DNA Methylation and Herpes Simplex Virus

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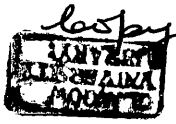

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Abbreviations

The abbreviations used in this thesis are in agreement with the recommendations of the editors of the Biochemical Journal (Biochem. J. (1992) **281**, 1-19) with the following additions.

BrdU	5′-bromo-2′-deoxyuridine
CAT	chloramphenicol acyl transferase
DMSO	dimethylsulphoxide
DTT	dithiothreitol
FITC	fluorescein isothiocyanate
m^5C	5-methylcytosine
m^5A	N^6-methyladenine
PCA	perchloric acid
PBS	phenylmethylsulphonylfluoride
PFU	plaque forming unit
RE	rat embryo
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
tk	thymidine kinase
TCA	trichloroacetic acid
TMPD	tetramethylpentadecane
HTF	*Hpa* II tiny fragments
KLH	keyhole lympet haemocyanin

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Summary

Infection of rat embryo (RE) cells with herpes simplex virus type 2 (HSV-2) inhibits the methylation of newly synthesised cellular DNA. This hypomethylation may be an important step in the transformation of cells by HSV-2 as various cell strains also show reduced levels of methylation in comparison to the parent RE cells. The mechanism of HSV infection induced hypomethylation and in addition, the relationship between this hypomethylation and the hypomethylation of transformed cell strains was investigated during the course of this work.

DNA methylase activity, as measured in an *in vitro* assay, is unaltered following HSV-2 infection. There is no increase in the rate of degradation of the methyl group donor, SAM and the ratio of SAM/SAH is also unaltered in infected cells. Moreover, cellular DNA in infected cells retains the ability to accept methyl groups. These results suggest, therefore, that despite the inhibition of DNA methylation, RE cells infected with HSV-2 appear to retain all the factors necessary for DNA methylation to take place.

The possibility that HSV infection inhibits DNA methylase activity *in vivo* by altering the intracellular ionic environment was investigated. However, we were unable to mimic infection induced hypomethylation by treating cells with high (0.24 M) concentrations of NaCl or with a variety of ionophores.

Immunofluoresence studies using anti-methylase antibodies showed that DNA methylase concentrates around the nuclear periphery following infection with HSV-2. This is in contrast to the sites of DNA replication which are distributed throughout the nucleus and this differential distribution leads us to speculate that the inhibition of DNA methylation following infection occurs as a result of DNA methylase being distanced from its substrate, newly synthesised DNA. In support of this theory we have demonstrated that a viral mutant which does not cause an inhibition of DNA methylation when infected into RE cells similarly does not cause the redistribution of DNA methylase. The mechanism of this redistribution is still

unknown but we hypothesise that DNA methylase may migrate to the nuclear periphery by association with cellular chromatin which undergoes nuclear margination and condensation at early times after infection.

RE cells can be transformed with the *Bgl* II n fragment of HSV-2 and these cloned cell strains have a genome-wide reduction in the level of methylation as compared to the parent RE cells. However, we have no evidence for inhibition of methylation within 48 hours following the transfection of RE cells with the *Bgl* II n or neighbouring fragments. This suggests that an inhibition of methylation is not the primary event in the transformation of RE cells by fragments of HSV-2 although it may be important in the maintenance of the transformed state.

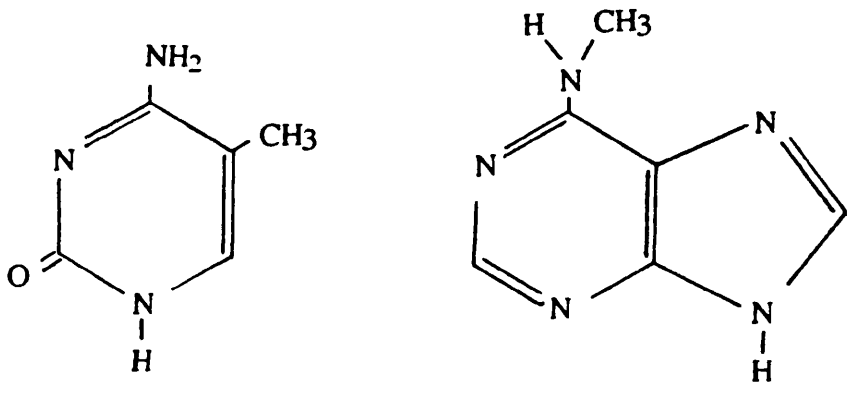
Chapter 1 Introduction

1.1 DNA Methylation

1.1.1 The Presence of 5-Methylcytosine in DNA

1.1.1.1 Modified Bases in DNA

The presence of the modified bases, 5-methylcytosine (m^5C) and N^6-methyladenine (m^6A) (see figure 1.1) in DNA, was first observed over thirty years ago by Hotchkiss, (1948), Wyatt, (1951) and Dunn and Smith, (1958). Since these initial findings, such bases have been found to occur in the genomes of almost all prokaryotes and eukaryotes to a greater or lesser extent.



5-methylcytosine **N^6-methyladenine**

Figure 1.1 5-Methylcytosine and N^6-Methyladenine

Prokaryotic DNA contains both m^6A and m^5C. A third modified base N^4-methylcytosine, the product of modification by the *Bcn* I methylase, has also been found in certain bacteria (Janulaitis *et al.*, 1983). The DNA of certain lower eukaryotes, such as protozoa, also contain both m^5C and m^6A whereas the DNA of higher eukaryotes contains only m^5C. The frequency of occurrence of m^5C in eukaryotes varies greatly with species. In vertebrates, 2-8% of all cytosines are methylated but in plants this figure may be as high as 40%. In contrast, the level of DNA methylation in insects is much lower for example, the level of m^5C is 0.95%

in locusts (Wyatt, 1951) and 0.17% in mosquitoes (Adams *et al.*, 1979b). Only the DNA of certain nematodes (Simpson *et al.*, 1986), yeast, fungi (reviewed by Magill and Magill, 1989) cellular slime moulds (Smith and Ratner, 1991) and surprisingly *Drosophila* (Pollack *et al.*, 1984) have been shown to contain negligible levels of m^5C.

Methylated bases have also been detected in the DNA of several viruses. Many types of bacteriophage have been shown to contain levels of m^5C or m^6A in their genomes comparable to that of their bacterial host. In contrast to this post replicative modification of bases, all the cytosine residues in the *Xanthomas* bateriophage, XP-12, are replaced by m^5C incorporated directly into DNA (Kuo and Tu, 1976). Similarly, hydroxymethylcytosine completely replaces cytosine in the DNA of T-even phages.

With the exception of frog virus 3 DNA (Wills and Granoff, 1980), DNA isolated from intact virions and free intracellular viral DNA from animal viruses does not contain m^5C. Animal virus DNA, however, may become methylated when integrated into the host chromosome e.g adenovirus (reviewed by Doerfler, 1991) or during latency and this will be discussed further in section 1.2.6.

1.1.1.2 The Distribution of 5-Methylcytosine

In animals, the highest proportion of m^5C occurs in the CpG dinucleotide with approximately 70% of such dinucleotides methylated. In plants m^5C is also found in the trinucleotide sequence CpNpG where N represents any base. The distribution of m^5C in these sequences, however, is far from random.

At the cellular level, there are tissue-specific differences in the levels of m^5C as measured in total genomic DNA from various tissues in the same organism (Vanyushin *et al.*, 1970,1973a and b; Ehrlich *et al.*, 1982; Gama-Sosa *et al.*, 1983). Tissue-specific levels of methylation have also been observed in the vicinity

of a number of specific genes, for example, in chicken and human globin genes (reviewed in Adams and Burdon, 1985). These tissue-specific differences were the first indications as to possible functions of DNA methylation which will be discussed later. In a more recent study Kochanek *et al.*, (1990) demonstrated that the DNA methylation status of the genes encoding human necrosis factor α and β in specific cell types showed remarkable interindividual concordance. This suggests a high degree of stability of cell type specific DNA methylation patterns.

At the genomic level, there are varying levels of DNA methylation in different classes of DNA. Genomic DNA can be fractionated according to its reassociation kinetics and there are a number of reports indicating that highly repetitive, rapidly annealing DNA is more highly methylated than moderately repetitive or unique sequence DNA (Drahovsky *et al.*, 1979; Ehrlich *et al.*, 1982).

The sites of methylation in animals, i.e. CpG dinucleotides, are themselves not arranged in a random manner throughout the genome. CpG is underrepresented in bulk vertebrate DNA and is present at only 10-20% of its expected frequency. The predicted frequency, however, does occur in specific regions of DNA of 0.5-3.0 kb and these clusters of CpG dinucleotides have been termed CpG islands or HTF regions (Bird, 1986). One important feature of CpG islands is that they are unmethylated in all tissues so far tested including the germline and are only found methylated on the inactive X-chromosome or in inactive genes in certain cells in culture.

1.1.1.3 Maintenance and *De Novo* Methylation

The patterns of DNA methylation described in the previous section can be stably inherited and transmitted from one generation to the next by a process called maintenance methylation.

Semi-conservative replication of a methylated DNA duplex produces a methylated strand paired with a non-methylated nascent strand (see figure 1.2). The enzyme DNA methyltransferase (DNA methylase) in some still yet unknown way recognises this so-called hemi-methylated site and transfers a methyl group to the cytosine on the daughter strand. This mechanism was first proposed by Holliday and Pugh, (1975) and Riggs, (1975) and was later confirmed by Wigler *et al.*, (1981) who demonstrated that DNA introduced into cells retains its pattern of DNA methylation over 25 generations.

