoprecipitated from mouse L929 cells. The cells were radiolabelled with ^{35}S-[L] Methionine, and DNA methylase was immunoprecipitated from cell extracts using polyclonal antiserum and Pansorbin (Section 2.2.1.14). Cells were harvested 18 h and 48 h after labelling, to observe the size of the enzyme present at log phase growth (18 h) and to determine if degradation of high molecular weight enzyme occurred as cell growth declined (48 h). The fluorograph in Figure 7 shows that the polyclonal antiserum immunoprecipitates 2 high molecular weight bands of 190 kD and 160 kD corresponding to the reported size of mouse DNA methylase.

Pre-immune serum failed to precipitate these proteins. No distinct difference is observed between the protein bands precipitated from cells after 18 h or 48 h and subsequent experiments to immunoprecipitate the enzyme from cells at various stages of cell growth also failed to reveal any substantial change in the size of the enzyme.

FIGURE 7

Fluorograph of DNA methylase immunoprecipitated from mouse L929 cells.

Track		Sample
1	)	5 ul 18 h cells immunoprecipitated
2	)	15 ul "
3	)	45 ul "
4	)	5 ul 48 h cells immunoprecipitated
5	)	15 ul "
5	)	45 ul "
7	)	15 ul 18 h cells + pre-immune serum
8	)	15 ul 48 h cells + pre-immune serum
9	)	15 ul 18 h Minus Antiserum
10	)	15 ul 48 h Minus Antiserum
11		Antiserum only
12		Pansorbin only


190
160
1
2
3
4
5
6
7
8
9
10
11
12

CHAPTER 4 : LIMITED PROTEOLYSIS OF DNA METHYLASE

4.1 The Effect of Limited Proteolysis on DNA Methylase Activity

4.1.1 Introduction

Stimulation of *de novo* methylation of DNA following limited proteolysis of mouse ascites DNA methylase was first reported by Adams et al (1983). In this Section, further data will be presented on:

(a) The breakdown pattern of the enzyme during proteolysis.

(b) The effect of varying degrees of proteolysis on enzyme activity.

(c) The assessment of CpG specificity of the enzyme following proteolysis.

4.1.2 Partial Proteolysis of DNA Methylase

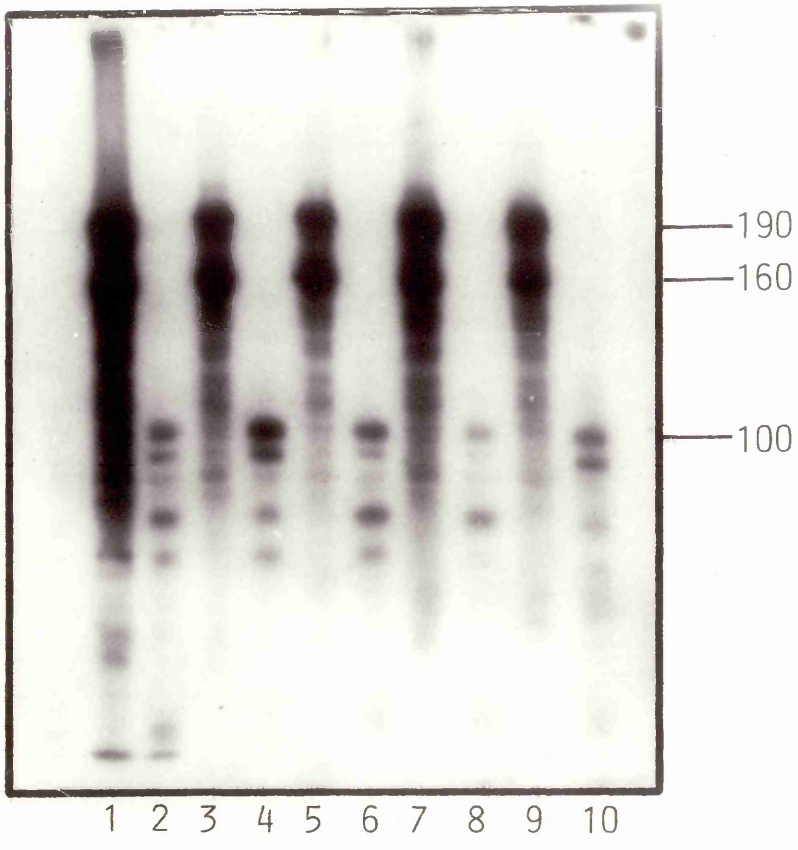
Purified, or partially purified, samples of DNA methylase were treated with trypsin as described in Section 2.2.2.1. Figure 8 shows a Western blot of paired untreated and trypsin-treated enzyme samples. The enzyme protein:trypsin ratio used in samples 2, 4, 6, 8 and 10 was 200:1. The incubation time with trypsin was 30 minutes at 37^{o}C in each case. Partial proteolysis of DNA methylase with trypsin was found to result in the gradual breakdown of the enzyme with the loss of the 190 kD and 160 kD species of enzyme, and the subsequent appearance of lower molecular weight, immunologically cross-reacting polypeptides. An increase in the specific activity of enzyme samples shown in tracks 1 and 7 was found to occur following treatment with trypsin (tracks 2 and 8 respectively;

FIGURE 8

Western blot of partially proteolysed DNA methylase.

Paired samples of untreated and trypsin-treated enzyme are shown: odd numbered lanes show untreated DNA methylase from Fraction IV (Track 1) and Fraction V (Tracks 3, 5, 7 and 9) of a standard DNA methylase purification. Even-numbered lanes show proteolysis of the sample in the preceding track. Approximately 20µg of each sample was subjected to SDS PAGE. The specific activity of each sample is given below:

Lane	Sample	Specific Activity (U/mg)
1	Fraction IV	464
2	Fraction IV Proteolysed	1401
3	Fraction V 25	2400
4	Fraction V 25 Proteolysed	1155
5	Fraction V 26	2059
6	Fraction V 26 Proteolysed	1967
7	Fraction V 27	1395
8	Fraction V 27 Proteolysed	2679
9	Fraction V 28	1904
10	Fraction V 28 Proteolysed	1406


190
160
100
1 2 3 4 5 6 7 8 9 10

see Figure 8 Legend) while samples in tracks 3, 5 and 9 showed some decrease in specific activity following proteolysis (Tracks 4, 6 and 10 respectively). As each of the proteolysed samples shown in Figure 8 were subjected to 30 minutes incubation with trypsin, it seemed possible that despite the standard enzyme protein:trypsin ratio applied, the individual enzyme samples had undergone varying degrees of proteolysis during this time.

To further investigate the effect of limited proteolysis on enzyme activity, a time course of enzyme incubations with trypsin was carried out. Again, the final enzyme protein:trypsin ratio was 200:1. The mixture was incubated at 37^{o}C and aliquots were removed after 1, 5, 10, 15, 20, 25, 30 and 40 minutes. The reaction was stopped by the addition of equimolar trypsin inhibitor. DNA methylase activity was assayed using native M. luteus DNA as the substrate. The size of the proteolysed enzyme was assessed immunologically, by Western blotting and probing with polyclonal antiserum and I^{125}-labelled protein A, as described in Section 2.2.1.13.

4.1.3 Stimulation of DNA Methylase Activity Following Limited Proteolysis

Figure 9A shows a Western blot of DNA methylase following treatment with trypsin over a period of 1-40 minutes. The graph in Figure 9B shows corresponding enzyme activity over the same period.

The substantial increase in enzyme activity initially observed is followed by a gradual drop in enzyme activity to below

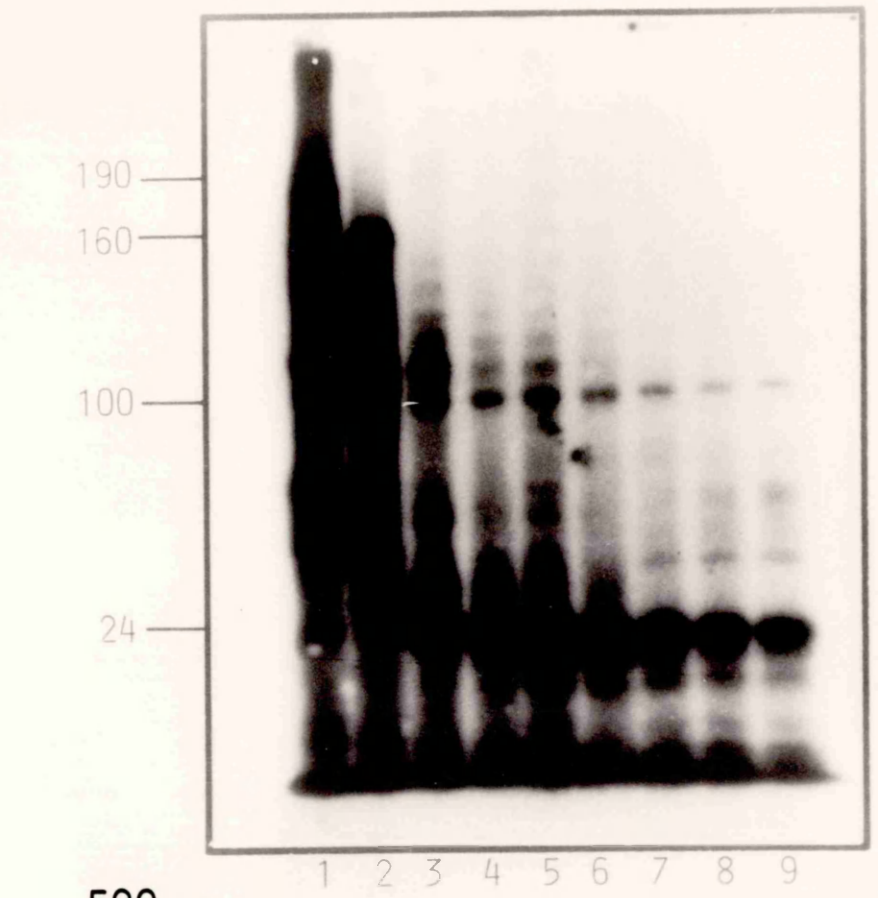
FIGURE 9A

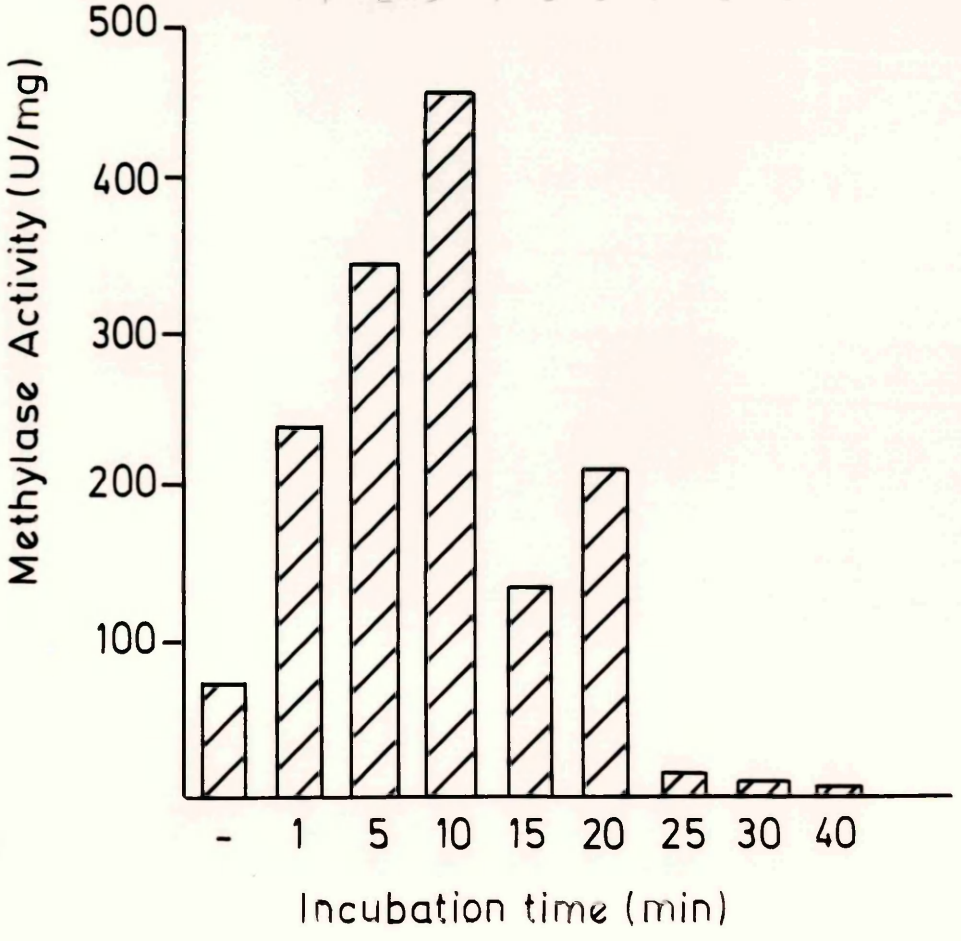
Western blot of DNA methylase subjected to partial proteolysis with trypsin over a period of 1-40 min.

Lane	Incubation Time with Trypsin
1	- (untreated DNA methylase)
2	1 min
3	5 min
4	10 min
5	15 min
6	20 min
7	25 min
8	30 min
9	40 min

FIGURE 9B

Specific activity of partially proteolysed DNA methylase using native M. luteus DNA as the substrate. Activities given correspond to the samples shown in Figure 7A.