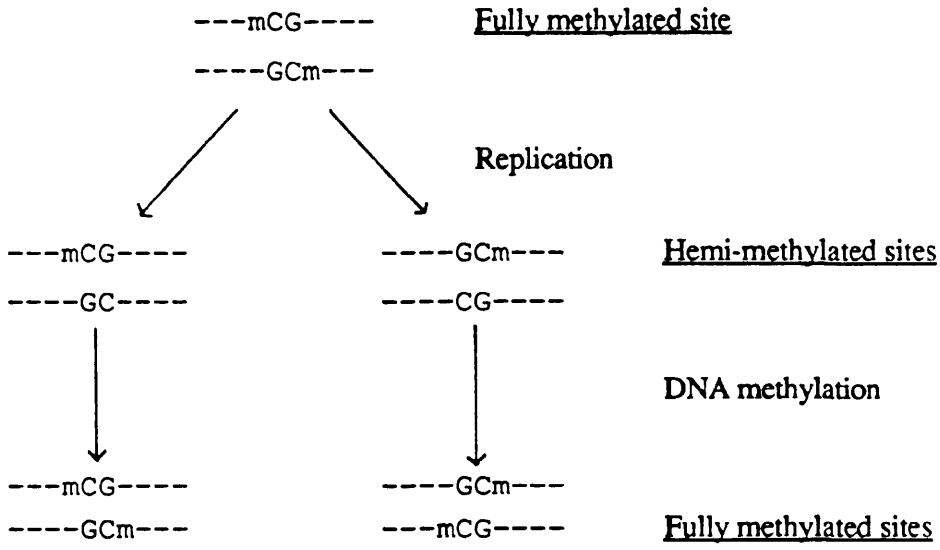


Figure 1.2 Maintenance Methylation

De novo methylation of previously unmethylated sites is widespread during gametogenesis and early embryogenesis and is thought to be important in the control of development (Monk, 1990; Cedar and Razin, 1990). Although the methylation pattern in somatic cells is relatively stable there is limited evidence to suggest that these cells also have the ability to carry out ***de novo*** methylations. Szyf *et al.*, (1989) for example, demonstrated that DNA fragments encoding the mouse steroid 21-hydroxylase gene are *de novo* methylated, in a sequence specific manner, when introduced into the mouse adrenocorticol tumour cell-line.

1.1.2 The Enzymatic Modification of Cytosine Residues in DNA

1.1.2.1 The Synthetic Reaction

DNA methylation is a post-replicative event. The enzyme DNA methyltransferase (DNA methylase EC 2.1.1.3.7) catalyses the transfer of a methyl group from the methyl group donor, S-adenosyl methionine (SAM), to the 5 position on the cytosine ring. The products of the reaction are 5-methylcytosine and S-adenosyl homocysteine (see figure 1.3.)

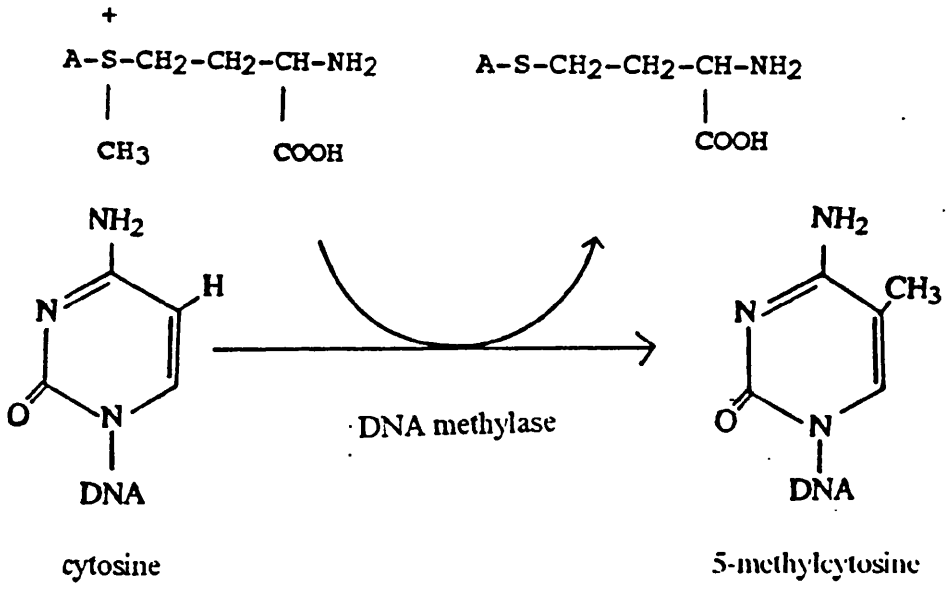


Figure 1.3 The Enzymatic Methylation of Cytosine in DNA

Santi *et al.*,(1983, 1984) proposed that the reaction probably involves the intermediate formation of an enzyme-DNA covalent link as this is the mechanism whereby other enzymes catalyse electrophilic substitutions at the 5 position of pyrimidine rings. This hypothesis has recently been confirmed by Chen *et al.*, (1991) who demonstrated that the prokaryotic DNA methylase, M.*Hae*III, forms a covalent link with a duplex oligonucleotide, containing the suicide substrate 5-fluoro-2′-deoxycytidine, when incubated together in the presence of SAM. The

DNA attachment site within the enzyme was found to be a cysteine residue that is contained within the Pro-Cys dipeptide motif that is found conserved in all known DNA methylases. This finding provides experimental verification for the hypothesis (Wu and Santi, 1987) that this Cys residue is the catalytic nucleophile.

DNA methylation takes place in the S-phase of the cell cycle. There is a short lag of one or two minutes between the incorporation of cytosine during replication and its first becoming methylated however, methylation is not completed for a period of several hours (Adams, 1971; Woodcock *et al.*, 1982). Isolated nuclei from cells incubated with [^{3}H-methyl]-SAM incorporate radioactive methyl groups into endogenous DNA at cytosine residues (Davis *et al.*, 1985). As isolated nuclei are not synthesising DNA this provides further evidence that methylation can continue even when DNA replication has ceased. This delay period is postulated to be important to allow certain reactions such as mismatch repair and chromosome segregation to occur.

1.1.2.2 DNA Methyltransferases

Mammalian DNA methyltransferases have been isolated from a number of sources including mouse ascites cells (Turnbull and Adams, 1976), HeLa cells (Roy and Weissbach, 1975), human placenta (Pfeifer *et al.*,1983) and mouse erythroleukemia cells (Bestor and Ingram, 1983).

DNA methylase is a large, single subunit enzyme found in the nucleus of the cell. Estimations of its molecular weight range from 50-200 kDa but this variation may partly reflect its sensitivity to proteolysis.

Eukaryotic DNA methylases have no cofactor requirements and are not stimulated by ATP or Mg^{2+}. The pH optimum for mammalian enzymes is between pH 7.5 and pH 8.0 and the K_m for S-adenosyl methionine is around 2 μM (Simon *et al.*, 1978). The K_m for DNA has been variously estimated between 0.5 μg per

ml and 70 μg per ml depending on the substrate used. *In vitro*, the enzyme preferentially methylates hemi-methylated DNA although it is also capable to a lesser extent of methylating CpG dinucleotides *de novo*.

There is still relatively little known about the mechanism of action of DNA methylase. Drahovsky and Morris, (1971) postulated that the enzyme, once bound to native DNA, processes along the DNA in a "random walk" manner, methylating cytosines along the way. This mechanism of one-dimensional diffusion would greatly enhance the chances of methylase finding its substrate as opposed to an association-dissociation mechanism. When native DNA is used as a substrate to measure DNA methylase activity *in vitro*, the reaction is inhibited by high salt (Adams *et al.*, 1979a). This suggests that the enzyme requires single-stranded regions with which to interact, a situation favoured by low salt and high temperature. Indeed, most mammalian enzymes prefer denatured to native DNA as a substrate *in vitro*. In contrast to these results however, single-stranded oligonucleotides have been demonstrated to be extremely poor acceptors of methyl groups (Carotti *et al.*, 1986; Smith *et al.*, 1991; Adams *et al.*, 1992). The difference between denatured DNA and single-stranded oligonucleotides is probably that denatured DNA has the ability to form transient duplex regions whereas the oligonucleotides do not contain significant self-complementary regions. These results suggest that although DNA methylase requires single-stranded regions with which to interact, the transfer of methyl groups to DNA occurs only in duplex regions.

As mentioned above, eukaryotic DNA methylase can carry out both maintenance and *de novo* methylations *in vitro*. In somatic cells, the primary methylation reaction occurring is that of maintenance whereas in non-differentiated cells *de novo* methylations are widespread, for example, during embryo development. There is no evidence for a separate enzyme in these non-differentiated cells hence both types of activity are speculated to be catalysed by the same methylase both *in vivo* and *in vitro*.

Adams *et al.*, (1983) demonstrated that the ability of DNA methylase, purified from Krebs II ascites cells, to add methyl groups to native unmethylated DNA i.e. *de novo* activity, was stimulated by proteolysis. They later proposed the following model to explain the control of the two types of DNA methylase activity (Adams *et al.*, 1986). In this model they envisage that the enzyme has two domains, one containing the active site and the other, a recognition domain which contains an allosteric site which inhibits the enzyme when it encounters an unmethylated symmetrical CpG site. Removal of this recognition domain by proteolysis would remove a constraint on the enzyme and allow it to methylate DNA *de novo*. Evidence to support this domain theory has come from Bestor *et al.*, (1988) who have cloned and sequenced the cDNA encoding DNA methylase from mouse erythroleukaemia cells. The predicted amino acid sequence appears to be organised into two structural domains. The 573 amino acid C-terminal domain is homologous to bacterial type II DNA methylases and is thought to be the catalytic domain. The N-terminal domain is 1000 amino acids long and contains a putative Zn-binding motif and may be the recognition domain envisaged in the model by Adams *et al.*, (1986).

1.1.3 Functions of DNA Methylation

1.1.3.1 DNA Methylation and Gene Expression

One of the major functions of DNA methylation in eukaryotes is postulated to be regulation of gene expression. In general terms, lack of methylation of a gene has been correlated with its expression. The first indications of this relationship came from studying the methylation patterns of tissue specific genes. Many studies demonstrated that the transcriptional control regions in tissues of expression were undermethylated relative to the same sequence in non-expressing tissues. The methylation patterns in these early studies were investigated using the isoschizomer restriction enzymes, *Msp* I and *Hpa* II in a technique developed by Waalwijk and Flavell, (1978). Both enzymes cleave the sequence CCGG however, *Hpa* II will not cut if the internal cytosine is methylated. Comparison of the size of the fragments obtained on digestion with these can therefore show the proportion of cytosines methylated in the CpG dinucleotide. The disadvantage of this approach however, is that only methyl groups in the recognition sequence CCGG are detected and this constitutes only a small fraction (4-6%) of the total number of cytosines in DNA.

One family of genes which has been extensively studied using this technique is the globin gene family. In chickens, the adult α globin genes are undermethylated in haemoglobin producing reticulocytes but they are heavily methylated in other tissues. Conversely, the foetal genes are undermethylated in the foetal liver which contains the cells actively producing foetal haemoglobin (Weintraub *et al.*, 1981; Haigh *et al.*, 1982). Other genes which also show tissue specific hypomethylation correlating to gene expression include the mouse metallothionine gene (MT-1) (Compere and Palmiter, 1981) and the rabbit β globin genes (Shen and Maniatis, 1980).

It should be noted however, that there are also a number of genes that show imperfect, patchy or no correlation at all between hypomethylation and gene expression. For example, the rat albumin and α fetoprotein genes in 18 day old rat foetal liver are both heavily methylated when at this stage they are also being transcribed (Kunnath and Locker, 1983). In some cases only certain sites exhibit tissue-specific patterns of DNA methylation. In the chicken ovalbumin gene, for example, a number of *Hpa* II sites are always methylated or never methylated irrespective of activity whereas some sites show variable methylation patterns being undermethylated in the expressing tissue (Mandel and Chambon, 1979; Kuo *et al.*, 1979). This demonstrates that only critical sites need be hypomethylated to mediate gene expression in tissue-specific genes. Apparent anomalies may, therefore, be at least partly due to the limitations of using *Hpa* II as a means of detecting methylated sites. This limitation has recently been overcome by the development of a method called genomic sequencing which can detect m^5C by chemical sequencing (Saluz and Jost, 1989) and this method has already been used to obtain detailed methylation profiles in a number of studies.

Although the types of experiments outlined above suggest that DNA methylation may be a mechanism for controlling gene expression, at least in some cases, they do not directly prove that DNA methylation of a gene is the cause of its suppression. More detailed evidence has come from the use of two different experimental approaches.

One approach is to use gene transfer. This involves methylating DNA *in vitro* and introducing it into cells. Yisreli *et al.*, (1988) showed that endogenous β globin genes in human fibroblasts are methylated and not expressed however, unmethylated copies of the same gene transfected into the same cell type are transcribed.

A second approach uses the demethylating agent 5-azacytidine. This compound is a cytosine analog and once incorporated into DNA it appears to bind irreversibly to DNA methylase and hence inhibits DNA methylation. Treatment of cells with this

drug has been shown to cause inactive genes to become active. For example, the inactive endogenous provirus gene in chicken AEV cells was turned on after the cells were exposed to 5-azacytidine and the corresponding gene sequences were found to be undermethylated (Groudine *et al.*, 1981).

Although the correlation between DNA methylation and gene expression has been established for more than 10 years only now are the mechanisms by which the signal is transduced beginning to be understood. It was generally assumed that the modification of cytosines residues in some way altered the binding of proteins involved with transcription machinery. However, it was not clear if this was caused directly by influencing specific protein:DNA interactions or whether DNA methylation indirectly inhibits the access of the transcription machinery for example, by causing alterations in chromatin conformation. Recent studies indicate that both mechanisms probably occur and that the different mechanisms depend on the gene involved.

Initial evidence to suggest that chromatin structure could be related to the level of methylation come from a study by Razin and Cedar, (1977) who showed that DNA containing m^5C was organised into chromatin that was relatively resistant to nucleases. Direct evidence that DNA methylation could cause DNA to adopt a nuclease insensitive structure came from a study by Keshet *et al.*, (1986) who showed that the chromatin conformation of a transfected DNA sequence was a consequence of its methylation status.

Antequera *et al.*, (1989) have shown that methylated CpGs in intact nuclei are preferentially inaccessible to restriction endonucleases that normally cleave at these methylated sites in naked DNA. They postulate that this effect is due to the presence of a protein they have previously purified and characterised called methylated CpG binding protein (MeCP) which has been shown to bind preferentially to methylated cytosines in a non-specific manner (Meehan *et al.*, 1989). Tazi *et al.*, (1990) examined the structure of a CpG island at the 5' end of housekeeping genes using a technique developed by Antequera *et al.*, (1989). This method is based on the fact

that complete digestion of nuclei with CpG cutting restriction endonucleases gives short oligonucleotides derived mainly from CpG islands whereas bulk chromatin remains intact. Analysing the protein content of the DNA derived from CpG islands they concluded that these islands differed from bulk chromatin in that they are nucleosome free and are bound by highly acetylated histone H3 and H4.

In addition to its effect on chromatin structure, DNA methylation has been shown to directly inhibit transcription by blocking the binding of positively acting transcription factors. For example, binding of the sequence specific transcription factors E2F and MLTF found in HeLa cells is inhibited by methylation of certain CpG residues (Kovesdi *et al.*, 1987; Watt and Molloy, 1988). However, methylation of the binding site for Sp1 does not directly block the binding of the Sp1 protein (Harrington *et al.*, 1988).

Proteins have also been isolated which require CpG methylation for binding, for example the protein MeCP discussed above. More recently Boyes and Bird (1992) have shown that this protein can repress transcription when bound to a variety of different gene promoters but this repression is most effective when bound to promoters containing a high density of methylated CpG sites. They postulate that this protein is involved in the inactivation of CpG island associated genes.

The above examples illustrate that the effect of CpG methylation is specific to each protein:DNA interaction and may be neutral, inhibitory or required for binding. However, despite these apparent contradictions, whenever methylation has an effect on protein:DNA interactions the net result is a repression of the gene.

1.1.3.2 DNA Methylation and X-Chromosome Inactivation

Normal diploid somatic cells in female mammals contain two X-chromosomes, twice as many as their male counterparts. However, to ensure that both have the same dosage of X-chromosome transcripts one of the female X-chromosomes is inactivated (or more than one in individuals with supernumerary X-chromosomes e.g. XXXY) to leave only a single active X.

X-chromosome inactivation occurs at a specific stage in embryogenesis. In the mouse this happens at 4-6 days gestation around the time of implantation. Both the maternal and paternal X-chromosomes have an equal probability of being inactivated but once this pattern is established the chromosomes retain their active or inactive state for the life of the individual. The only exception to this is found in the germ cells. Although the primordial germ cells undergo X-chromosome inactivation this inactive chromosome is then reactivated shortly before mieosis (reviewed by Gartler and Riggs, 1983; Lyon, 1989).

Initiation of inactivation is generally thought to begin at an X-chromosome inactivation centre and spread bidirectionally along the chromosome. Brown *et al.*, (1991) have now narrowed the location of the human X-chromosome inactivation centre to a region within band Xq13. They speculate that it may bind a trans-activating factor which would inactivate this region on the active X-chromosome.

Riggs (1975) was first to propose that DNA methylation may play a role in the inactivation process. Since then, several X-chromosome genes have been shown to have a strong correlation between inactivity and hypermethylation of the 5′ region. This includes the CpG rich islands at the 5′ regions of housekeeping genes which are never methylated in autosomal genes or in the germ-line. Examples of genes showing these correlations are hypoxanthine phosphoribosyltransferase (HPRT), glucose-6-phosphate dehydrogenase (G6PD) and phosphoglycerate kinase (PGK) (reviewed by Monk 1990). DNA methylation however, does not appear to be the initial step by which the gene inactivation occurs. Lock *et al.*, (1987) have

demonstrated that methylation of the HPRT gene on the inactive X-chromosome occurs after the inhibition event. Thus, it has been proposed that DNA methylation may be an important factor in the maintenance of the active state once it has been established.

Further evidence to support the theory that DNA methylation is important for maintaining the inactive state has come from studies using 5-azacytidine. The reactivation of genes on the inactive X-chromosome by 5-azacytidine was first reported by Mohandas *et al.*, (1981). They used the drug to reactivate the HPRT gene in mouse-human hybrid cells that only contained an inactive X-chromosome. More recently, Pfeifer *et al.*, (1990) have used PCR aided genomic sequencing to determine the methylation status of the PGK gene in a similar human hybrid cell line after treatment with 5-azacytidine. On the inactive chromosome, 117 out of 121 CpG sites were methylated near the promoter start site whereas the same sites on the active chromosome or on the chromosome of clones reactivated with 5-azacytidine were completely unmethylated.

In comparison to the work on somatic cells, relatively little is known about the DNA methylation events in oogenesis. Singer-Sam *et al.*, (1992) have demonstrated that a critical Hpa II site in the promoter region of the PGK gene, critical in maintaining the X-inactivation in somatic cells, is unmethylated in female germ cells well before the inactive X-chromosome is reactivated. They speculate that this site is never methylated in these primordial germ cells. Hence, the germ-line inactivation-reactivation cycle may not involve methylation. This observation is consistant with the suggestion by Monk *et al.*, (1987) that the germ-line cells are sequestered from the rest of the embryo early in development and hence avoid methylation.

1.1.3.3 DNA Methylation and Cellular Differentiation

The process of development and cellular differentiation involves the complex control of gene activity. DNA methylation, because it is a means of regulating gene expression in a heritable manner, has been suggested to be an important epigenetic mechanism involved in the unfolding of the developmental program (reviewed by Cedar and Razin, 1990; Holliday, 1989).

In cultured cells, agents that can inhibit DNA methylation have been shown to induce differentiation. For example, Christman *et al.*, (1977) showed that treatment of Friend erythroleukemia cells with ethionine results in their differentiation to haemoglobin producing cells. Ethionine has been shown to lead to the synthesis of undermethylated DNA and indeed, Christman *et al.*, (1977) demonstrated that the DNA isolated from these cells is hypomethylated compared to uninduced cells. More recently this group have also shown similar effects using the drug 5-azacytidine (Christman, 1984).

Taylor and Jones, (1979) have also shown that 5-azacytidine can induce cellular differentiation. Cultured mouse embryo cells exposed to the drug differentiate into three distinct types of muscle cell. The expression of a single gene, called *MyoD 1*, has recently been shown to be sufficient to convert these cells to the differentiated phenotype (Davis *et al.*, 1987). Jones *et al.*, (1990) have shown that induction with 5-azacytidine also results in the hypomethylation of this *MyoD 1* gene. These experiments on cultured cells suggest, therefore, that DNA methylation may be important in establishing and maintaining the differentiation of cells during development.