190
160
100
24
1 2 3 4 5 6 7 8 9


Methylase Activity (U/mg)
500
400
300
200
100
- 1 5 10 15 20 25 30 40
Incubation time (min)

the level of the control: After a 1 minute incubation of DNA methylase with trypsin, the specific activity of the enzyme on native DNA is seen to increase by greater than three fold, rising to a four fold increase after 5 minutes and a six fold increase after 10 minutes' incubation. Between 15 and 20 minutes, enzyme activity begins to decline and thereafter the activity measured is considerably lower than that of the untreated enzyme. This is presumably due to more extensive proteolysis of the enzyme, causing a break in molecular configuration.

From the autoradiograph in Figure 9A, the corresponding degree of breakdown of DNA methylase following SDS polyacrylamide gel electrophoresis and Western blotting is shown. After incubation with trypsin for 1 minute, the 190 kD band present in the untreated enzyme sample is no longer visible, and after 5 minutes the 160 kD protein band has also been replaced by a series of lower molecular weight polypeptides, with bands notably evident at 100 kD and 24 kD. After 30 minutes' incubation, the 100 kD band is seen to diminish with the predominant species now the 24 kD polypeptide band.

The degradation pattern of proteolysed DNA methylase observed upon SDS gel electrophoresis may not reflect the true state of the enzyme as it undergoes limited proteolysis: it is likely that treatment with trypsin, introducing nicks into the amino acid sequence, does not immediately cause the enzyme to dissociate into discrete fragments. The initial effect of introducing breaks in the polypeptide chain is likely to be a more general loosening of the domain structure of the enzyme.

The loss or spatial distancing of a regulatory domain (possibly for CpG recognition, or for the recognition of hemi-methylated sites in DNA) may account for the increased level of DNA methylase activity observed following limited proteolysis. The structure of DNA methylase may therefore initially remain relatively intact *in vitro*, although the conditions of SDS PAGE would suffice to cause dissociation of the partially proteolysed enzyme. As proteolysis proceeds and a large number of breaks are introduced, more extensive breakdown of the enzyme may occur *in vitro*. The complete dissociation of enzymatic domains may account for the negligible levels of enzyme activity observed following prolonged exposure of DNA methylase to trypsin.

4.1.4 Specificity of DNA Methylase Following Partial Proteolysis

One possible explanation for the increase in activity of the proteolysed enzyme is that it may be less stringent with regard to the CpG site specificity characteristic of the native enzyme. To investigate this further, ΦX174 R.F. DNA was methylated using untreated and trypsin-treated DNA methylase (Section 2.2.2.2). Controls included the same reaction procedure carried out in the absence of S-adenosyl-L-[Methyl-^{3}H] methionine. Following methylation, the DNA was isolated and digested with methyl sensitive restriction enzymes. Table 4 describes the properties of the restriction enzymes used:

TABLE 4

Summary of Restriction Enzyme Properties

Enzyme	Recognition Site	Recognition Blocked	No. of Sites in ΦX174 RF DNA
Hpa II	CCGG	C^mCGG	5
Msp I	CCGG	^mCCGG	5
Alu I	AGCT	AG^mCT	24
Eco RII	CCA/TGG	C^mCA/TGG	2

The use of these enzymes allows identification of 5-m Cytosine residues in the dinucleotide sequences CC, CA, CT as well as CG. Following restriction enzyme digestion of the DNA, fragments were separated by agarose gel electrophoresis.

4.1.5 Methylation and Restriction Enzyme Digestion of ΦX174 RF DNA

Bacteriophage ΦX174 RF DNA was methylated and digested with restriction enzymes as described in Section 2.2.2. Following assay (see Section 2.2.2.2) the percentage methylation was calculated by:

$$\frac{\text{p Moles 5-mC incorporated}}{\text{p Moles CpG in ΦX174 DNA}} \times 100$$

where ΦX174 RF DNA contains 15 p Moles of CpG. Methylation of the DNA with native DNA methylase proved difficult as the DNA was

frequently degraded, or otherwise undetectable, following restriction digestion and agarose gel electrophoresis. This effect may have been due to the presence of exogenous nucleases in the enzyme preparation, despite the presence of 1 mM EDTA (known to inhibit many nucleases) during preparation and storage of the enzyme. The presence of active nucleases could cause substantial degradation of DNA during the 4 h incubation with DNA methylase. No such degradation is observed upon methylation of DNA with trypsin-treated enzyme, indicating perhaps that trypsin inactivates nuclease activity. This in itself could lead to an apparent increase in methylase activity.

An alternative explanation is that enzyme-DNA aggregates form upon incubation of DNA with native DNA methylase, with subsequent loss of DNA following phenol extraction. Proteolysis of the incubation mix after the addition of SDS but prior to isolation of DNA, should cause dissociation of such complexes and resolve this possibility.

4.1.6 Restriction Digest Analysis of Methylated ΦX174 RF DNA

Figures 10, 11 and 12 show the restriction patterns obtained following electrophoresis of DNA digested with Alu I and Eco RII (Figure 10), and Hpa II and Msp I (Figures 11 and 12). No difference in the restriction pattern could be detected between methylated and unmethylated DNA digested with Alu I, indicating that methylation of CpT does not occur to a measurable extent when the overall level of DNA methylation is 13%.

Eco RII consistently failed to cleave either methylated

FIGURE 10

Restriction of ΦX174 RF DNA by Alu I and Eco RII.

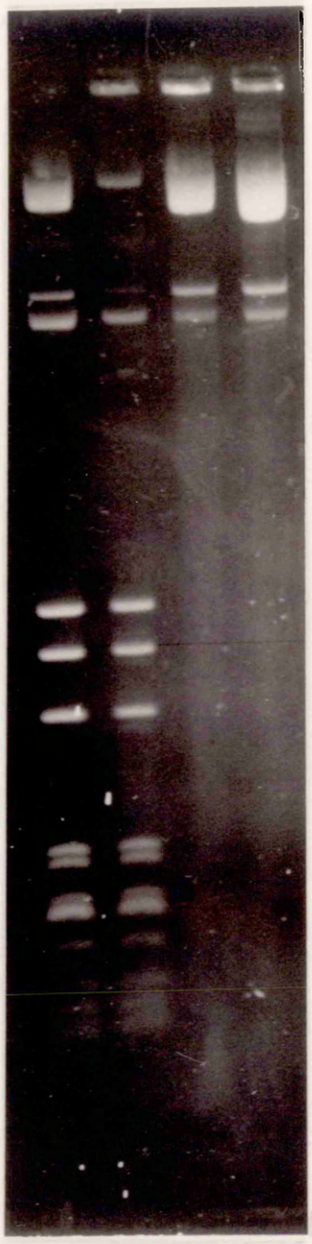
Track

1 Methylated ΦX174 RF DNA digested with Alu I.
2 Unmethylated ΦX174 RF DNA digested with Alu I.
3 Methylated ΦX174 RF DNA digested with Eco RII.
4 Unmethylated ΦX174 RF DNA digested with Eco RII.

Digestion of ΦX174 RF DNA methylated to 13%, with the restriction enzymes Alu I and Eco RII.

Tracks 1 and 2 show a comparison of DNA methylated using partially proteolysed DNA methylase (Track 1) and with the same enzyme in the absence of S-Ado Met (Track 2). The restriction pattern obtained upon digestion of DNA with Alu I is the same in each case, indicating no substantial protection of CpT sites by methylation.

Tracks 3 and 4 illustrate the inability of Eco RII to cleave methylated DNA (Track 3) or DNA mock-methylated in the absence of S-Ado-Met (Track 4), probably due to inherent methylation of these sites during propagation of the phage in a dcm^+ host strain of E. coli.



1 2 3 4

or unmethylated DNA: it is therefore not possible at this stage to exclude the occurrence of CpA methylation. Resistance of ΦX174 RF DNA to restriction with Eco RII may be due to dcm methylation of the phage DNA. Self-methylation is a mechanism by which bacteriophage can overcome the restriction modification system of the infected host cell. Dam methylation refers to methylation of Adenine in the sequence GATC, while dcm methylation results in modification of Cytosine in the sequence CC(A/T)GG, which is the recognition site for restriction with Eco RII.

Figures 11 and 12 show the restriction pattern obtained following digestion of ΦX174 RF DNA methylated to 5% and 27% respectively, using partially proteolysed DNA methylase (Tracks 1 and 2) and incubated with the same enzyme in the absence of S-Ado Met (Tracks 3 and 4). Tracks 1 and 3 show digestion with Hpa II, and Tracks 3 and 4 show digestion with Msp I. In Figure 11, some inhibition of Hpa II digestion (Track 1) is evident in comparison with the restriction pattern in the unmethylated control (Track 3). Several extra bands are visible in Track 1, indicating that methylation of CpG has occurred, blocking the action of Hpa II on its target site. No inhibition of Msp I is observed in Track 2 when compared with the control (Track 4).

In Figure 12, inhibition of Hpa II is again observed (Track 1). In addition, the restriction pattern of Msp 1 on methylated DNA (Track 2) appears to correspond closely to that of methylated DNA cleaved with Hpa II. However, it is possible that on this occasion Msp I failed to cut for reasons other than

FIGURE 11

Restriction enzyme digestion of methylated and unmethylated ΦX174 RF DNA.

Track 1 : Hpa II digestion of ΦX174 RF DNA methylated to 5%.

Track 2 : Msp I digestion of ΦX174 RF DNA methylated to 5%.

Track 3 : Hpa II digestion of unmethylated ΦX174 RF DNA.

Track 4 : Msp I digestion of unmethylated ΦX174 RF DNA.

Some inhibition of Hpa II digestion is evident in Track 1, indicating CpG methylation. No inhibition of Msp 1 is evident in Track 2.

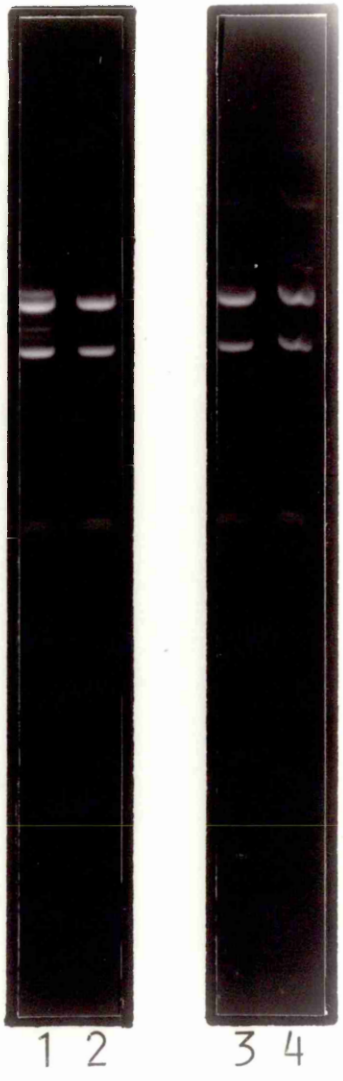

1 2
3 4

FIGURE 12

Restriction enzyme digestion of methylated and unmethylated ΦX174 RF DNA.

Track 1 : Hpa II digestion of ΦX174 RF DNA methylated to 27%.

Track 2 : Msp I digestion of ΦX174 RF DNA methylated to 27%.

Track 3 : Hpa II digestion of unmethylated ΦX174 RF DNA.

Track 4 : Msp I digestion of unmethylated ΦX174 RF DNA.


1 2 3 4

methylation of the 5' C in its recognition sequence, as the control digestion with unmethylated DNA also shows considerable failure to cleave (Track 4).

4.1.7 Fluorography of ^{3}H Methylated ΦX174 RF DNA

Figures 13 and 14 show fluorographs corresponding to the restriction digests show in Figures 11 and 12. A comparison of these fluorographs showing the restriction patterns of DNA methylated to 5% (Figure 13) and 27% (Figure 14) indicates that there is a greater level of inhibition of Hpa II action in the latter case. While there also appears to be some inhibition of Msp I (Track 2) it is not possible to correlate this solely to methylation of CpG sites in view of Figure 12 (Track 4). The specificity of the proteolysed enzyme for CpC sites therefore remains undetermined.

The difficulty of methylating ΦX174 DNA to a level of greater than 30% presents problems in the interpretation of results. It is possible that the proteolysed enzyme retains a sequence preference for cytosine residues in the dinucleotide CpG and methylates other sites at a lower frequency, undetectable at low overall levels of DNA methylation.

The nature of the proteolysed enzyme "activation" observed is unresolved at this stage. Further studies on the DNA recognition site of the proteolysed enzyme are required before it will be possible to state with certainty that the increased activity is not due to a loss of DNA site specificity by the enzyme.

FIGURE 13

Fluorograph of the restriction digests shown in Figure 11.

Following electrophoresis, agarose gels were incubated in 1 M Salicylic acid/1 M NaOH pH 6, for 1-2 h at RT. The gels were then washed in dH_2O, dried and autoradiographed.

Track 1 : Hpa II digestion of ΦX174 RF DNA methylated to 5%.

Track 2 : Msp I digestion of ΦX174 RF DNA methylated to 5%.