To investigate the role of methylation in embryonic development, attempts have been made to analyse m^5C levels at various stages of cellular differentiation. The most extensive study has been undertaken by Monk *et al.*, (1987) who used a highly sensitive technique based on restriction digestion with *Hpa* II and *Msp* I to perform comparative estimations of genome wide methylation in germ cells and in

early mouse development. In accordance with previous studies, they showed that oocytes are under methylated compared to overall methylation levels in sperm DNA and DNA methylation in the 8-cell embryo was compatible with the presence of undermethylated maternal and methylated paternal DNA. During further preimplantation development, at around the blastocyte stage, the level of methylation then fell. This is followed, at around the time of implantation and X-chromosome inactivation, by a progressive increase in *de novo* methylation. This *de novo* methylation appears to occur independently in embryonic and extraembryonic lineages. Monk, (1990) also speculates that foetal germ cell DNA is delineated before this period of extensive *de novo* methylation and hence escapes this process.

It is generally accepted that the methylated ground state that is established is then maintained with demethylation being associated with the onset of expression of specific genes in different tissues at different times as differentiated cells become committed to their specialised functions. There is, however, very little experimental evidence as to the methylation fate of specific genes as a function development and as to whether the global alterations seen preceed or occur as a consequence of gene expression. Possible mechanisms for establishing these different patterns of methylation during development will be discussed later in section 1.1.5.

Another important role of DNA methylation in development that has emerged in recent years is the parental imprinting of genes.

Genomic imprinting is an epigentic mechanism that confers functional differences on certain autosomal genes depending on the sex of the parent from which they were inherited. In this respect, neither the maternal nor the paternal genome is totipotent and normal development only occurs in the presence of germ-line DNA from both parents (reviewed by Surani *et al.*, 1990).

DNA methylation has been suggested to be an important epigenetic mechanism underlying imprinting and there is now evidence which shows that the methylation status of transgenes in transgenic mice can be dependent on whether they are

inherited from the mother or the father. For example, Swain *et al.*, (1987) created a transgenic mouse strain bearing elements of the Rous sarcoma virus long terminal repeat. This gene was shown to be expressed in the heart only if inherited from the male parent but if inherited from the female parent it was not expressed at all. Interestingly, this pattern of expression correlated precisely with the imprinted methylation state. Paternal inheritance correlated with transgene undermethylation and tissue specific expression whereas maternal inheritance correlates with methylation and lack of expression.

One important property of any epigenetic imprinting mechanism is that it must be completely reversible to allow the genetic material to switch between paternal and maternal forms each generation. Chaillet *et al.*, (1991) have followed the methylation status of maternal and paternal transgenes as they moved through various stages of development to investigate if DNA methylation correlates with these imprinting alterations. They found all methylation is erased in primordial germ cells then as the male and female germ cells mature, they then undergo *de novo* methylation to the appropriate parental form. After fertilisation, the maternal pattern is maintained whereas the methylation pattern in sperm undergoes further modification during embryogenesis.

1.1.3.4 Additional Possible Functions of DNA Methylation

DNA methylation, as well as its role in modulating gene expression and the consequence of this on X-chromosome inactivation and cellular differentiation, has also been speculated to function in a variety of other roles, outlined below.

(i) Recombination

DNA methylation is speculated to have a function in the control of recombination. The highly repetitive sequences where recombination events occur

are more highly methylated than other genomic sequences and therefore, DNA methylation may be acting as a protective mechanism against deleterious recombination. Indeed, during mieosis when satellite DNA is undermethylated the frequency of recombination is enhanced (Adams and Burdon, 1985).

As discussed in section 1.1.4.3, m^5C is unstable and can readily deaminate to thymine which results in a G:C → A:T mutation. In a recent paper, Kricker *et al.*, (1992) propose that DNA methylation induced mutagenesis is a means to diversify duplicated sequences and hence protect against potentially damaging recombination mediated chromosomal rearrangements. Reducing the degree of similarity of sequences by only a few percent sharply reduces the frequency of recombination and thus they postulate that the small diversification of sequence resulting from methylation may be sufficient to control recombination. To support their hypothesis, Kricker *et al.*, (1992) carried out sequense analysis and demonstrated that repeated sequences in mammals preferentially experience a high frequency of transition mutations at sites of cytosine methylation.

(ii) Restriction/Modification

DNA methylation has also been postulated to be involved in a host defense mechanism similar to the restriction/modification system in prokaryotes (discussed in Doerfler, 1992). However, no sequence specific endonucleases have ever been isolated in mammals that are also inhibited by methylation. In addition, mammalian genomes are only partly methylated and therefore a large amount of the genome would be unprotected and subject to restriction.

(iii) Mismatch Repair

In prokaryotes there is only a short delay between replication and the methylation of nascent DNA. However, during this delay it has been demonstrated that the presence of m^6A on only the parental strand directs a mismatch repair

system to the unmethylated nascent strand (Jones *et al.*, 1987). In eukaryotes the lag period before the completion of methylation is longer but it is also postulated to play a role in guiding the strand selection of repair systems in a manner similar to that seen in prokaryotes. Evidence to support this theory has come from a study by Hare and Taylor, (1985). They transfected hemi-methylated SV40 DNA into cells and found that mismatch repair only occurred on the unmethylated strand.

(iv) DNA Replication

DNA methylation has been demonstrated to play a role in the initiation of replication in certain prokaryotes such as *E.Coli* and plasmid P1. The origin of replication in the P1 plasmid replication system contains a number of copies of the sequence GATC, the recognition site for *E.Coli* DNA adenine methylase and it has been demonstrated that replication of this plasmid requires the adenine methylation of this sequence (Bakker and Smith, 1989). Although there is limited evidence to suggest that cytosine methylation may also be involved in replication in prokaryotes (Billin, 1968) there is little evidence supporting the role of methylation in the control of replication in eukaryotes.

1.1.4 DNA Methylation and Carcinogenesis

It is reasonably clear that, in certain circumstances, DNA methylation can influence gene expression. Therefore, it is conceivable that aberrant alterations in DNA methylation could lead to abberrant changes in gene expression which could lead to cancer.

Two different approaches have been used to address the question of whether DNA methylation is involved in carcinogenesis. Firstly, do tumour cells have altered levels of methylation compared to normal cells. Secondly, does treatment of cells with carcinogens lead to alterations in DNA methylation and hence, are demethylating agents, therefore, carcinogenic.

1.1.4.1 Patterns of DNA Methylation in Tumours and Tumour Cell Lines.

The level of DNA methylation in various tumours was investigated by Gama-Sosa *et al.*, (1983). Using HPLC, they examined the level of m^5C in the bulk DNA of 103 human tumours which included samples representing a variety of stages of tumour development. They found that not only was the level of DNA methylation reduced in these tumours but also that the level of DNA methylation decreased going from primary malignant to metastatic tumours. Similar observations have since been published by a number of groups. Feinberg *et al.*, (1988) showed unequivocal hypomethylation of DNA from human colonic tumour cells and found an average reduction of 8-10% in the level of m^5C in all colon adenomas and adenocarcinomas. However, they found similar levels of methylation in benign and malignant tumours indicating that hypomethylation occurs early in the development of these tumours.

Similar studies have also been carried out on the levels of DNA methylation in cultured tumour cells. These results are less clear-cut than those obtained for tumours and have shown that although some tumour cell lines are hypomethylated (Arnaud *et al.*, 1985; Macnab *et al.*, 1988) this is not always the case, with some cell lines showing no decrease or even showing increases in methylation (Kautianen and Jones, 1986; Flatau *et al.*, 1983). The intepretation of these results is, however, complicated by the difficulty in obtaining the appropriate controls and the fact that there is evidence to suggest that DNA methylation patterns can change when cells are cultured.

The above studies analysed the DNA methylation levels in bulk DNA from tumour cells and cell lines. However, the effect of DNA methylation on gene expression may be mediated by only a small number of specific CpG sites in the genome. A number of groups have, therefore, chosen to compare the DNA methylation status of specific genes in normal and tumour cells. Feinberg and Vogelstein, (1983a and b) and Goelz *et al.*, (1985) demonstrated that a number of genes were hypomethylated in all 23 neoplastic growths from the human colonic tumours they examined. The genes investigated included growth hormone, γ-globulin, α-chorionic gonadotropin and γ-crystallin The data obtained suggested that hypomethylation was a consistent characteristic of human colonic cancers, a finding later confirmed by Feinberg *et al.*, (1988) who demonstrated the hypomethylation of bulk DNA from similar tumours.

The DNA methylation status of specific genes from cultured tumour cell lines has also been studied. Achten *et al.*, (1991) investigated the methylation status of a number of genes includin several proto-oncogenes in normal human lymphocytes and a series human cell lines derived from lymphomas and leukaemias. Comparison of the DNA methylation patterns in a given DNA segment showed the tumour cell lines to have changes in these patterns in several instances. However, these changes were complex and included both increases and decreases in DNA methylation.

De Bustros *et al.*, (1988) have also discovered apparent contradictions to the observation that transformation and malignancy is associated with hypomethylation. They have examined the DNA methylation of a number of markers on chromosome 11 in a number of human lung and colon carcinoma and leukaemic cells. They discovered that the 5' region of the calcitonin gene to be hypermethylated, including sites within a CpG island , and the short arm of chromosome 11 in general to be a hot spot for hypermethylation in neoplastic cells. Interestingly, this chromosome region is thought to harbour several putative tumour supressor genes. Baylin *et al.*, (1987) have found similar increases in methylation of the 5' region of the calcitonin gene in a number of human lymphoid and acute myeloid malignancies.

In conclusion, therefore, the correlation between DNA methylation and tumourigenesis may not be as simple as early studies suggested. The recent discovery of tumour supressor genes (reviewed by Marshall, 1991) has demonstrated that tumour progression is a complex process which may involve both the activation of and inactivation of important genes and hence any alteration in DNA methylation levels could still lead to abberrant changes in gene expression which could contribute to the transformation process.

1.1.4.2 The Effect of Carcinogens on DNA Methylation

Further evidence to support the hypothesis that DNA methylation is important in carcinogenesis has come from findings that certain chemical carcinogens can interfere with DNA methylation both *in vivo* and *in vitro*.

Alkylating agents such as N-methyl-nitrosourea (MNU) and N-acetoxy-N-2-acetylaminofluorene (NAAAF) are electrophilic agents. These can react with nucleophilic sites in DNA to give alkylated bases, for example, 7-methyl guanine. Wilson and Jones, (1983) showed that DNA modified in this way was a poorer substrate for DNA methyltransferase than native, unmodified DNA.