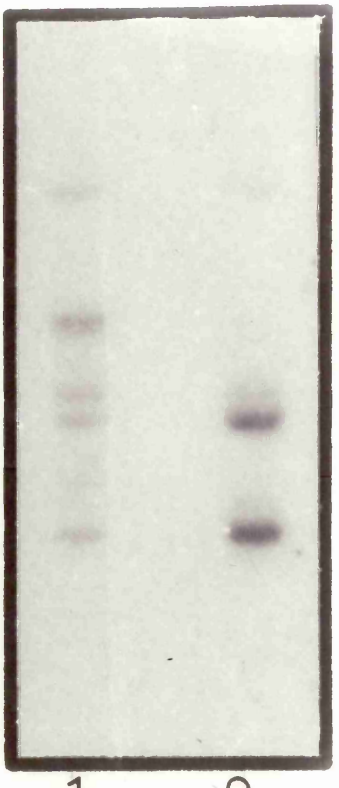

1
2

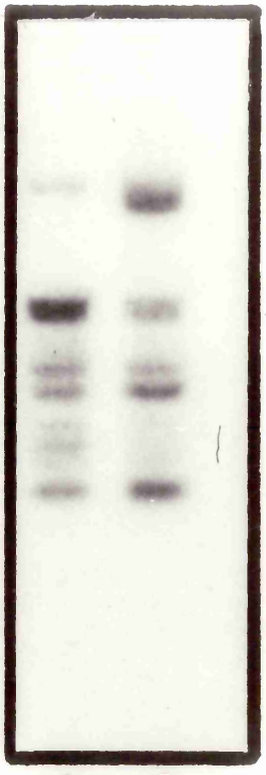
FIGURE 14

Fluorograph of the restriction enzyme digests shown in Figure 12.

Following electrophoresis, agarose gels were incubated in 1 M Salicylic acid/1 M NaCH pH 6, for 1-2 h at RT. The gels were then washed in dH_2O, dried and autoradiographed.

Track 1 : Hpa II digestion of ΦX174 RF DNA methylated to 27%.

Track 2 : Msp I digestion of ΦX174 RF DNA methylated to 27%.


1
2

CHAPTER 5 : PRODUCTION AND PRELIMINARY CHARACTERISATION OF RAT MONOCLONAL ANTIBODIES AGAINST DNA METHYLASE

Chapter 5 : Production and Preliminary Characterisation of Rat Monoclonal Antibodies Against DNA Methylase

5.1 Introduction

The task of preparing monoclonal antibodies against DNA methylase was undertaken to provide a more precise immunological tool to study characterisation of the enzyme in greater detail. In this section the production techniques used to generate monoclonal antibodies, and details of the preliminary characerisation of antibody-secreting hybrid clones will be discussed.

5.2 Choice of Animals and Cell Lines

The rat system was chosen primarily because of the nature of the antigen - in these studies, mouse DNA methylase. In general, a greater response is elicited in the host by a foreign antigen. DA rats were used as recipients for the antigen, and the rat myeloma line Y3.Ag 1.2.3 (Y3) was used as the fusion partner for spleen cells in all fusion experiments. The Y3 line is derived from the LOU/C strain of rats (Bazin et al, 1973). Y3.Ag 1.2.3 is an azaguanine resistant derivative of the myeloma R210.RCY3, and secretes kappa light immunoglobulin chains. Azaguanine resistance is selected as a means of isolating $HPRT^-$ cells. 8-azaguanine is a cytotoxic base analogue which is incorporated into DNA via HPRT. Cells in which only the main purine biosynthetic pathway is functional and which therefore lack HPRT can continue to grow in the presence of 8-azaguanine, while cells operating both the main and salvage synthetic pathways will die.

5.3 Procedure for the Isolation of Monoclonal Antibodies

In the development of a protocol for the production of monoclonal antibodies against DNA methylase, several variations of procedures were considered to establish optimum fusion conditions. Davidson et al (1976) and Klebe and Mancuso (1981) have reported that most successful fusions are performed using polyethylene glycol (PEG) in the molecular weight range 600 - 6000. In these studies 50% PEG 1500 supplied by Boehringer in 75 mM Hepes buffer pH 7.5 was used as the fusing agent. The reported optimum pH for fusions lies between 7.55 - 8.20 (Klebe, 1981) while 75 mM Hepes is thought to be the most satisfactory buffer (Sharon, 1980). DMSO is reported to enhance fusion frequency (St Groth and Scheidegger, 1980). In a comparative study of fusions carried out using PEG 1500 in the absence and the presence of 5% DMSO, it was found that a greater proportion of fused cells resulted from the latter approach.

As PEG is toxic to cells, the amount of PEG 1500 used and the length of time it remained in contact with the cells proved critical. 1 ml of PEG 1500 per 1.5 x 10^8 cells (typical fusion) proved sufficient for a successful fusion. Incubation times of greater than 90 seconds frequently proved fatal to most of the cells.

The fusion procedure used to generate hybridomas is given in Section 2.2.3.4.

5.4 Hybridoma Techniques

5.4.1 Selection of Fused Cells in HAT Medium

To eliminate non-fused myeloma cells from the fusion mixture, selective pressure against the parent myeloma (Y3.Ag 1.2.3) was introduced by maintenance of the cells in medium containing Hypoxanthine, Aminopterin and Thymidine (HAT medium). The absence of the enzyme Hypoxanthine-Guanine phosphoribosyl transferase (HPRT) from the parent myeloma line forms the basis for selection of hybridomas in HAT medium. Aminopterin, a folic acid antagonist, blocks the main biosynthetic pathway of purine and pyrimidine synthesis.

Salvage pathways by which exogenous nucleosides are utilised for synthesis of deoxynucleotide triphosphates, involve the enzyme Thymidine Kinase (TK) in the pyrimidine pathways and HPRT in the purine pathway. TK catalyses the conversion of exogenous thymidine to dTMP, subsequently phosphorylated to dTTP. In the purine salvage pathway the conversion of hypoxanthine and guanine to IMP, the precursor of both AMP and GMP, is dependent on the enzyme HPRT. Cells lacking this enzyme are unable to grow in HAT medium as both metabolic pathways for purine biosynthesis are blocked. Normal spleen cells remain viable in culture for only a few days, hence only fused cells possessing HPRT activity and the ability to divide indefinitely will grow.

It is essential to eliminate unfused myeloma cells from the mixed culture as soon as possible, as rapidly growing

myeloma cells can easily dominate cultures containing initially slow growing hybridomas.

5.4.2 Maintenance of Hybridoma Cultures

In general, colonies of fused cells were visible 7-10 days after the fusion, although all plates were monitored for several weeks to detect initially slow-growing hybridomas. The cells were maintained in HAT medium for 4-5 weeks, before transfer to medium containing HT for one week to ensure the complete removal of aminopterin. Following this the cells were maintained in normal growth medium.

Approximately 3 weeks after the fusion, when hybrid cells had grown to near confluency, supernatants were removed from the wells and tested for specific antibody content by ELISA assay. Positive wells were subcloned several times and re-tested by ELISA. At each stage of cloning, aliquots of cells were frozen to safeguard against loss of secretion of specific antibody, or culture contamination. Occasionally, subclones initially established to be positive with regard to secretion of specific antibody were later found to be indistinguishable from control supernatants when tested by ELISA, indicating that some reversion did occur. In general, however, cells which were positive on the parent plate retained this identity throughout cloning.

5.5 Specificity of the Monoclonal Antibodies

5.5.1 Tests for Monoclonal Antibody Specificity

From a large number of established clones, 6 hybridomas were selected for characterisation on the basis of their rapid growth and positive assay by ELISA. Several tests were selected to determine whether the antibodies produced were specifically directed against DNA methylase, namely:

(A) Inhibition of enzyme activity.

(B) Immune precipitation of the enzyme.

(C) Immunodetection of DNA methylase on Western blots.

Initially, crude supernatants from hybrid clones were used in these tests and appeared to cause inhibition of enzyme activity. However, controls of unfused myeloma supernatants gave a similar result, indicating that some component in the cell medium was the most likely cause of this observation. It therefore proved necessary to isolate immunoglobulins from the cell supernatants to use in these tests.

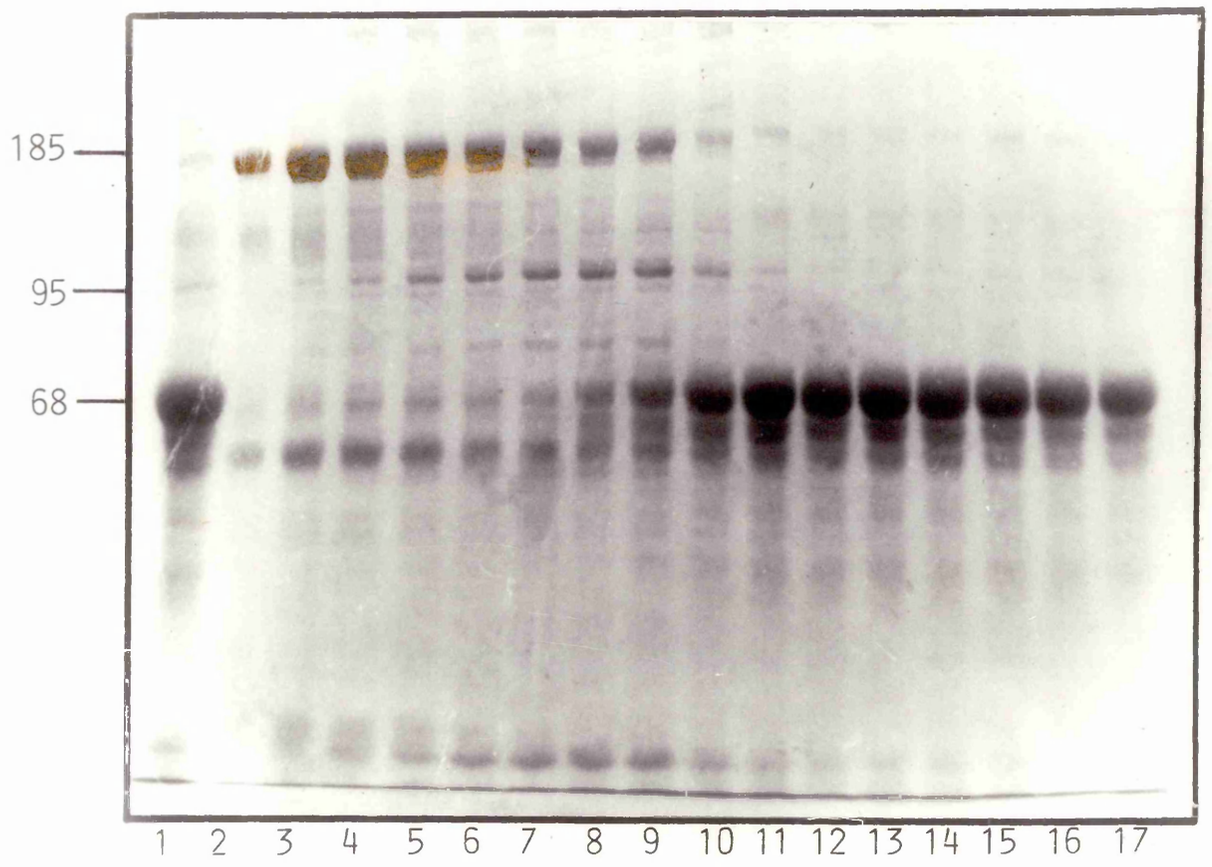
Purification of monoclonal immunoglobulin from supernatants is described in Section 2.2.3.11. Following precipitation in ammonium sulphate and fractionation on QAE Sephadex-A50, samples were assayed by ELISA to determine the peak immunoglobulin containing fraction. Immunoglobulins from 6 hybrid clones: 4B, 4E, 4H, 5G, 6E, 8E were prepared in this way. Figure 15 shows SDS gel electrophoresis of the QAE Sephadex fractionation of immunoglobulin 4B.

FIGURE 15

Coomassie Stained Gel of Monoclonal Immunoglobulin 4B, fractionated from the hybridoma cell supernatant.

Track	Sample
1	Markers: 185 kD, 95 kD, 68 kD
	ml Eluted from QAE
2	6
3	7
4	8
5	9
6	10
7	11
8	12
9	13
10	14
11	15
12	16
13	17
14	18
15	19
16	20
17	21

Tracks 2-17 show 1 ml samples collected from QAE Sephadex. Optimum activity (as measured by ELISA assay) was contained in the sample shown in Track 10.


185
95
68
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

5.5.2 Fractionation and Titration of Monoclonal Immunoglobulins

Figure 16 shows the elution profile of purified immunoglobulins from cell supernatants following fractionation on QAE Sephadex A50. Individual 1 ml fractions collected were tested by ELISA assay (absorbance measured at 492 nm) and the protein content was measured by the method of Bradford (1976). As a control, immunoglobulin from unused complete medium containing 10% (v/v) FCS was isolated and assayed by the same procedure. The maximum ELISA reading of the control sample was found to be 0.2, while immunoglobulins isolated from hybrid clones show maximum values ranging from 0.50 - 0.84. Maximum Ig activity (as measured by ELISA assay) eluted before the major protein peak in each sample.

To give an approximate measure of the degree of antibody affinity for the enzyme, peak immunoglobulin fractions were titrated. Antibody dilutions ranged from 1/2 - 1/128. Figure 17 shows the titration of immunoglobulin from samples 8E, 4E, 4H and 6E.