Treatment of cells with chemical carcinogens has also been shown to decrease DNA methylation levels *in vivo*. Ivarie and Morris, (1986) showed that the alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) could increase by some 50-100 fold the reversion frequency of a mutant HeLa cell line harbouring a hypoxanthine phosphoribosyl transferase gene (HPRT) silenced by methylation. Lapeyre *et al.*, (1981) also showed that hepatocarcinomas induced by three different carcinogens were undermethylated compared to normal liver samples. Some carcinogens have also been shown to have the opposite effect on DNA methylation i.e. they increase the level of methylation within modified DNA. For example, Farrance and Ivarie, (1985) showed that methylation of poly [d(C-G).d(C-G)] with ethyl methane sulphonate (EMS) stimulates the methyl accepting ability of the DNA.

If carcinogens exert their effect by altering the pattern of DNA methylation then it is reasonable to hypothesise that agents which induce these methylation changes should also be carcinogenic.

5-azacytidine has been widely used as a demethylating agent and in the past it has been used to treat leukemia. However, there is now growing evidence to show that it is a highly potent carcinogen and is more highly efficient at inducing a variety of tumour types than many other well known carcinogens. Initial studies carried out by Benedict *et al.*, (1977) demonstrated that 5-azacytidine induced the transformation of 10T1/2 cells in culture. A number of studies later confirmed these studies including Hsio *et al.*, (1985) who also showed that transient exposure to 5-azacytidine increased the cells sensitivity to further transformation by the chemical carcinogen benzo[a]pyrene. Several studies have also demonstrated that alteration of methylation patterns using 5-azacytidine can induce changes in tumour diversification and can control progression to the metastatic phenotype. For example, Olsson *et al.*, (1985) showed that clones of murine Lewis lung carcinoma cells selected for non-metastatic phenotype could be converted to a metastatic phenotype by brief exposure to 5-azacytidine.

As well as its effect on DNA methylation, 5-azacytidine has been shown to have the ability to induce chromosomal aberrations (Benedict *et al.*, (1977) and it, therefore, may not exert its carcinogenic effect directly through demethylation, however the concentrations shown to cause these chromosomal abberations are in excess of those shown to be maximally effective at causing cellular transformation.

1.1.4.3. 5-Methylcytosine as an Inherent Mutagen

In the previous two sections (1.1.4.1;1.1.4.2) the possibility that carcinogenesis is correlated to alterations in gene expression which arise through alterations in DNA methylation was discussed. However, there is also now accumulating evidence to suggest that m^5C is itself inherently mutagenic.

5-methylcytosine can readily deaminate to thymine and the resulting T:G mismatches cause C:G→A:T transition mutations. Sved and Bird, (1990) have recently calculated that methylation of cytosine increases its risk of undergoing such a transition by a factor of 12. Vertebrates have developed a repair system to repair T:G mismatches preferentially back to C:G (Brown and Jiricny, 1987). Weibaur and Jiricny, (1989) have recently isolated the 200 kDa protein responsible for this repair system which appears to function by excising the thymidine monophosphate and filling in the resulting gap to generate G:C. This system however, does not appear to be 100% efficient as C→T transitions account for a high proportion of all DNA polymorphisms with around 30-40% of all human germ line point mutations thought to be methylation induced (Cooper and Youssoufian, 1988). This high rate of transition mutations resulting from methylation of CpG dinucleotides is thought to be the reason that CpGs are under-represented in bulk DNA. CpG islands are thought to have escaped this fate as they are never methylated in the germ line.

A number of groups have investigated the role of m^5C as a mutagen in specific human diseases. For example, Koeberl *et al.*, (1990) have studied mutations in the

factor IX gene in heamophilia patients and have shown that transitions at CpG dinucleotides were elevated 24-fold relative to transitions at other sites. Another gene which has been extensively studied is the p53 which is found to be altered in half of all human tumours. In colon cancer, there are two hot spots for mutation. These mutations all represent C→T or G→A transitions at CpG sites which are shown to be methylated in somatic cells (Rideout *et al.*, 1990). Mutations in the p53 gene appear to be different in different tumour types and may be the result of both m^5C induced mutations and direct-acting environmental carcinogens (reviewed in Jones *et al.*, 1992). Further analysis of the mutations observed in cancers may go some way to determing the cause of the mutations in specific cell types which in turn may prove useful for the prevention of cancer in future.

1.1.5 Establishment and Alterations in DNA Methylation Patterns

As disscussed in the previous sections, there is now considerable evidence to suggest that DNA methylation may act as a "locking" mechanism for gene expression in somatic cells. In tissue-specific genes, there is a well established correlation between undermethylation of a gene and its expression. On the other hand, housekeeping genes have CpG islands at their 5′ end which are unmethylated in all tissues and which appear to make them permanently available to be transcribed. These distinctive patterns of methylation are inherited from one generation to the next by the action of DNA methylase which recognises the methylation status of the the parental strand and methylates the corresponding site on the daughter strand in a process called maintenance methylation (see section 1.1.1.3). Although this mechanism of maintaining methylation patterns is now widely accepted there is still relatively little known about how patterns of methylation can be changed.

1.1.5.1 *De Novo* Methylation

De novo methylation of DNA is most clearly seen during gametogenesis and early development. During early stages of development there are periods of *de novo* methylation which result in the methylation of almost all tissue specific genes (discussed in section 1.1.3.3). However, despite this high level of *de novo* activity there is no evidence to suggest the presence of an additional DNA methylase and the reaction is thought to be catalysed by the same DNA methylase that is also capable of the maintenance reaction (Bestor *et al.*, 1988). As discussed in section 1.1.2.2, the basis for the specificity of the DNA methylase reaction is still unknown but it is postulated that DNA methylase may have a recognition domain which inhibits its *de*

novo methylation activity. There is limited evidence to support this theory however virtually nothing is known about the control of such a process.

Although there is widespread *de novo* methylation during development this process does not modify every sequence type and in particular CpG islands appear to remain unmodified (Kolsto *et al.*, (1986). The 5′ end of the Thy-1 gene contains a CpG island which has been shown to remain hypomethylated during the development of transgenic mice (Kolsto *et al.*, 1986). Szyf *et al.*, (1990) transfected embryonic stem cells (a totipotent cell line isolated from blastocytes with known *de novo* methylation activity) with either a control plasmid or a construct containing a 214 base pair region from the 5' Thy-1 promoter sequence. The Thy-1 CpG island was shown to be protected from methylation whereas the control plasmid was extensively modified. Interestingly, the 214 base pair fragment was also able to protect flanking sequences from *de novo* methylation. Szyf *et al.*, (1990) therefore speculated that this DNA fragment contains a sequence that binds a protein which in some way then inhibits the methylation of neighbouring island sequences. These results demonstrate that elements other than DNA methylase *per se* are likely to be involved in determining the specificity of the DNA methylase reaction.

Alterations in DNA methylation levels are also seen when cells are cultured. When first put into culture there is an initial rapid loss of m^5C (Wilson *et al.*, 1983). However, the immortal cells which emerge appear to have undergone *de novo* methylation and are found to have methylated CpG islands (Jones *et al.*, 1990; Antequera *et al.*, 1990). Again, the mechanism whereby *de novo* methylation is switched on in these cells, where it was previously repressed, is unknown.

In a recent paper, Szyf, (1992) proposed the following mechanism in an attempt to explain the mechanism of this non-specific spreading of *de novo* methylation. He speculates that *de novo* methylation of a gene is initiated at a centre of methylation (CM), similar to the proposed X-inactivation centre (discussed in section 1.1.3.2). The binding of protein factors to this centre enhances the affinity of DNA methylase

which then methylates the sites adjacent to the CM. This *de novo* methylation is further spread by the binding of methylated DNA binding proteins to these newly methylated sites which causes the methylation of further adjacent sites.

1.1.5.2 Demethylation

If, during early development, widespread *de novo* methylation occurs to establish what is termed a methylated ground state then the activation of tissue specific genes must correlate with programmed demethylation events.

Yisreli *et al.*, (1986) used DNA mediated gene transfer to transfect *in vitro* methylated α-actin gene constructs into fibroblasts and L8 myoblasts, a cell line which is used as a model system for studying the activation and structure of muscle specific genes. Although DNA methylation significantly inhibited the expression of the α-actin gene in fibroblasts, both methylated and unmethylated constructs were expressed to the same extent in myoblasts. In these myoblasts, the methylated α-actin construct was found to have undergone site-specific demethylation whereas the methylation status of the gene introduced into fibroblasts remained unaltered. Similar results have been found in other transfection studies. For example, genes encoding the immunoglobulin κ chains undergo demethylation specifically in β-lymphocytes and the rat insulin gene has been shown to under go cell-type specific demethylation in rat insulinoma cell line (reviewed by Frank *et al.*, 1990). In all of the above studies, demethylation corresponded to the expression of the gene and the resulting methylation status resembled that of the endogenous gene in the same tissue. It would appear, therefore, that at least some differentiated cells retain the ability to recognise and demethylate a gene, presumably by the same mechanism that is used during development.

As well as these demethylation events correlating with the activation of tissue specific genes there is also evidence to suggest that a mechanism exists to

demethylate CpG islands. Frank *et al.*, 1991 introduced a methylated hamster adenine phosphoribosyltransferase gene (APRT) into mouse oocytes and analysed the methylation status of the gene in the resulting transgenic mice. The entire 5′ CpG island of the APRT gene was found to be demethylated whereas the 3′ end of the gene remained methylated, the resulting pattern of methylation being essentially identical to that of the endogenous hamster gene. They also demonstrated that this demethylation event was restricted to embryonic cells as the APRT gene remained fully methylated when introduced into fibroblasts but was demethylated following transfection into F9 carcinoma cells. Although CpG islands are always unmethylated in all tissues and in the germ-line such activity may be required to reactivate the X-chromosome during oogenesis.

The mechanism of demethylation has long been assumed to occur passively. Razin and Riggs (1980) proposed that if, following replication of fully methylated DNA, the resulting hemi-methylated site in some way failed to be methylated then a second round of replication would result in a totally unmethylated symmetrical site in 50% of cells (see figure 1.4).