5.5.3 The Use of Monoclonal Immunoglobulin to Neutralise DNA Methylase Activity

Section 2.2.3.13 describes the procedure used to determine whether monoclonal immunoglobulins can inactivate DNA methylase activity. Varying amounts of monoclonal antibody were incubated with DNA methylase to allow binding of specific antibody to the enzyme. The mixture was then assayed as described in Section 2.2.1.10.2. Controls included enzyme incubated with

FIGURE 16

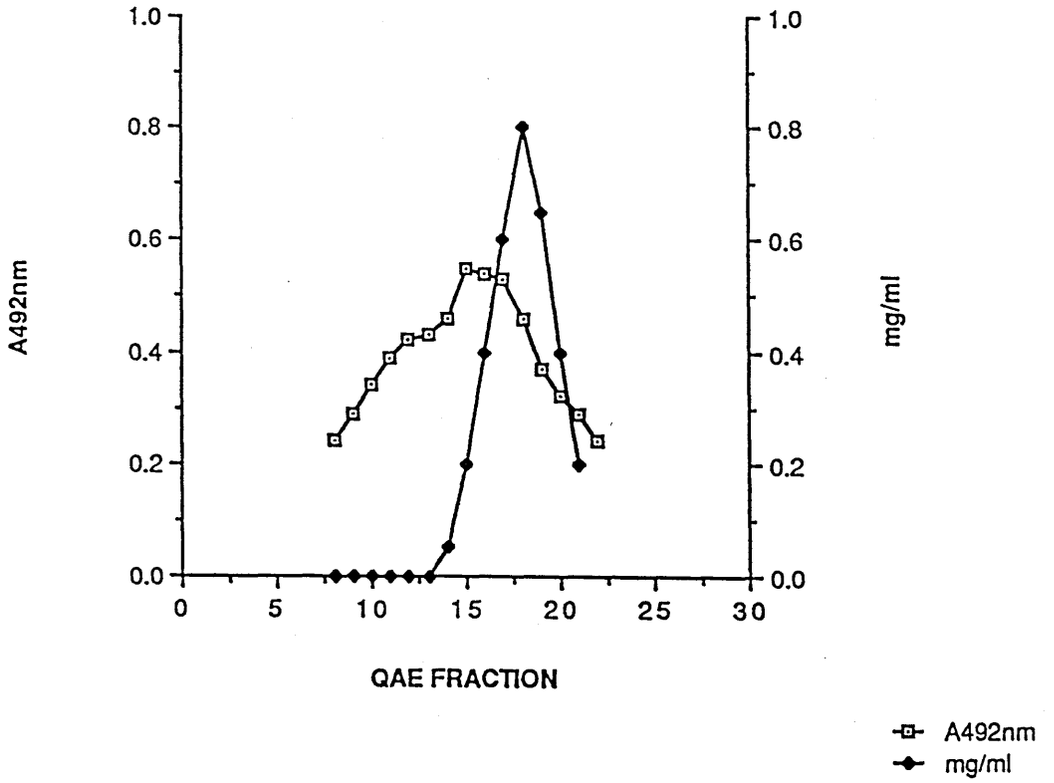
Elution of Monoclonal Immunoglobulin from QAE Sephadex A-50.

Graphs show the degree of interaction of the fractionated monoclonal immunoglobulin with DNA methylase as measured by ELISA assay (A492 nm), and the corresponding protein content of the samples.

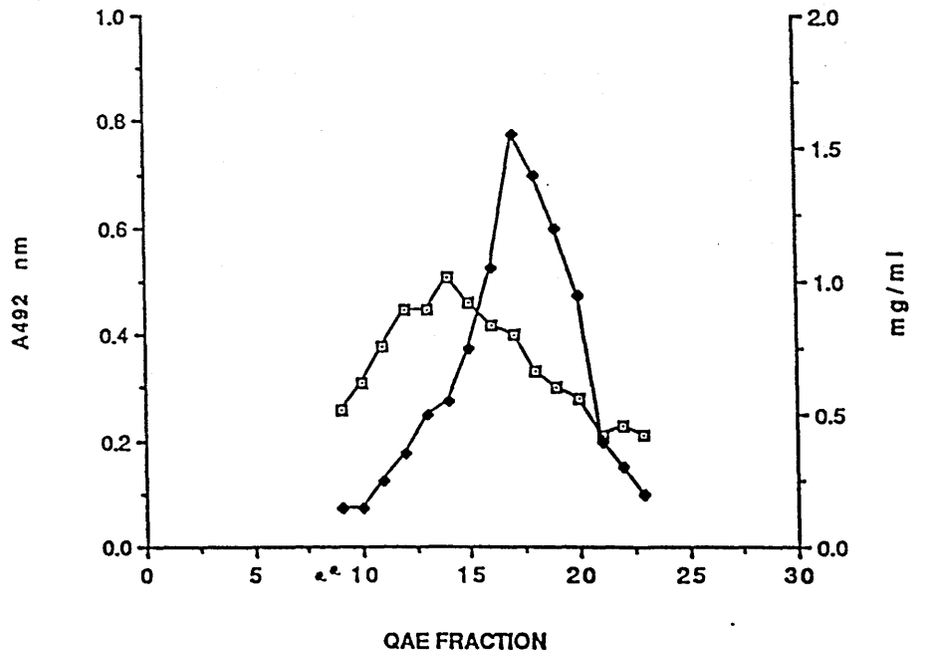
Graph	Immunoglobulin
A	4H
B	5G
C	4E
D	6E
E	4B
F	FCS Ig

Figure 16

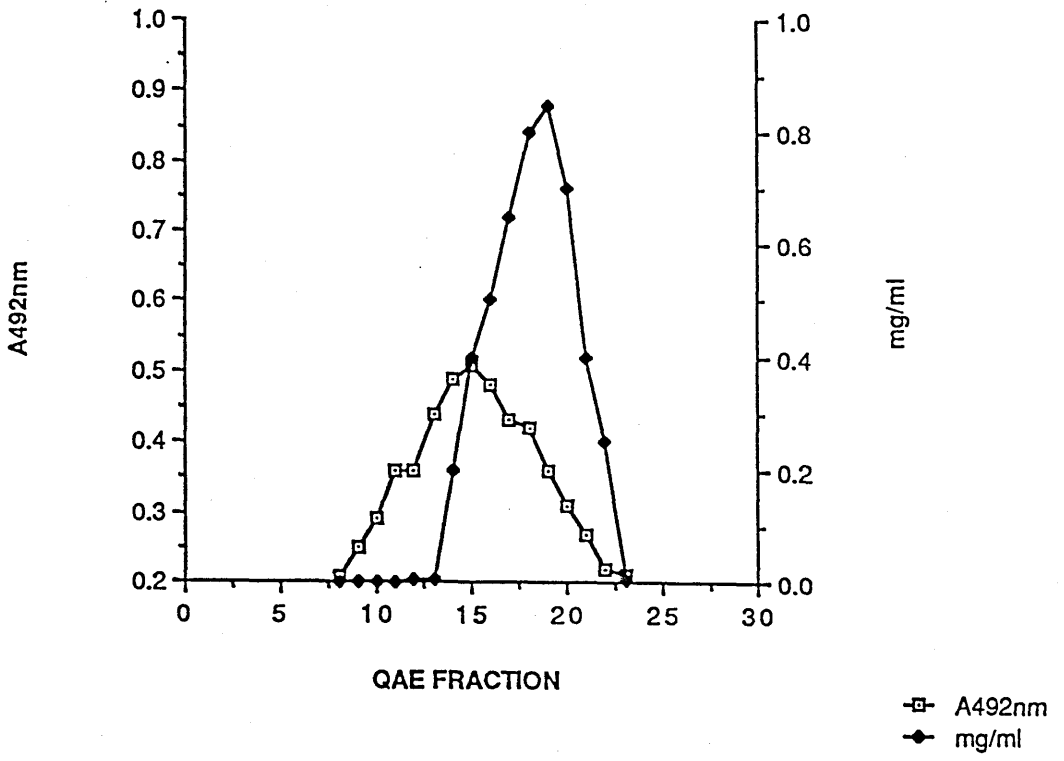
(A)


1.0
0.8
0.6
0.4
0.2
0.0
A492nm
mg/ml
0
5
10
15
20
25
30
QAE FRACTION
A492nm
mg/ml

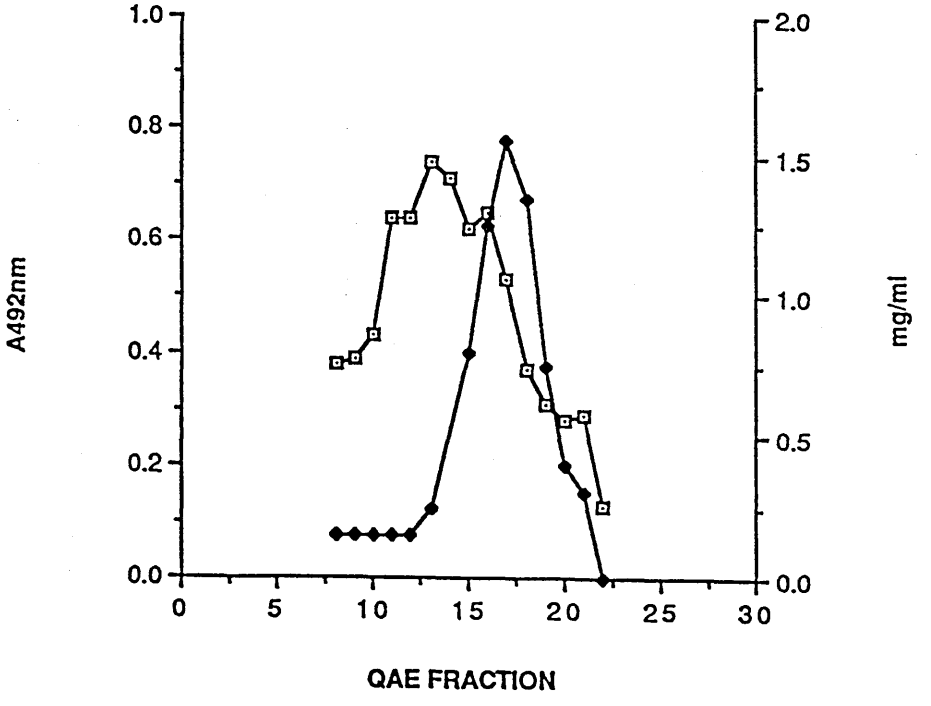
(B)


1.0
0.8
0.6
0.4
0.2
0.0
A492 nm
2.0
1.5
1.0
0.5
0.0
mg/ml
0
5
10
15
20
25
30
QAE FRACTION

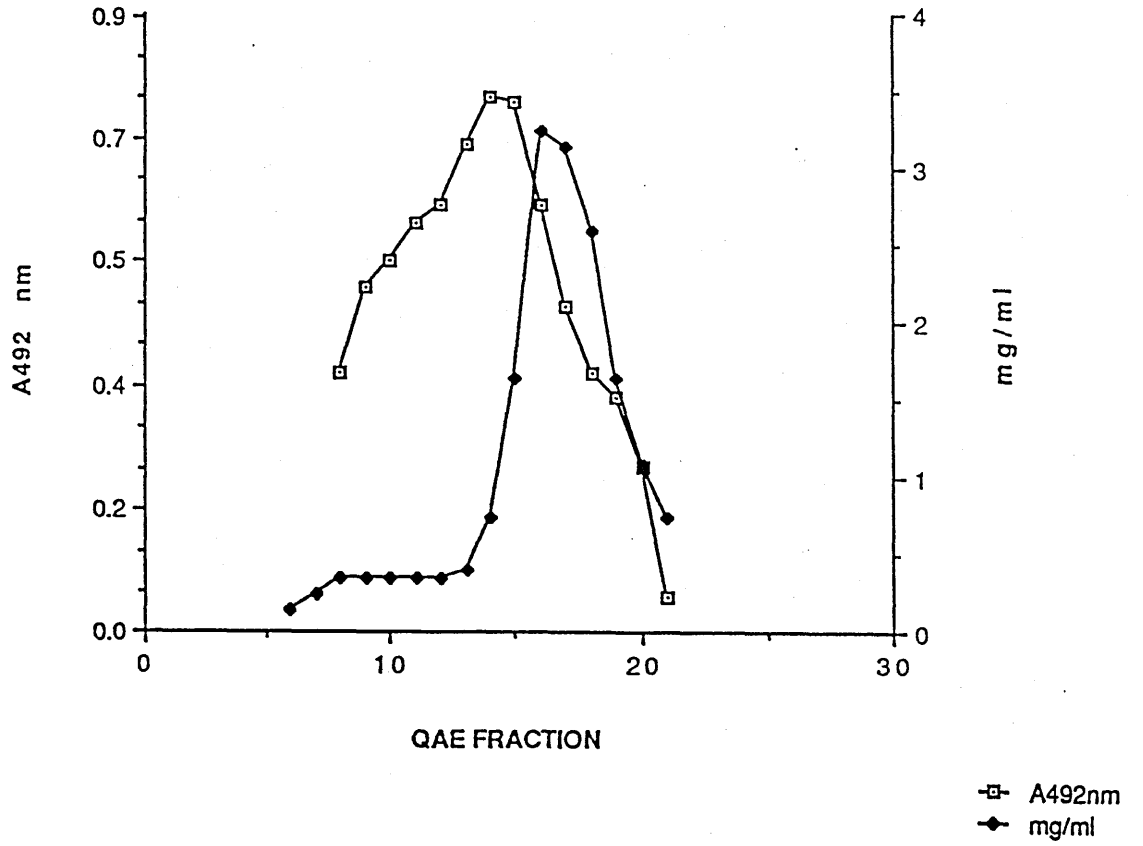
(C)