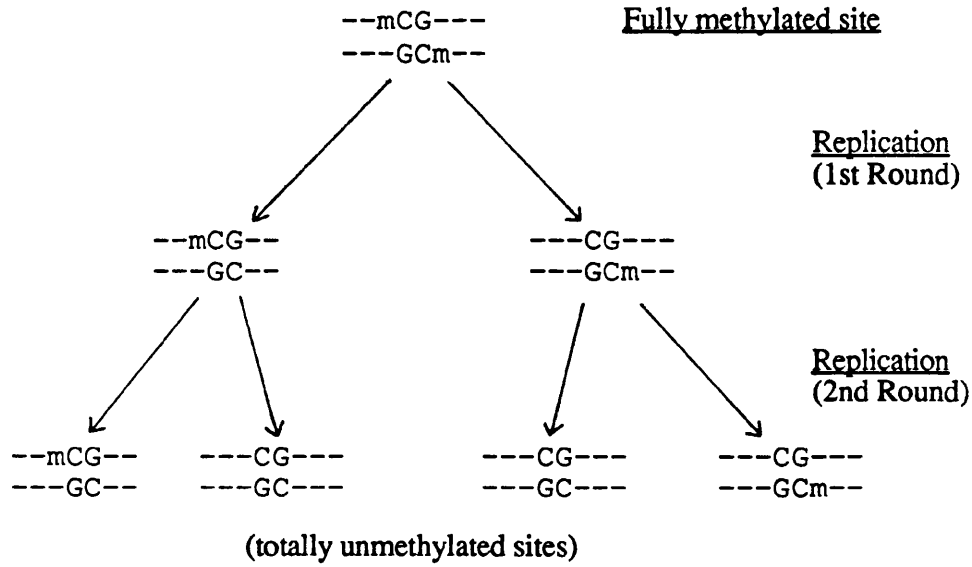


Figure 1.4 Passive Demethylation

Although this mechanism may well exist it is a slow process and there is now growing evidence to suggest that demethylation can also occur by an active process which happens in a much shorter timescale without the need for DNA replication. For example, Paroush *et al.*, (1990) followed up the studies of Yisreali *et al.*, (1986) discussed above and showed that the activation and demethylation of the α-actin gene introduced into myoblasts occurred in two stages. Within 2 hours of transfection the construct was found to be hemi-methylated and the methyl group on the second strand was not removed until at least 48 hours later. The removal of both methyl groups occurred in the absence of DNA replication suggesting an active demethylation event. Their results also showed that transcription of the gene only occurred after it became fully demethylated.

A similar two stage demethylation event is seen at two sites within the oestrogen responsive element (ERE) of the vitellogenin gene in embryonic liver cells following activation with oestradiol (Saluz *et al.*, 1986). Demethylation of the upper strand correlates well with the induction of vitellogenin mRNA synthesis which occurs within one day of oestradiol treatment whereas demethylation of the lower strand lagged by about 24 hours. Again, the obseved demethylation occurs in the absence of DNA replication.

Active demethylation events have also been observed in mouse erythroleukaemia cells where DNA methylation falls transiently after stimulation of the cells with hexamethylene bisacetamide, a chemical capable of inducing differentiation in these cells. A demethylase type activity where the methyl group only is removed from m^5C is improbable as this reaction is considered thermodynamically impossible. Razin *et al.*, (1986) postulated that the active, genome-wide, demethylation occurs by a mechanism whereby m^5C is specifically replaced by cytosine. They presented evidence to support a proposal that this replacement was caused by a transglycosylase reaction which replaces m^5C with a cytosine residue without breaking the phosphodiesterase backbone of DNA. In contradiction to this, however, Adams *et al.*, (1990) have presented evidence to

show that this demethylation is more likely due to a very short patch, excision repair-like mechanism.

Hughes *et al.*, (1989) have purified a protein called NHP1 which binds to a site within the ERE of the chick vitellogenin gene. This protein shows a preference for methylated DNA and makes nicks in the DNA near its binding site. It is speculated that this ubiquitous protein plays a role in the active demethylation of DNA in this system and it may function by facilitating a similar short patch excision repair-like system which replaces m^5C residues with cytosine.

1.2 Herpes Simplex Virus

1.2.1 Morphology of Herpes Simplex Virus

1.2.1.1 Classification

Herpes simplex virus is a member of the *Herpesviridae* family of eukaryotic double-stranded DNA viruses. This family can be divided into three subfamilies α, β, and γ based on host range, tropism, length of reproductive cycle and the characteristics of latency (Roizman, 1982).

The two serotypes of herpes simplex virus, HSV-1 and HSV-2 belong to the α subfamily. Members of this family cause epithelial lesions and have the ability to become latent in nervous tissue, most often in ganglia. This group is also characterised by having a short reproductive cycle (<24 hours) and a broad host range. HSV-1 is the best studied example of the group and is generally associated with facial and oral lesion whereas HSV-2 infection is associated with genital lesions.

1.2.1.2 Virion Structure

The herpes virion has four distinct morphological elements.

(i) An electron-opaque core which contains the double-stranded DNA genome wrapped around a proteinaceous matrix in the shape of a torus (Furlong *et al.*, 1972; Nazerian, 1974).

(ii) An icosahedral nucleocapsid, consisting of 162 capsomeres, approximately 100 nm in diameter, which encloses the DNA-containing core (Wildy *et al.*, 1960).

(iii) An electron-dense, amorphous material called the tegument which immediately surrounds the nucleocapsid (Roizman and Furlong, 1974).

(iv) An outer lipid bilayer envelope from which several viral glycoproteins protrude (Wildy, 1986).

1.2.1.3 Genome Organisation

The HSV genome consists of a linear double-stranded DNA molecule. The genome of HSV-1 has been completely sequenced and consists of 152 260 base pairs with a G+C content of 68.3% (McGeoch *et al.*, 1988). The HSV-2 genome has not been completely sequenced but its genome is estimated to be 50% homologous to HSV-1 (Kieff *et al.*, 1972) with a G+C content of 69% (Goodheart *et al.*, 1968). Hybridisation studies have shown the homologous regions to be colinear (Davison and Wilkie, 1983) and this has been more recently confirmed by the sequencing of a number of specific genes (discussed in McGeoch *et al.*, 1991). The regions of least homology between the two genomes include the R_L region region (see below) and McGeoch *et al.*, (1991) propose that this may be because it is the most recently evolved major element of HSV and is still in a state of relatively rapid change.

The HSV genome consists of two linked components designated L and S. Each of these two components consists of a unique sequence (U_L and U_S) bracketed by inverted repeats as shown in figure 1.5. The long repeat R_L and short repeat R_S are distinct. In addition, the molecule also contains a 400 base pair direct repeat sequence termed the *a* sequence which is present at the genomic termini and which is also present in the opposite orientation at the "joint" between the L and S segments.

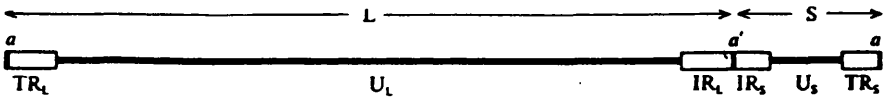


Figure 1.5. Genomic Organisation of HSV

For a description of the various sequence elements see text.

The L and S segments can invert relative to each other and DNA extracted from wild-type virions consist of an equimolar mixture of four populations of sequence-orientation isomers. By convention, one isomer chosen arbitrarily is designated as the prototype (P) for the purposes of genomic maps (reviewed by Roizman, 1979).

1.2.2 Viral Protein Synthesis

Productive infection with HSV involves the expression of three sets of viral genes, immediate early (IE or α), early (E or β) and late (L or γ) in a coordinated, sequential manner (Honess and Roizman, 1974). The regulation of expression of these genes occurs primarily at the level of transcription which is carried out by the host cell RNA polymerase II (Costanzo *et al.*, 1977).

Immediate early genes are transcribed immediately following infection and do not require *de novo* protein synthesis. Instead, initiation of viral gene expression requires the *virion polypeptide* Vmw 65 (Campbell *et al.*, 1984) also known as VP16, ICP 25 or as TIF as well as the cellular transcription factor Oct-1 and at least one other cellular transcription factor (Gerster and Roeder, 1988; Kirstie *et al.*, 1989; Katan *et al.*, 1990). The *cis*-acting regulatory sequences required to control expression of IE genes have been located in the 5′ flanking region of these genes and the following motifs, important for transcriptional activation, have been identified.

(i) A "TATA" box sequence at approximately -25 base pairs 5′ of the transcriptional start site.

(ii) Multiple Sp1 binding sites (Jones and Tijan, 1985).

(iii) GC rich sequences homologous to the core of the SV40 enhancer sequence (Preston and Tannahill, 1984).

(iv) Far-upstream sequences (usually beyond -110 base pairs) containing at least one copy of the the consensus sequence TAATGARAT (Gaffney *et al.*, 1985) which show enhancer type properties.

Vmw 65 does not appear to independently bind to DNA. Rather, it forms a complex with the cellular transcription factor Oct-1 that, in turn, binds to the TAATGARAT motif (O'Hare and Goding, 1988; Preston *et al.*, 1988; McKnight *et*

al. 1987). This transcriptional complex also involves at least one other cellular factor (Xiao and Capone *et al.*, 1990; Katan *et al.*, 1990). However, the precise mechanisms by which the complex forms, binds to DNA and stimulates transcription are still unknown.

The gene sequence of the Vmw 65 homologue in HSV-2 has recently been determined (Greaves and O'Hare, 1991) and the deduced protein sequence shows a high degree of sequence conservation (86%) with HSV-1. In addition, the HSV-2 protein also forms a transcription complex with Oct-1 and the TAATGARAT motif and displays powerful transactivating properties.

The HSV genome encodes 5 immediate early gene products, four of which are known to affect the expression of later viral genes. These gene products are named according to their apparent molecular weight (Vmw) as measured by SDS-PAGE or by "infected cell polypeptide" (ICP) number and the properties and functions of these proteins are briefly summarised below.

Immediate Early Gene 1

This gene encodes the protein known as ICP0 or Vmw 110. Shortly after synthesis, this protein is phosphorylated and localises to the nucleus, where it assumes a granular location throughout the duration of the infection (Pereira *et al.*, 1977; Knipe and Smith, 1986). Although it has been shown to bind to chromatin in infected cell nuclei there is no direct evidence that it binds directly to DNA (Hay and Hay 1980).

Vmw 110 can stimulate expression of all classes of HSV genes as well as other, non-HSV, eukaryotic genes in transient expression assays (O'Hare and Hayward, 1985a; Everett, 1986; Sekulovich *et al.*, 1988). Mutant viruses which have deletions in Vmw 110 are still able to grow, although at a low multiplicity of infection the mutants are slightly impaired for lytic growth (Stow and Stow, 1986; Sacks and Shaffer 1987). Thus Vmw 110 is not essential for productive infection

although it is required for fully effecient growth. Vmw 110 is also a requirement for the reactivation of HSV from the latent state (Leib *et al.*, 1989).

In a recent study Cai and Schaffer, (1992) using mutants in Vmw 110, demonstrated that Vmw 110 upregulates E and L gene expression but not the expression of IE genes during productive infection. However, in the absence of Vmw 65, when cells were transfected with infectious DNA, Vmw 110 may play a role in activating IE genes during the early stage of reactivation from latency which proceedes in the absence of virion-associated proteins.

Immediate Early Gene 2

This gene encodes the protein known as Vmw 63 or ICP 27 and like the product of the IE 1 gene it is a phosphoprotein which is localised throughout the nucleus for the duration of the infection (Knipe and Smith 1986; Knipe *et al.*, 1987). Analysis of several different types of mutants have shown that Vmw 63 is essential for viral growth and it appears to be required to repress expression of certain IE and E genes as well as being an absolute requirement for the transcription of late genes.(Sacks *et al.*, 1985; McCarthy *et al.*, 1989; Rice *et al.*, 1989).

The mechanism by which this protein carries out these various functions is still poorly understood but transfection assays have shown that it may mediate its action via interactions with and modifications of Vmw 175 and other viral or cellular proteins (Everett, 1986; Rice and Knipe, 1988; Sekulovich *et al.*, 1988; Su and Knipe, 1989; McMahan and Schaffer 1990). It has also been reported to be able to bind DNA (Hay and Hay, 1980) but it is unclear if this is related to its function.

Immediate Early Gene 3

The product of the IE 3 gene is the phosphoprotein Vmw 175 or ICP 4 and it is the best characterised of all the IE gene products. Vmw 175 translocates to the nucleus shortly after synthesis (Pereira *et al.*, 1977). Initially it assumes a diffuse distribution within the nucleus but at later times it is localised in globular structures

which correspond to the sites of HSV DNA replication (Knipe *et al.*, 1987). The structure and composition of these replication complexes will be discussed in more detail later (section 3.7). This differential location may reflect different mechanisms by which Vmw 175 may influence gene expression at two different periods of the replicative cycle.

A number of studies using both temperature sensitive and deletion mutants have shown that Vmw 175 can down-regulate the expression of all 5 IE genes and up-regulate the expression of E and L genes (Preston, 1979; Dixon and Schaffer, 1980; DeLuca *et al.*, 1985). These findings have been further supported by cotransfection studies where the Vmw 175 has been shown to transactivate transcription from E or L promoters but inactivates transcription from IE promoters (Everett, 1984; DeLuca and Schaffer, 1985; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985a and b).

Smith and Schaffer, (1987a and b) have shown that the HSV-1 and HSV-2 homologues of Vmw 175 are are functionally interchangeable despite a number of biochemical differences.

Vmw 175 has been purified and shows DNA binding activity. Faber and Wilcox, (1986) showed that the protein binds to the consensus sequence ATCGTNNNNYCGRC. However, although Muller, (1987) detected binding of Vmw 175 at this site in the 5′ region of the IE 3 gene, other studies have shown Vmw 175 can also bind to other sequences within this promoter region (Kirstie and Roizman, 1886; Michael *et al.*, 1988). The reason for these apparent anomalies is unclear but it is reasonable to suppose that the binding of the Vmw 175 to its own gene promoter is the mechanism whereby Vmw 175 autoregulates its expression.

The mechanism by which Vmw 175 causes transactivation of E and L genes is still not clearly understood and as yet there is no correlation between the sequences defined for Vmw 175 binding and transactivation.

Immediate Early Genes 4 and 5

The gene products of IE 4 (Vmw 68 or ICP 22) and IE 5 (Vmw 12 or ICP 47) are not required for viral replication (Post and Roizman, 1981; Mavromara-Nazos *et al.*, 1986) and the exact function of these proteins in viral growth is still unknown.

Early Genes

Transcription of early genes is dependent on the prior expression of IE proteins (Honess and Roizman, 1974,1975; Preston, 1979). E mRNAs are first detected about 3 hours post-infection (p.i) and reach maximum levels 5-7 hours p.i (Wagner, 1985). Early proteins include the proteins required for viral DNA replication as well as those responsible for altering the pool sizes of nucleoside triphosphates (e.g. ribonucleotide reductase and thymidine kinase)

The regulation of expression of E genes is not as well understood as the regulation of IE genes. Sequence comparison of the E promoters gD, *tk* and ICP 8 have identified the following promoter elements (Su and Knipe, 1987)

(i) A "TATA" box at about -25 base pairs 5′ to the mRNA start site.

(ii) A distal upstream promoter region containing A+T rich sequences and G+C rich inverted sequences. Two of these G+C regions fit the consensus binding sequence for SP1 and, indeed, the *tk* promoter has been shown to be able to bind this transcription factor (Jones and Tijan, 1985).

(iii) A "CCAAT" box at -80 base pairs in the *tk* promoter only.

None of the above sequence motifs are unique to HSV E promoters. Therefore, cellular transcription factors may be required to cooperate with viral proteins to activate transcription from these promoters but the precise mechanism of E gene expression is still unknown.

The late genes (L or γ) can be divided into two groups. The EL or γ_1 genes which require 3-5 hours of viral protein synthesis and whose expression, although not dependent on DNA replication, is stimulated by it and the true late genes (L or γ_2) whose expression has an absolute requirement for viral DNA synthesis (Holland *et al.*, 1980; Wagner, 1985).

1.2.3 HSV Replication

The HSV genome contains both *cis* and *trans*-acting elements which are necessary for its replication. The *cis*-acting are two distinct but similar DNA sequences which function as replication origins. These are termed ori_L, of which there is one copy in the U_L portion of the viral genome (Weller *et al.*, 1985) and ori_S which maps to the R_S region and hence is present at two copies per genome (one in TR_S and one in IR_S) (Stow, 1982).

The trans-acting elements have been identified using an assay based on the transient replication of plasmids containing ori_L or ori_S and seven HSV genes have been identified and sequenced which are necessary for origin dependent HSV replication in this assay (McGeoch *et al.*, 1988; Wu *et al.*, 1988). All seven have now been assigned functions and these are outlined briefly below.

Two of the genes, UL30 and UL29, encode the previously characterised proteins DNA polymerase (Purifoy *et al.*, 1977) and the single-stranded DNA-binding protein ICP8 (Weller *et al.*, 1983) respectively. The UL 42 gene has been identified as encoding the 65K DNA-binding protein ICP 34/35 (Parris *et al.*, 1988) which is an accessory factor to DNA polymerase (Gallo *et al.*, 1988). The three proteins encoded by the genes UL5, UL8 and UL52 have been shown to interact to form a functional helicase-primase complex (Crute *et al.*, 1989) and the final essential gene UL9 has been identified as encoding the ori_S binding protein (Olivio *et al.*, 1988)

As well as the seven proteins described above, viral replication in resting cells appear to require additional viral gene products. HSV encodes the enzyme ribonucleotide reductase, an enzyme responsible for the production of DNA precursors. This enzyme is composed of two subunits, the large subunit RR_1 designated ICP6 for HSV-1 and ICP10 for HSV-2 and the small subunit RR_2 designated UL40 for HSV-1. Viral mutants in the HSV-1 ICP6 gene are normal for replication in growing cells but DNA replication is reduced in resting cells

(Goldstein and Weller, 1988) and similarly ICP10 expression is required for HSV-2 replication only in resting cells (Smith *et al.*, 1992). The viral thymidine kinase gene is also not required for viral replication in growing cells but is essential for normal DNA replication in resting cells (Jamieson *et al.*, 1974). These viral enzymes duplicate normal cellular genes, however, HSV has probably evolved these homologues of cellular enzymes in order to allow them to replicate in non-dividing cells where these enzymes are not normally active.

Replication of HSV appears to occur in the nucleus in large globular structures termed replication complexes (reviewed by Knipe et al., 1990). Immunofluorescence studies have shown that one of the seven proteins essential for viral DNA replication, the major DNA-binding protein ICP8, is associated with replicating viral DNA and is essential for the formation of these complexes (De Bruyn-Kops and Knipe, 1988). In addition to ICP8, the viral DNA polymerase and the HSV alkaline nuclease, a protein necessary for the production of progeny virus, have also been shown to co-localise with viral DNA replication (Bush *et al.*, 1991; Thomas *et al.*, 1992) as have replicating cellular DNA and various host cell replication proteins (Wilcock and Lane, 1990).

The complete mechanism of HSV DNA replication is still not understood but electron microscopic studies and studies on the physical forms of DNA throughout the replicative cycle suggest that HSV DNA replicates by a rolling circle mechanism (reviewed by McGeoch, 1987).

1.2.4 Effect of HSV Infection on Host Macromolecular Synthesis

HSV infection results in the inhibition of both host DNA and protein synthesis (reviewed in Fenwick, 1984).

Cellular DNA synthesis is inhibited at 2.5 hours by as much as 80% (Roizman, 1969). The mechanism of this inhibition is still unknown and has not been mapped to any specific HSV protein or gene. A diagnostic feature of HSV infection is the displacement of cellular chromatin from the nuclear interior and its condensation at the nuclear membrane, an event which probably occurs between 0–1 hours p.i. (Dargan and Subak-Sharpe, 1983). It has been suggested that this margination of chromatin may remove the majority of it from nuclear sites of DNA replication and hence result in the inhibition of cellular DNA synthesis (Kaplan and Ben-Porat, 1963; Knipe, 1990)

In comparison to the shut-off of cellular DNA synthesis, the shut-off of protein synthesis has been more extensively studied. This occurs in two stages, the primary phase does not require viral gene expression whereas a late shut-off function reduces the remaining level of host protein synthesis and requires the expression of viral genes (Fenwick and Clark, 1982). Both of these effects are mediated by the product of the virion host shutoff (*vhs*) gene of HSV which maps within the UL 41 open reading frame (Kwong *et al.*, 1988). The product of the *vhs* gene encodes a protein that is a structural component of the virion and early in infection it causes the inhibition of cellular protein synthesis by inducing the degradation of host mRNA (Strom and Frenkel, 1987; Kwong and Frenkel, 1989). The *vhs* protein also degrades viral mRNA from all three classes of viral genes (Strom and Frenkel, 1987; Oroscar and Read, 1989) and hence, it appears to have a non-selective mRNA degradation function although the way it carries out this function is still unknown.