A492nm
mg/ml
QAE FRACTION
A492nm
mg/ml

(D)


A492nm
mg/ml
QAE FRACTION

(E)


A492 nm
mg/ml
QAE FRACTION
A492nm
mg/ml

(F)

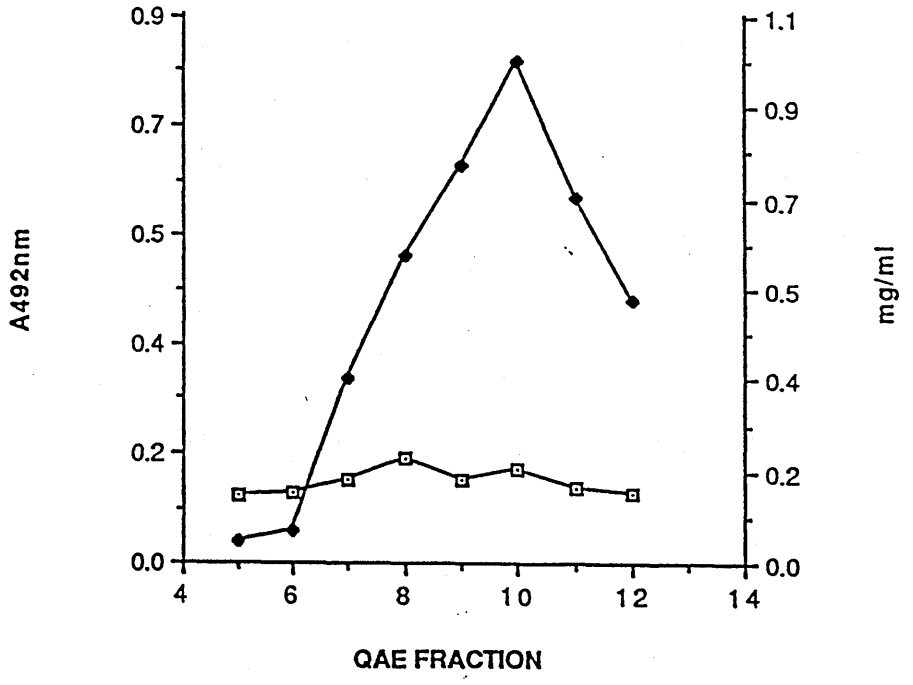

A492nm
mg/ml
QAE FRACTION

FIGURE 17

Titration of Monoclonal Immunoglobulin.

To determine the affinity of the purified immunoglobulin for DNA methylase, samples showing maximum Ig activity (as measured by ELISA, see Figure 16) were titrated.

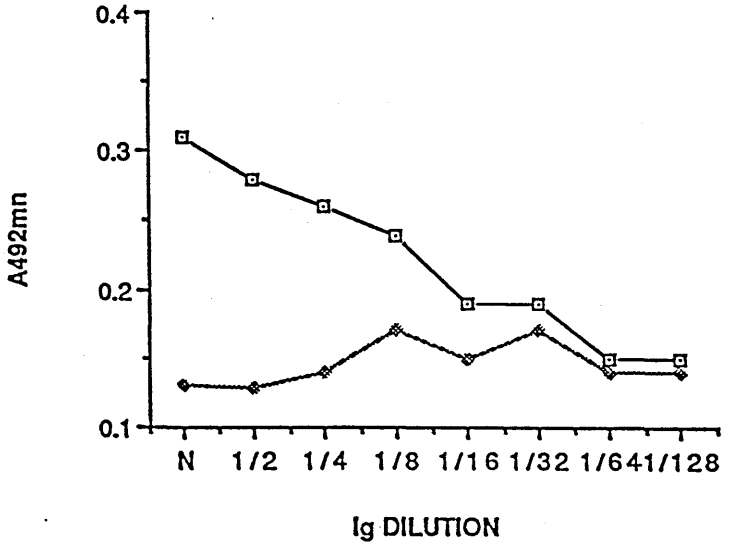
Graphs in Figure 17 show ELISA results of the peak immunoglobulin fraction at dilutions ranging from 1/2 to 1/128 (N = Neat, undiluted Ig).

Ig isolated from unused cell culture medium containing foetal calf serum was used as the control. The titres of the purified immunoglobulin fractions fell between Ig dilutions of 1/32 and 1/64.

Figure	Sample
A	Titration of Ig 8E
B	Titration of Ig 4E
C	Titration of Ig 4H
D	Titration of Ig 6E

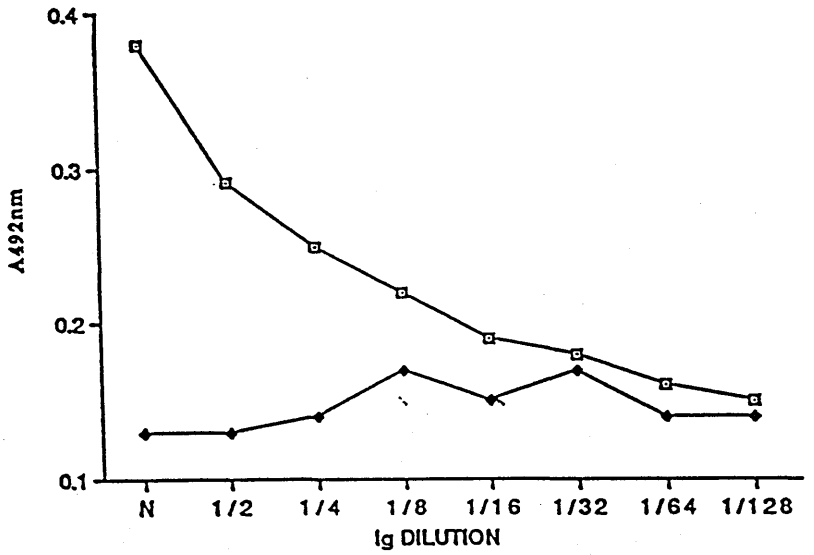
Figure 17

(A) TITRATION OF IMMUNOGLOBULIN 8E

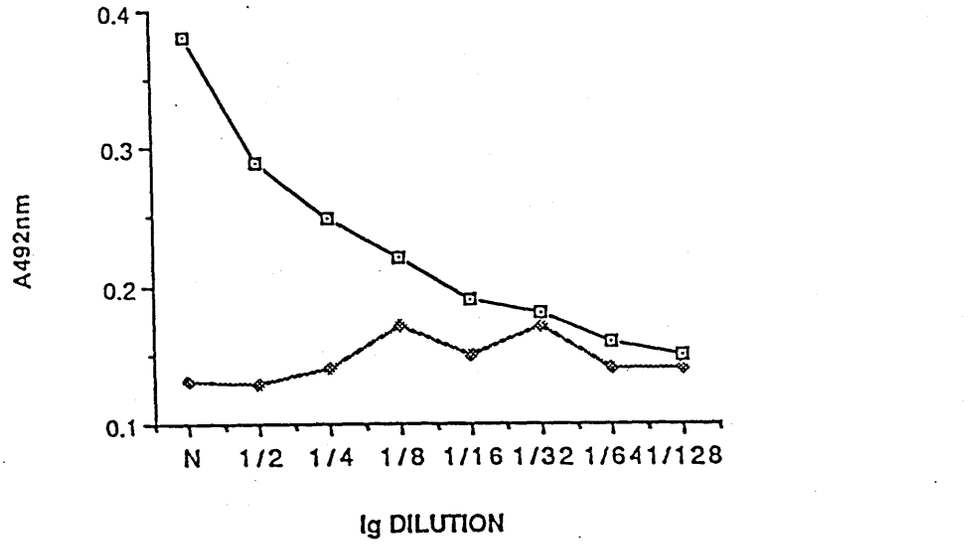


Mono Ig
Control

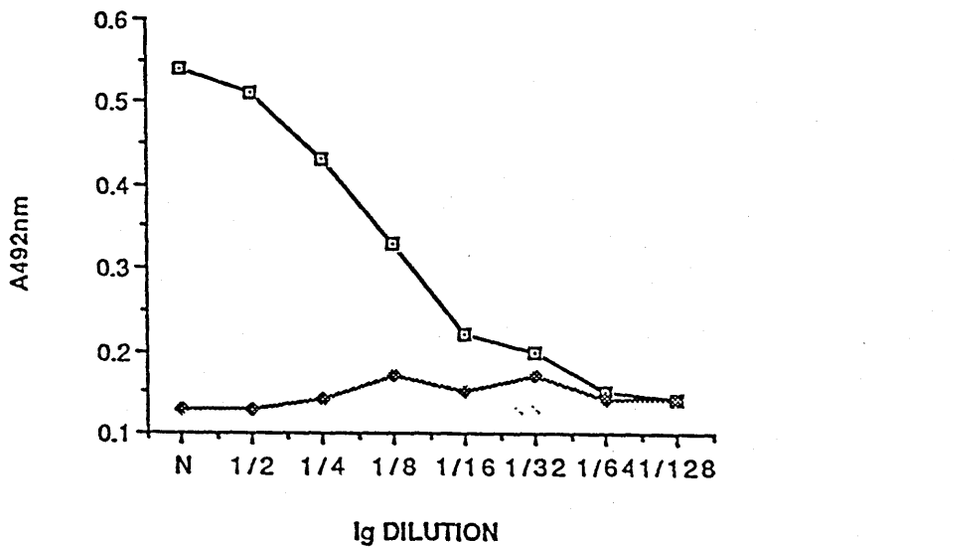
(B) TITRATION OF IMMUNOGLOBULIN 4E



(C) TITRATION OF MONOCLONAL 4H



(D) TITRATION OF IMMUNOGLOBULIN 6E



immunoglobulin isolated from unused culture medium and enzyme incubated in PBS prior to assay.

Figure 18 show the enzyme assay results following preincubation with various immunoglobulins. No marked inhibition of enzyme activity was detected. In some cases (namely Figure 18 B, C and D) stimulation of enzyme activity was observed although presumably this is not immunoglobulin related.

5.5.4 Precipitation of DNA Methylase by Monoclonal Immunoglobulin

Precipitation of DNA methylase by purified monoclonal immunoglobulins is described in Section 2.2.3.14. DNA methylase was pre-incubated with varying amounts of immunoglobulin to allow formation of an immune complex. The mixture was centrifuged and the supernatant assayed in a standard DNA methylase assay. Figure 19 show the assay results obtained. Few samples showed values below the level of the control, indicating that the enzyme did not appear to have formed a precipitable complex with the immunoglobulin, and again there appears to be stimulation of enzyme activity in some cases.

The failure either to precipitate the enzyme from solution using monoclonal immunoglobulins, or to inactivate DNA methylase activity, will be discussed in Chapter 6.

FIGURE 18

Inhibition of DNA Methylase by Monoclonal Immunoglobulin.

Varying amounts of monoclonal immunoglobulin were pre-incubated with DNA methylase for 12 h at 4°C to allow binding of specific antibody to the enzyme. The enzyme - Ig samples were then assayed as described in Section 2.2.1.10.2. The control sample "C" on each graph shows enzyme activity following pre-incubation of DNA methylase with PBS prior to assay.

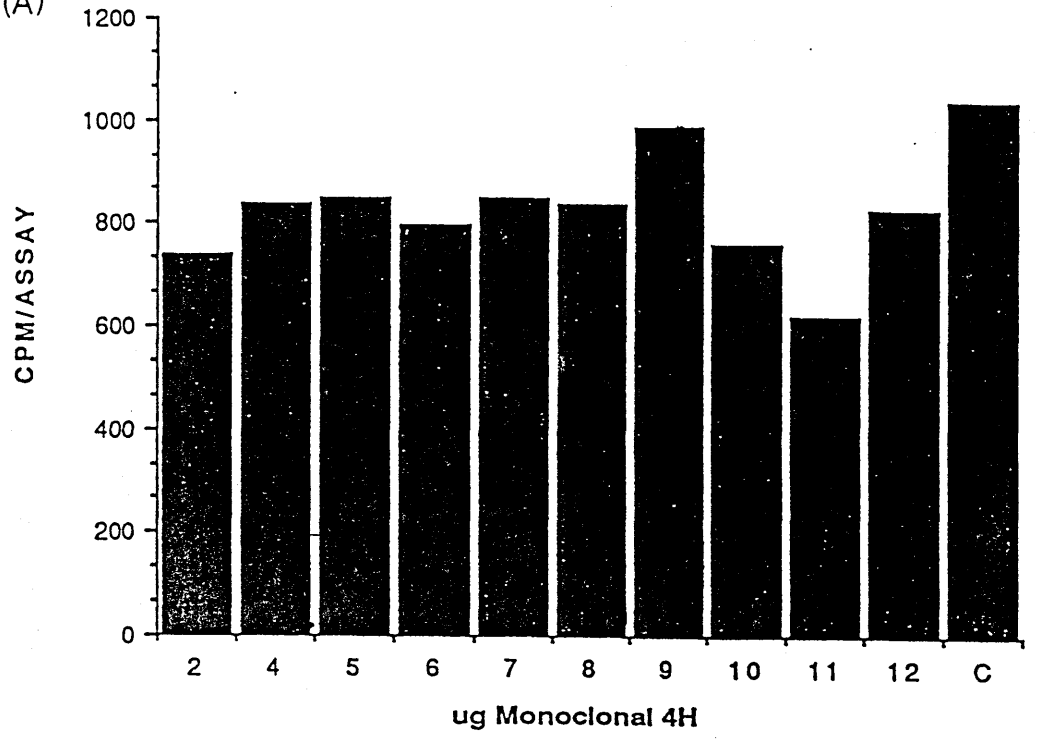
Graph

A	Enzyme pre-incubation with Ig 4H
B	Enzyme pre-incubation with Ig 4B
C	Enzyme pre-incubation with Ig 4E
D	Enzyme pre-incubation with Ig 5G

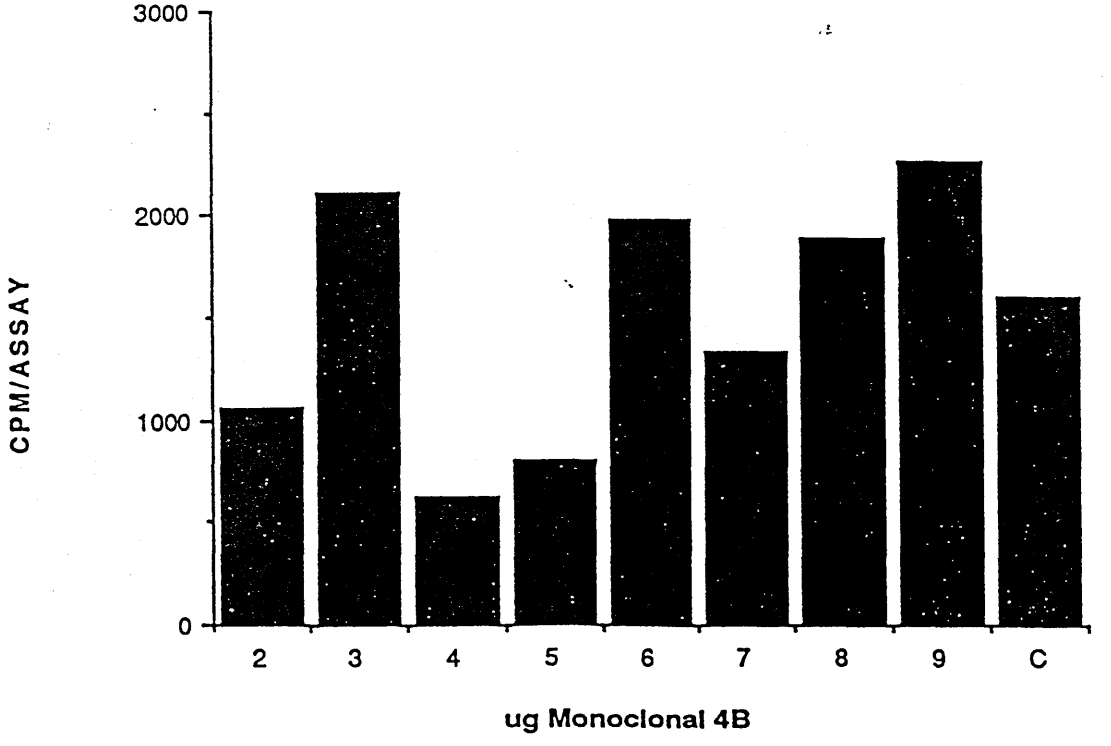
Figure 18

INHIBITION OF DNA METHYLASE BY MONOCLONAL IMMUNOGLOBULIN

(A)

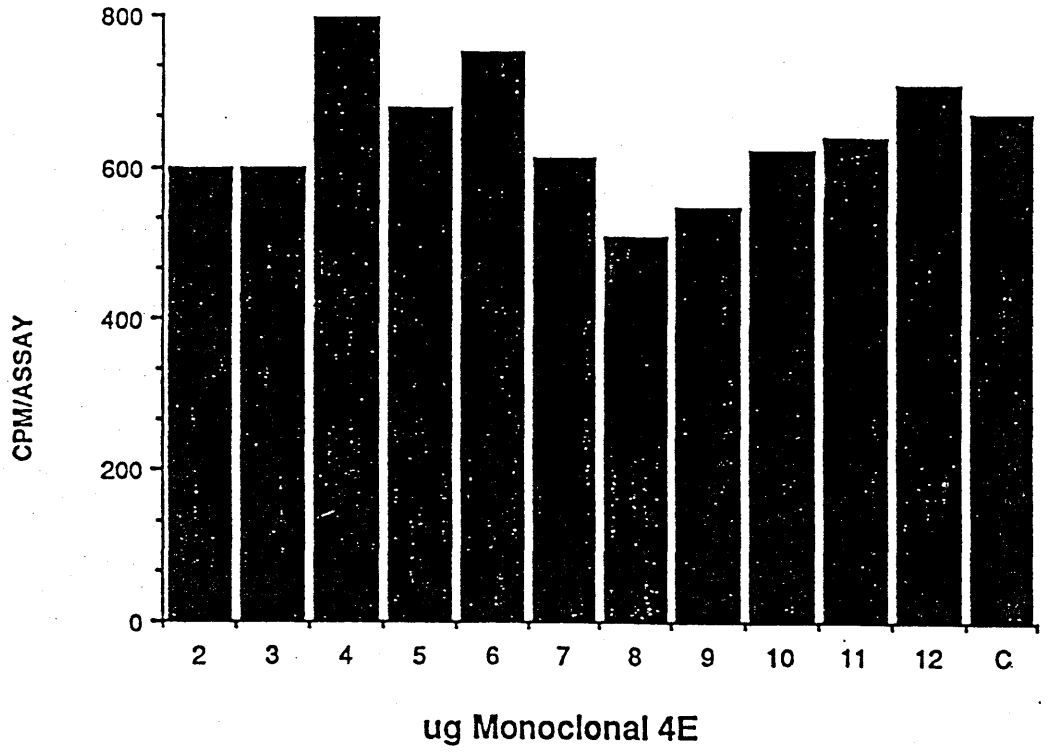


(B)



INHIBITION OF DNA METHYLASE BY MONOCLONAL IMMUNOGLOBULIN

(C)



(D)

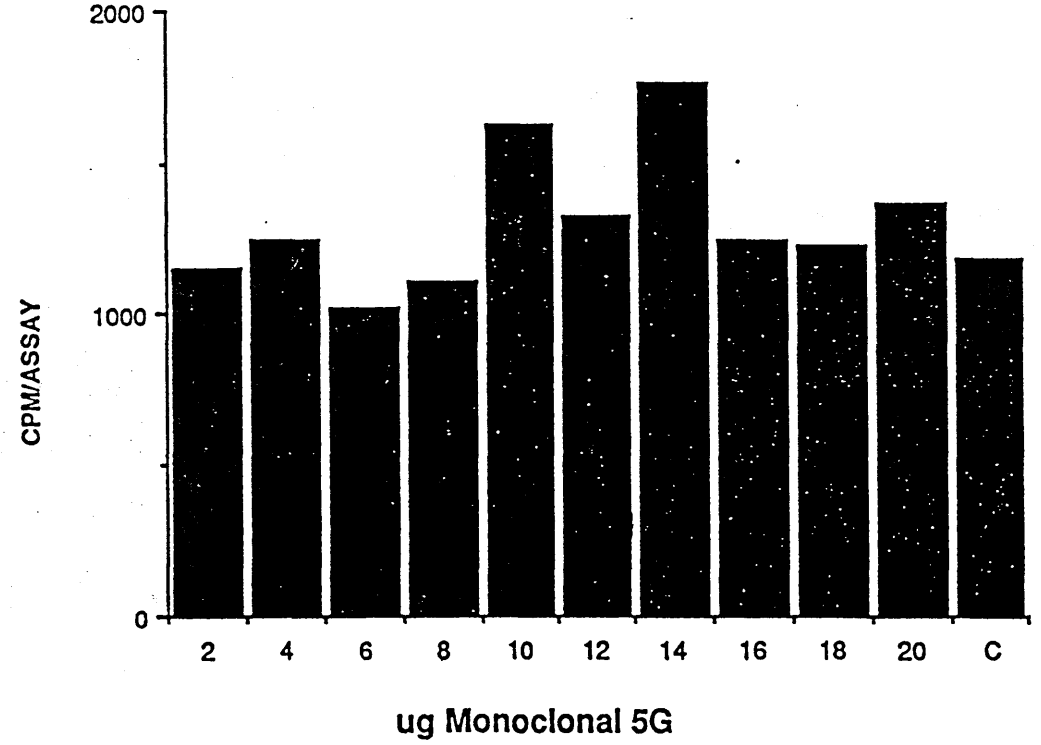


FIGURE 19

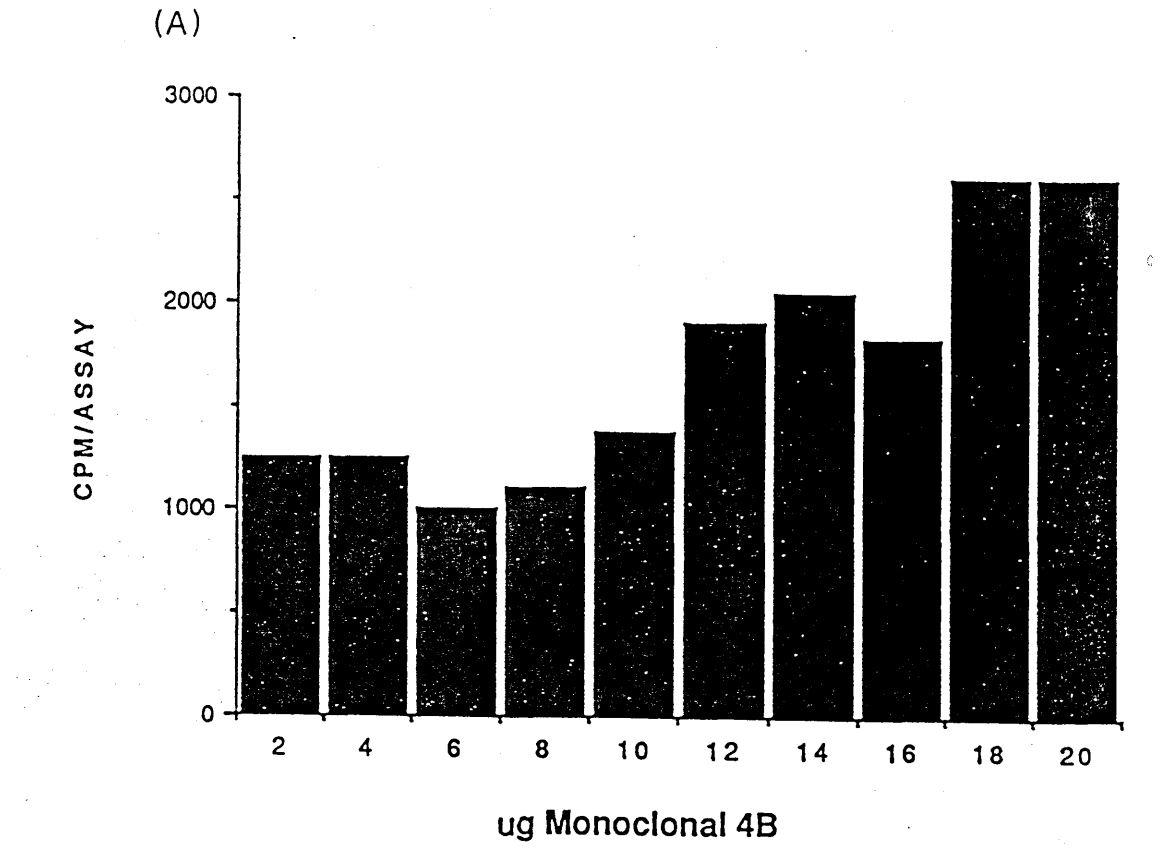
Precipitation of DNA Methylase by Monoclonal Immunoglobulin.

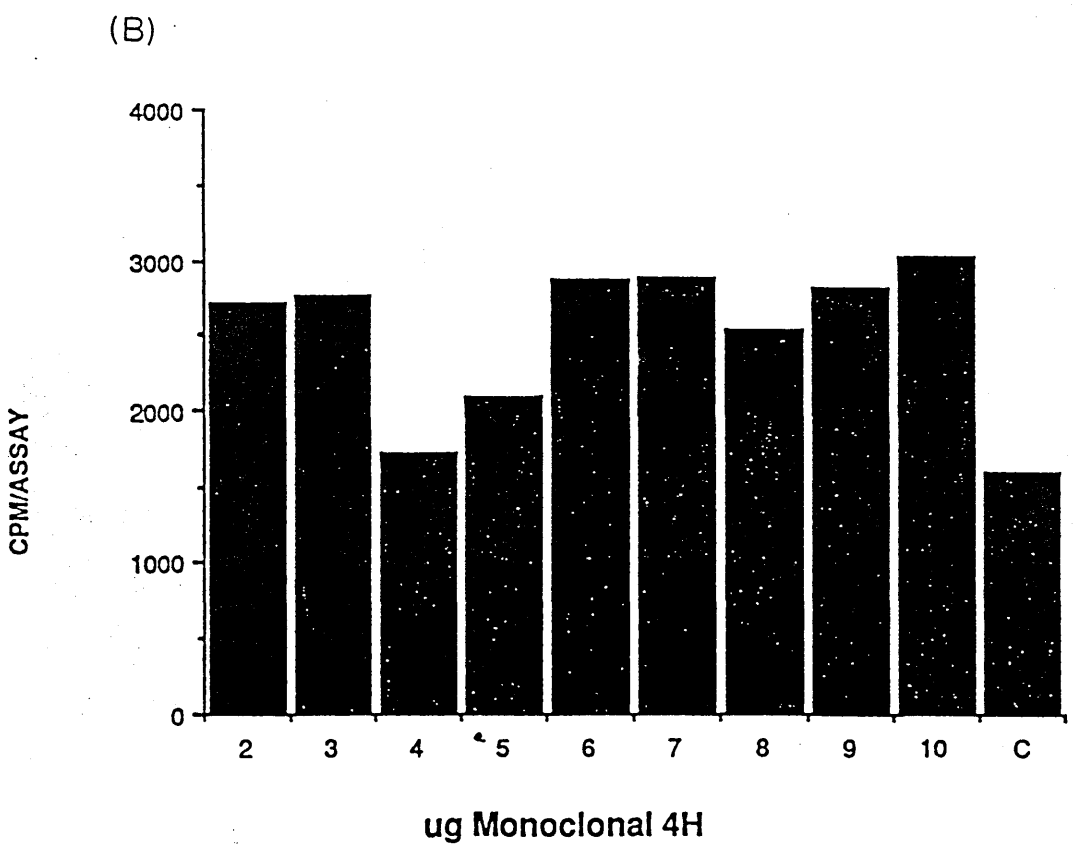
DNA methylase was pre-incubated with purified monoclonal Ig to allow formation of immune complexes. The mixture was centrifuged and the supernatant was assayed for enzyme activity as described in Section 2.2.1.10.2. The control sample "C" on graph B shows the activity of enzyme pre-incubated in PBS. This control sample refers to the experiments shown in graphs A and B.

Graph

A	Immunoprecipitation of enzyme by Ig 4B
B	Immunoprecipitation of enzyme by Ig 4H

Figure 19


(A)
3000
2000
1000
0
CPM/ASSAY
2
4
6
8
10
12
14
16
18
20
ug Monoclonal 4B


(B)
4000
3000
2000
1000
0
CPM/ASSAY
2
3
4
5
6
7
8
9
10
C
ug Monoclonal 4H

5.5.5 Immunodetection of DNA Methylase by Monoclonal Immunoglobulin

Monoclonal immunoglobulins were used to probe DNA methylase hybridised on a nitrocellulose membrane (Section 2.2.3.15). Attempts to illuminate the enzyme on the membrane using purified immunoglobulin and I^{125}-labelled protein A were unsuccessful. It is unclear whether this was due to a low specificity of the antibody molecules for the denatured protein, the absence of a sufficient number of antibody molecules to react with the enzyme or a lack of specificity of the antibodies for DNA methylase. The implications of this result will be discussed at length in Section 6.6.

Since protein A interacts with only a minor subclass of rat IgG species, it is possible that an alternative detection method will prove succesful in identifying interactions of DNA methylase on Western blots with specific monoclonal antibodies . Rabbit anti-rat whole Ig can be used as a linker molecule, creating a sandwich of monoclonal Ig - anti rat Ig -I^{125} protein A. By this means a wider range of monoclonal antibody species may be identified, as anti-rat whole Ig interacts with the four rat IgG subclasses and IgM.

CHAPTER 6 : DISCUSSION

6.1 Introduction

Since the discovery of the enzymatic methylation of DNA by Burdon et al. (1967) various studies have been undertaken to purify and characterise DNA methylase from a wide range of mammalian sources. The difficulties of this task are reflected in the fact that, to date, many structural features of the enzyme and its mode of catalysis remain incompletely characterised. The strategy underlying the use of antibodies in the present work is outlined in Section 1.5.

6.2 Factors Influencing Antibody-Antigen Interactions

Interactions between an antibody and a protein antigen occur subject to a variety of factors influencing the degree of complementarity between the two molecules: The molecular shape of the interacting molecules, surface charges, hydrogen bonding and hydrophilic/hydrophobic interactions all contribute to the potential for antibody-antigen binding. Mobility and accessibility of surface determinants may influence the antigenicity of the molecule, although it is likely that all accessible regions will be potentially antigenic. Antigenic determinants on protein antigens are surface structures which may be formed by contiguous or non-contiguous amino acid sequences. Antibody-antigen interactions may effect significant structural changes in the antibody or antigen, or there may be no detectable alteration, as both flexible and rigid structures can be antigenic. The mechanism of antibody-antigen interaction can take the form of a rigid "lock and key" mechanism, where the antibody and antigen surfaces complement one another prior to binding (Fischer,

1894), illustrated in the binding of the D1.3 Fab lysozyme complex (Amit et al, 1986). Alternatively, an "induced fit" mechanism may operate, whereby some degree of conformational change in either or both of the interacting molecules contributes to the final binding configuration. For antibodies this may involve movement of the hypervariable loops or possibly major movement of the two variable domains of the antibody relative to one another (Davies et al, 1988).

Some significant conformational change in the antigen is also thought to accompany binding (Getzoff et al, 1987, Geysen et al, 1987). It seems likely that some conformational changes in both antibody and antigen molecules will occur as their surfaces are brought into contact. The antibody combining site is formed by the juxtaposition of the V_H and V_L domains which are joined by non-covalent forces. Interaction of these sites with antigen may result in the positioning of V_H relative to V_L such that the complementarity of the antibody for the antigen is increased.

6.3 Antibodies as Probes

The decision to use both polyclonal and monoclonal antibodies as probes to study DNA methylase was based on the premise that each type of antiserum has distinct advantages for the detection and analysis of antigen under different *in vitro* and *in vivo* conditions. While some degree of non-specific interactions may occur using polyclonal antiserum, the specificity of monoclonal antibodies for single determinants on the antigen may prove impracticable for detection by some screening methods, with polyclonal antibodies

providing a more comprehensive signal. In other cases the specificity of monoclonal antibodies for antigen can be used to advantage in the precise detection of antigenic determinants, without interference from non-specific antibody-antigen interactions, often inevitable using polyclonal antiserum.

Occasionally, unexpected cross reactivity of monoclonal antibodies can occur (Lane et al, 1982). This may be due to the detection of structurally similar epitopes in two antigens, or to multispecificity of the antibody combining site, enabling it to stably bind structurally unrelated antigens through different bonding interactions.

Relatively high yields (up to 1mg/ml) of antibody can be obtained from polyclonal antiserum and used to detect antigen by conventional immunological assays, e.g. ELISA, immunoprecipitation, Western blotting, radioimmunoassay and immunocytochemistry. However, despite extensive purification of the antigen prior to immunisation, antibodies against minor contaminants may still arise, resulting in some non-specific cross reactions by the antiserum. Highly immunogenic contaminants may result in non-specific antibodies outnumbering the required specific antibodies.

6.4 Comparison of Monoclonal and Polyclonal Antibody Interactions

Polyclonal antiserum, by definition, consists of a heterogeneous population of hundreds or thousands of clonal products

which are capable of interacting with many or all of the antigenic determinants accessible on the antigen surface (Benjamin et al, 1984). Binding conditions for polyclonal antibodies are generally effective over a broad range of pH (4-9) and salt concentrations (0-1M NaCl). Within the population, antibodies differ widely in their affinity for the target antigen. In contrast, monoclonal antibodies consist of an homogenous clonal product, with antibodies usually binding at a single unique site on the target antigen. A monoclonal antibody has a uniform affinity for the antigen. The range of conditions over which monoclonal antibodies are operative is often more discrete than that described for polyclonal antibodies, though it may vary from one monoclonal antibody population to another.

The reactivity of immune serum generated against native protein antigen is often much weaker when tested on the denatured protein (Arnon, 1973) although in general, polyclonal antiserum contains some antibodies which can recognise the denatured protein. The formation of three dimensional lattice structures, formed by cross-linking between polyclonal antibodies and antigen does not generally occur on incubation of antigen with a single species of monoclonal antibody (with the exception of antigen molecules exhibiting two or more identical binding sites). In addition, because the antigen is bound by interaction to only one monoclonal antibody, the affinity of the antibody for antigen is critical. Monoclonal antibodies with affinities less than 10^7 mol^{-1} may prove to be unsuccessful in immunoprecipitation of antigen. Screening techniques for hybridomas will detect antibodies with an affinity of 10^6 mol^{-1} or greater (Lane, 1982) therefore not all monoclonals

will be suitable for use in immunoprecipitation assays.

Cross-linking and immunoprecipitation of antigen can usually only be achieved using two or more species of monoclonal antibodies. The use of pooled monoclonal antibodies incorporates the best properties of polyclonal and individual monoclonal antibodies, combining a high degree of specificity with the ability to form stable immune complexes. The use of selectively pooled combinations of monoclonal antibodies is likely to provide the best reagent for immunoprecipitation of antigen.

6.5 Interaction of Monoclonal Antibodies with DNA Methylase

The failure to precipitate DNA methylase using a single species of monoclonal antibody most likely reflects the specificity of the antibodies for single non-repetitive determinants on the enzyme. Neutralisation of enzyme activity by a single species of monoclonal antibody would only be expected to occur in cases where the antibody was directed against an epitope near the active site, or if binding of the antibody prohibited the enzyme from binding DNA or S-Ado Met. Adams et al (personal communication) have recently demonstrated that a pool of four monoclonal antibodies can cross-link DNA methylase and inhibit enzyme activity by 100%, while the individual monoclonal antibodies reduce enzyme activity by less than 50%. The use of a secondary antibody binding species can enhance the precipitation of small immune complexes formed between monoclonal antibodies and antigen. Protein A, synthesised by Staphylococcus aureus is commonly used in the detection or purification of antibodies. This protein binds to the Fc

region of many IgG molecules with high affinity and specificity. Pansorbin, a standardised extract of Staphylococcal protein A has proved useful in the detection of polyclonal antibodies against DNA methylase.

Although rabbit IgG has a high affinity for protein A, the majority of rat immunoglobulins have a much lower affinity in comparison (Rousseaux et al, 1981; Nilsson et al, 1982) thus limiting its use in the detection of rat monoclonal antibodies. Anti-immunoglobulin or protein G (Pharmacia) are likely to prove effective substitutes for protein A in the binding and detection of rat monoclonal antibody-antigen complexes.

6.6 Immunoblotting with Monoclonal Antibodies

The problem of detecting denatured antigen with uniform affinity monoclonal antibodies directed against the non-denatured protein has been mentioned in Section 6.4. The heterogeneity of a polyclonal antibody population is such that small changes in the structure of the antigen, such as glycosylation, generally have little effect on antibody binding capacity. Even after denaturation of the antigen, a proportion of the antigenic determinants remain in a sufficiently recognisable state as to be bound and precipitated by antibodies (Burnette, 1981). Monoclonal antibodies, however, with their single specific recognition site for the antigen, may recognise or fail to recognise the denatured protein in an "all-or-none" response.

The configuration of proteins following Western transfer is speculative; Some proteins may partially renature following SDS polyacrylamide gel electrophoresis. A number of studies have shown that some GTP-binding proteins can renature following SDS PAGE and transfer to nitrocellulose (McGrath et al, 1984, Schmitt et al, 1986, Lapetina and Reep, 1987). During Western transfer, proteins may partially regain their native conformation as SDS, migrating to the anode, is effectively lost from the sample.

Since an antigenic determinant may be composed of as little as four or five amino acids, antibodies directed against short stretches of primary protein sequence may still recognise the partially denatured antigen. It is possible, therefore, that some monoclonal antibodies will detect proteins after Western transfer.

The lack of reaction of the monoclonal antibodies against DNA methylase in immunoblotting experiments again highlights the specificity of antibody recognition, and the problems of identifying denatured antigen by this procedure. Only six monoclonal antibodies have so far been tested in this detection system: It is possible that on screening of a larger number of the available monoclonal antibodies some identity of enzyme determinants will be achieved.

6.7 Immunocytochemical Detection of Antigen

Immunocytochemical techniques may also prove useful in localising different forms of DNA methylase present in cells at various stages of development.

In terms of both labelling intensity and specificity monoclonal antisera react equally as well as polyclonal antisera in immunocytochemistry studies, giving cleaner reactions than polyclonal antisera with no background staining (Mason et al, 1983). Visualisation of specific antibody is achieved by the attachment of "labelling" molecules. Commonly used labels include fluorochromes such as fluorescein, and enzymes. Specific antibody may be detected by the direct binding of the fluorescent label or enzyme to the antibody. Alternatively the enzyme may be bound to an intermediate such as protein A, or a similar antibody binding molecule. A third technique involves the attachment of enzyme to the biotin-avidin detection system (Boorsma, 1983). In this system biotin molecules are coupled to antibodies, with avidin used as a high affinity

secondary reagent. The coupling of avidin to fluorochromes or enzymes creates an antibody-biotin-avidin-label complex. This method has the advantage that secondary antibody is not required.

The use of fluorescent or enzyme conjugated monoclonal antibodies should facilitate the precise detection of DNA methylase in different cellular fractions, and identify the presence or appearance of the enzyme in cells stimulated to divide or differentiate.

6.8 Characterisation of DNA Methylase in Mouse and Other Species

Monoclonal antibodies directed against specific epitopes on DNA methylase may be useful in identifying the size and distribution of the enzyme in a variety of mouse tissues, and, by cross reactivity studies, in the comparison of DNA methylases isolated from different species.

Immunoblotting and probing of DNA methylase from mouse ascites cells with polyclonal antiserum and ^{125}I protein A has identified two bands of purified mouse enzyme of 185-190 kD and 160 kD. The smaller protein is believed to be a product of proteolytic digestion of the 185 kD enzyme (Adams et al, 1988). In contrast, immunoblotting of DNA methylase isolated from adult and newborn mouse liver identifies a single band of 100 kD, although some undegraded enzyme may be present in the nucleus of newborn mouse liver cells.

Greater than 80% of DNA methylase activity in newborn mouse liver is found tightly bound within the nucleus, as described in Section 1.3.1, while only 20% of the total enzyme activity is present in this fraction in the adult mouse tissue. A change in the distribution pattern of mouse liver DNA methylase, together with an increase in degradation of the enzyme occurs as the rate of cell division declines with the maturation of liver function (Adams et al, 1989). Studies on the distribution of DNA methylase in mouse tissues have shown that the 190 kD species is present in rapidly growing cells, such as spleen, while the 100 kD species of enzyme is found in slower growing tissues such as liver, kidney and gut (Adams et al, 1988).

Degradation and redistribution of DNA methylase has also been shown to occur in mouse L929 cells entering stationary phase; Immunoblotting of enzyme isolated from these cells again detects a 100 kD species, in comparison with the 190 kD protein identified in extracted nuclei and in the nuclear matrix fraction of log phase cells (Adams et al, 1988).

The use of monoclonal antibodies to precipitate DNA methylase from mouse tissues and cultured cells should give a more precise indication of the number of cross reacting species of enzyme present *in vivo*, and also the number of bound proteins.

Interaction of monoclonal antibodies with DNA methylase from different species will give an indication of the degree

of evolutionary conservation not only between mammalian methyltransferases but between methyltransferases from a variety of vertebrate and invertebrate tissue.

Studies have already shown that polyclonal antiserum raised against mouse ascites DNA methylase also interacts with the enzyme from many other vertebrates and plants (Adams et al, 1988). Mammalian cells generally show protein bands of 180-190 kD with some smaller degradation products, similar to the pattern shown with the mouse ascites enzyme. Polyclonal antiserum detects a 200 kD protein in frog spleen, although the major cross-reacting proteins in other frog tissues are 135 kD and 148 kD, in agreement with the size of the native enzyme detected in Xenopus tissue culture cells (Adams, 1981). Protein bands of 140 kD and 120 kD have been detected in chick tissues while in pea shoot nuclei the presence of a 115 kD protein is indicated.

The use of monoclonal antibodies as probes will confirm the indentity of the cross reacting material and will allow indentification of antigenic determinants of DNA methylase which have been specifically conserved throughout evolution.

6.9 Studies on MEL Cell Differentiation

As described in Section 1.2.4, mouse erythroleukaemia cells (MEL cells) are reported to undergo a genome-wide transient demethylation, following the induction of differentiation by agents such as DMSO (Razin et al, 1988). The mechanism by which this alteration in the 5-mC content of the DNA is effected is unclear.

Bestor and Ingram (1984) have described the presence of three similar but distinct forms of DNA methylase in uninduced MEL cells, with each species of enzyme associated with a particular state of cell proliferation. A 190 kD enzyme was reported in rapidly growing cells, a 175 kD protein in late log phase cells and a 150 kD enzyme in stationary cells. It is possible that the 175 kD and 150 kD proteins are the products of degradation of the 190 kD enzyme found in actively dividing cells. DNA methylase activity is very low or absent from non-growing cells and presumably the enzyme becomes degraded following cessation of new DNA synthesis.

During induction of differentiation, pre-existing DNA methylase may also undergo degradation to a lower molecular weight, inactive state, thus perhaps accounting for the transient hypomethylation observed in these cells. However, transient hypomethylation is observed 12-20 hours after the induction of differentiation when a new round of replication is occurring. It is possible therefore that the observed decrease in methylation levels is merely due to the presence of increased levels of newly synthesised, unmethylated DNA.

Using antibodies as probes it should be possible to detect any change in the size or distribution of DNA methylase in cells undergoing differentiation. This would involve pulse labelling of MEL cells with L-[^{35}S]-methionine and subsequent immunoprecipitation of DNA methylase from whole cell extracts, nuclei and salt extracted nuclei at various intervals between the initiation of induction and terminal differentiation. In this way it should be possible to determine the half life of the enzyme in differentiating cells in comparison with the rate of breakdown of DNA methylase in non-induced MEL cells.

6.10 Affinity Purification of DNA Methylase

In addition to *in vitro* and *in vivo* characterisation of DNA methylase, monoclonal antibodies may be the key to developing a simpler and more effective purification strategy.

Partially purified DNA methylase has been used to methylate SV40 DNA *in vitro* to determine the effect of methylation at specific sites within the promotor region on the binding of transcription factors and the transcription of a cloned marker gene (Bryans and Adams, 1989, in preparation). To perform these and other experiments designed to investigate the role of DNA methylation in the control of gene expression, large amounts of purified active enzyme are required. The use of an affinity column of monoclonal antibodies cross-linked to CNBr Sepharose may provide the basis for a rapid and highly effective purification procedure.

As each monoclonal antibody can bind two molecules of DNA methylase, one mole of purified monoclonal antibody should immobilise two moles of the enzyme.

Monoclonal antibodies against human placental DNA methylase have already been used to affinity purify DNA methylase from mouse P815 mastocytoma cells (Pfeifer et al, 1985) achieving a 10,000 - 15,000 fold purification over the nuclear homogenate. DNA methylase has proved to be highly unstable and susceptible to degradation during purification, despite the use of protease inhibitors at all stages. In addition, low concentrations of purified enzyme often prove unstable during storage at -20^{o}C despite the presence of 50% (v/v) glycerol and PMSF. The ability to specifically concentrate milligram amounts of DNA methylase on an affinity column should result in the isolation of highly purified enzyme at a suitably stable protein concentration for use in *in vitro* methylation experiments.

Although some considerable difficulties were encountered in the use of a polyclonal antibody affinity column, it is hoped that a monoclonal affinity column will prove more effective. While DNA methylase will be specifically retarded on the column, enzyme molecules will be bound at just one point of contact by a single antibody molecule. The interacting forces between antibody and antigen may therefore be easier to overcome than in the more complex situation using a polyclonal affinity column, whereby the enzyme, interacting with many antibodies, became irreversibly cross-linked.

6.11 Limited Proteolysis: An Overview

Limited proteolysis describes the restricted sequential peptide bond cleavage in a protein substrate following nucleophilic attack by a specific protease. The specificity of proteolytic cleavage arises from the close fit required between the substrate and the active site of the attacking protease for the successful hydrolysis of the peptide bond.

Trypsin is classified as a serine protease, one of a group of homologous proteolytic enzymes exhibiting amino acid sequence and structural similarities. Particularly, the active sites of the serine proteases lie in similar non-polar crevices in each molecule (Matthews et al, 1967; Shotton and Watson, 1970; Stroud et al, 1971) and are characterised by a serine residue at position 195 in each case. Although trypsin can potentially cleave any lysyl or arginyl bond in a protein substrate, limited proteolysis is generally expected to primarily affect surface regions of the native protein substrate which are readily accessible to the protease, as opposed to internal domains of the peptide chain (Naslin et al, 1973).

Limited proteolysis serves as a mechanism for controlling many physiological functions *in vivo*, and the activation of enzymes by this means is a feature of several biological systems: Trypsin itself is activated from its precursor trypsinogen by limited proteolytic cleavage, as are other digestive enzymes including pepsin and chymotrypsin. Blood coagulation is controlled via a cascade of proteolytic activations of zymogens, including prothrombin and fibrinogen, and insulin is proteolytically activated

from proinsulin. Thus a variety of physiological functions can be induced via limited proteolytic cleavage of peptide bonds in precursor proteins.

The activity of trypsin and other pancreatic proteases is physiologically controlled by specific protease inhibitors. These proteins are analogues of true trypsin substrates, which have evolved in parallel with the enzyme and which bind tightly to the active site, through a combination of interactions with specific residues and hydrogen bonding (Stroud et al, 1975). The half life of the trypsin:trypsin inhibitor complex formed is usually several months (Stryer, 1981).

The addition of equimolar amounts of trypsin inhibitor to trypsin following limited proteolysis *in vitro* should therefore result in a stable inactivation of the enzyme, with further proteolytic action effectively blocked.

6.12 *In Vitro* Proteolysis of DNA Methylase

Proteolytic digestion of DNA methylase by trypsin is thought to result in the sequential hydrolysis of particular peptide bonds in the enzyme. The effect of this hydrolysis will presumably be, firstly, an increase in the number of peptide chains comprising the protein, accompanied by a greater flexibility of reactivity between distant functional amino acid residues.

A conformational change in the enzyme structure may be

responsible for the observed increase in activity of the proteolysed enzyme *in vitro*. Alternatively, the loss of a catalytic regulatory domain by proteolytic removal or by spatial distancing from the active site following hydrolysis, may result in the methylation of hitherto unmethylated sites in DNA; This may involve random CpG methylation, or a loss of CpG specificity by the enzyme, with ensuing methylation of CpA, CpC and CpT dinucleotides. Evidence supporting this latter hypothesis has come from nearest-neighbour analysis of DNA *de novo* methylated with proteolysed enzyme: Results have indicated that only 43% of methyl groups are added to cytosine in the dinucleotide CpG. 24% of cytosines in CpC dinucleotides were found to be methylated, as were 20% of cytosines in CpA and 13% of cytosines in CpT (Adams et al, 1988).

Trypsin-treated DNA methylase shows a reduced tendency to aggregate on incubation, even in the presence of high concentrations of DNA, in contrast to untreated mouse ascites DNA methylase. Methylation of cloned DNA using untreated enzyme declines after several hours incubation, possibly due to enzyme aggregation. The proteolysed enzyme shows no such loss of activity, and near saturation levels of methylation can be achieved (Adams et al, 1988).

The significance of the stimulation in enzyme activity observed following limited proteolytic degradation of DNA methylase is currently unresolved. It remains possible that the increased incorporation of methyl groups in DNA is due to non-specific methylation of dinucleotides other than CpG. Such events are not

unprecedented: Simon et al (1980) have shown a reduced specificity of the rat liver enzyme for CpG sites, demonstrating that CpA and CpT sequences are also methylated. Methylation of cytosine in CpA, CpC and CpT *in vivo* has also been reported by Gruenbaum et al, 1981; Grafstrom et al, 1985; Nyce et al, 1986; and Sneider, 1980; although CpG dinucleotides show the highest level of modifications. Further studies on the methylation of DNA using proteolysed and unmodified enzyme should resolve this question. An increase in the level of *in vitro* methylation attainable may be possible if affinity purification of DNA methylase using monoclonal antibodies is successful.

In the event that some loss of specificity is conclusively demonstrated, it may be possible to identify a domain of the enzyme which normally prohibits non-specific methylations. The binding of monoclonal antibodies to such a domain may be found to alter the specificity of the enzyme from (predominantly) CpG to CpN.

A further use of *in vitro* limited proteolysis of DNA methylase lies in the identification of the active site of the enzyme: N-ethyl-maleimide (NEM) binds S-H groups and has been shown to inactivate DNA methylase, indicating the presence of an S-H group close to the active site (Turnbull and Adams, 1976). The use of ^{14}C-labelled NEM to bind DNA methylase, following by proteolysis and immunoprecipitation of the enzyme-NEM complex may reveal the location of the catalytic centre of the molecule.

6.13 Studies on Mouse Embryo Development

Tissue specific methylation patterns in eukaryotic DNA are believed to be established during embryogenesis, when cells show evidence of increased levels of *de novo* methylation (Jahner et al, 1982). DNA methylases isolated from various mammalian tissues are capable of methylating both unmethylated and hemi-methylated DNA substrates, suggesting that one enzyme species is capable of performing both *de novo* and maintenance methylation *in vivo* (Pfeifer, 1983; Grunwald, 1984). The finding that partial proteolysis of DNA methylase *in vitro* often leads to an increase in the *de novo* activity of the enzyme invites the conjecture that some form of limited proteolysis may occur *in vivo* to modulate the twin activities. DNA methylase isolated from somatic cells shows a preference for hemi-methylated DNA as a substrate. Proteolysis of the enzyme may cause an increase in *de novo* activity by removing a domain which normally limits the activity of DNA methylases to hemi-methylated sites on DNA. Removal of a domain which recognises 5-mC on the parental DNA strand would release the enzyme from the constraint of acting only at hemi-methylated sites, leaving it free to methylate all available CpG sites. Thus, embryos exhibiting *de novo* methylation activity might be expected to possess a form of the enzyme related to that formed by partial proteolysis of the mouse ascites enzyme *in vitro*.

An immunological approach provides a simple and sensitive means for the detection of DNA methylase in embryonic cells. The use of specific monoclonal antibodies will reveal the extent of cross reactions between the enzyme present in adult somatic cells and its embryonic counterpart(s).

A further *in vitro* approach is to study the properties of DNA methylase in mouse teratocarcinoma cells: F9 cells are murine embryonal carcinoma cells which can only form derivatives of extra embryonic tissue. These cells are capable of *de novo* methylation, demonstrated by the methylation of proviral Moloney murine leukaemia DNA made from input M-MuLV RNA (Gautsch and Wilson, 1983). Isolation of DNA methylase by immunoprecipitation will enable a comparison of the size and location of the enzyme present in F9 cells with that isolated from rapidly dividing adult mouse tissue. The results of these and similar studies with mouse erythroleukaemia cells will clarify the importance of DNA methylase at different cell cycle stages, and reveal the changes in DNA methylase levels as cells undergo differentiation.

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