1.2.5 HSV and Cellular Transformation

Both HSV-1 and HSV-2 are able to induce the morphological transformation of normal diploid mammalian cultured cells, a phenomenon first demonstrated by Duff and Rapp, (1971, 1973) who observed transformation after exposing hamster embryo cells to the entire inactivated HSV genomes.

Three distinct regions of the HSV genome have since been identified which are sufficient to independently initiate transformation when transfected into cultured rodent cells (reviewed by Macnab, 1987). One of these, called MTR I (morphological transforming region I) is found in HSV-1 and maps to the restriction fragment

Bgl II i (0.32 to 0.42 map units) (Camacho and Spear, 1978; Reyes *et al.*, 1979). In HSV-2 the transforming regions have been localised to within the *Bgl* II n fragment (0.58 to 0.62 map units) (Reyes *et al.*, 1979; Galloway and McDougall, 1981) and to the *Bgl* II c fragment (0.43 to 0.58 map units) (Jariwalla et al., 1983) and these are termed MTR II and MTR III respectively (see figure 1.6).

Although these HSV sequences appear to be required to initiate the transformation process these are not necessarily retained within the cell and hence HSV is proposed to act by a "hit-and-run" mechanism which does not require the continued expression of a virus encoded transforming protein (Skinner *et al.*, 1976; Galloway and McDougall, 1983; Cameron *et al.*, 1985). A number of different mechanisms have been proposed for the initiation of neoplastic transformation by HSV in a "hit-and-run" type manner and these include the following:

(i) DNA Structure of Transforming Regions

Galloway *et al.*, (1984) have identified a 737 base pair fragment within the *Bgl* II n fragment which has morphological transforming ability but which does not appear to specify a viral protein. Analysis of the DNA sequence of this small fragment revealed a region which could be proposed to form a stem-loop structure

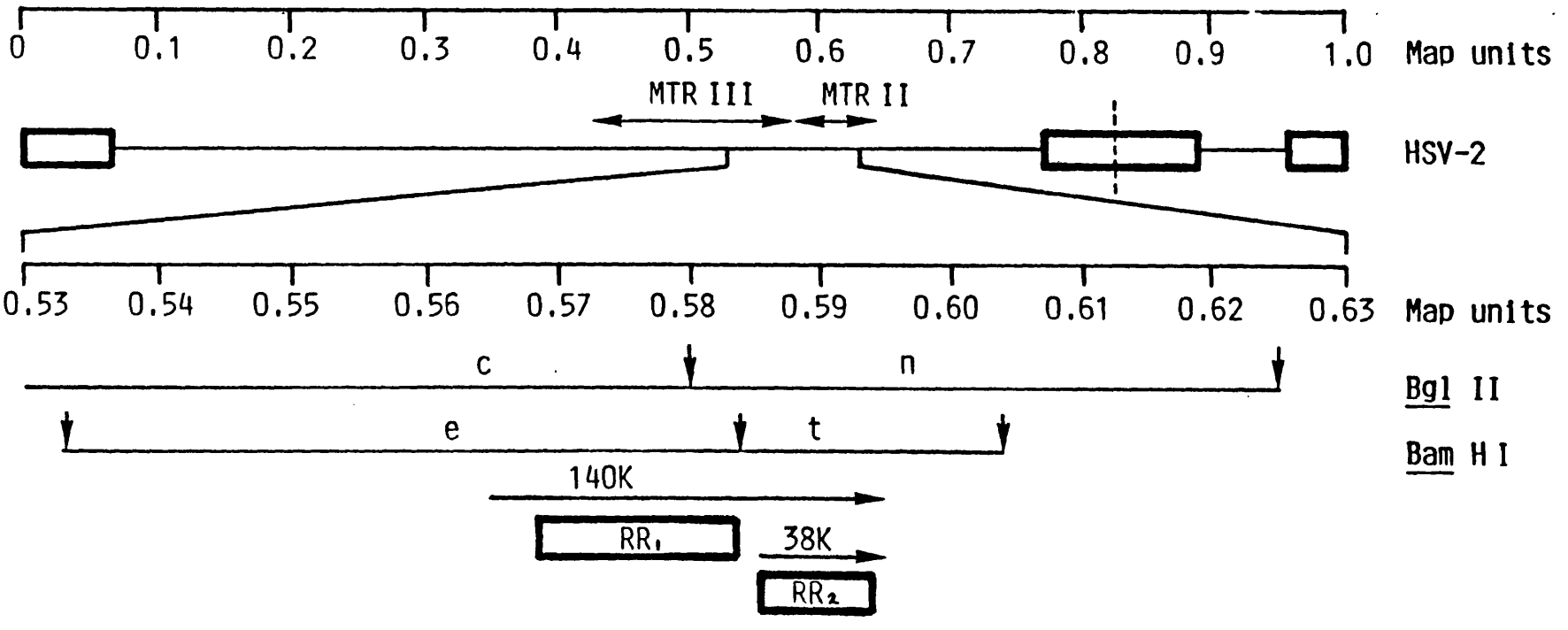


Figure 1.6 Transforming Regions of HSV-2

flanked by direct repeats similar to an insertion sequence-like element. Galloway *et al.*, (1984) hypothesised that this structure may cause transformation by activating a cellular oncogene or it may function as an enhancer element or as a mutagen. Similarly, Jones *et al.*, (1986) proposed that a 481 base pair within the *Bgl* II c fragment also contains an IS-like sequence. However, there is no direct proof as yet that these structures are important. In addition, neither of these structures are present in the corresponding regions of HSV-1 and may indeed be present at other regions of the genome which do not cause transformation (reviewed in Macnab, 1987).

(ii) Ribonucleotide Reductase

A second possibility is that the viral enzyme ribonucleotide reductase is involved in the transformation process as it maps within the region containing both MTR II and MTR III (see figure 1.6). The viral ribonucleotide reductase resembles the mammalian enzyme in that it consists of two non-identical subunits. The large subunit, RR_1, M_r 140 000 (ICP6 in HSV-1 and ICP10 in HSV-2) is mostly encoded by *Bgl* II c but the carboxy terminus lies in the *Bgl* II n fragment. The small subunit, RR_2, M_r 38 000, is entirely encoded by the *Bgl* II n fragment (McLauchlan and Clements, 1983; Bachetti *et al.*, 1984; Frame *et al.*, 1985) (see figure 1.6)

Although the entire RR_2 subunit maps within the *Bgl* II n fragment, no HSV specified ribonucleotide reductase activity has been detected in cells transformed with the *Bgl* II n fragment (Cameron *et al.*, 1985). Hayashi *et al.*, (1985) have detected the RR_1 subunit, using monoclonal antibodies, as far as passage 33 in cells transformed with the *Bam*H I e fragment which encodes the entire RR_1 subunit. However a subfragment of *Bam*H I e which encodes neither the amino nor carboxy terminus of RR_1 has also been shown to transform Rat-2 cells (Jariwalla *et al.*, 1986).

Unlike the mammalian enzyme, it has recently been discovered that the HSV RR_1 subunit also possess serine/threonine specific kinase activity with similar properties to growth factor receptor kinases (Chung *et al.*, 1989, 1990)

Smith *et al.*, (1992) transfected cells with expression vectors containing either the RR_1 or the RR_1 protein kinase domain and demonstrated that both are able to transform cells to a neplastic phenotype. Alterations of growth factor receptor kinase activity is believed to be involved in tumourigenicity (Yarden and Ullrich 1988) and Smith *et al.*, (1992) speculate that the protein kinase domain may be functioning in a similar manner. The RR_1 protein kinase domain maps within the *Bam* H I e and *Bgl* II c fragments discussed earlier and for these fragments this protein may prove to be an important transforming protein. However, this is clearly not the only mechanism of HSV DNA transformation as fragments which lie outside this protein kinase domain e.g. the *Bgl* II n fragment, can also independently cause transformation.

(iii) Altered expression of Cellular Genes

Macnab *et al.*, (1985) demonstrated that antisera, raised against HSV-2 rat embryo cells, immunoprecipitated a set of proteins from HSV transformed cells but not from control uninfected cells. These proteins were also precipitated with serum from tumour bearing animals or with a mouse monoclonal antibody, TG7A and peptide mapping confirmed these to be indistinguishable from cellular proteins. In addition, these proteins were also found in other transformed and immortalised cells. Macnab *et al.*, (1985) therefore proposed that these proteins, which accumulate on HSV infection and which are expressed in transformed cells, may be important in the initiation of of transformation and that induction of the genes encoding these proteins by HSV may be a mechanism by which HSV initiates the transformation process.

A set of four polypeptides have been identified, a 90 kDa doublet, a 40 kDa and a 30 kDa species but only the 90 kDa doublet has been extensively studied. In a

recent study, Hewitt *et al.*, (1991) demonstrated that the upper and lower 90 kDa proteins were not induced by heat shock, glucose starvation, tunicamycin or calcium ionophore and hence are not related to the stress proteins, heat shock protein 90 (HSP 90) or the glucose regulated protein, GRP 94 as was previously speculated (Macnab *et al.*, 1985). In addition, infection of the HSV-2 transformed cell-line Bn5T with HSV-2 increases the amount of the upper 90 kDa polypeptide (U90) by 2.5 to 5 fold whereas the amount of the lower 90 kDa protein remains unchanged. From these results, Macnab *et al.*, (1985) propose that the U90 polypeptide is a novel transformation specific protein which can be induced by HSV-2 infection.

The means by which HSV-2 infection induces these cellular polypeptides is still unclear. Infection of rat embryo cells with HSV-2 causes the undermethylation of host cell DNA and cell strains derived from HSV transformed cells also show reduced levels of methylation in comparison to the parent cells. This hypomethylation may, therefore, be a possible mechanism whereby HSV-2 can activate the expression of these cellular genes (Macnab *et al.*, 1988).

1.2.6 DNA Methylation and Herpesviruses

In section 1.1.4.3 it was indicated that methylation of CpG dinucleotides is an inherently mutagenic process and that this phenomenon is probably resposible for CpG supression, and hence the relative excess of TpG and its complement CpA, in vertebrate genomes. The exception to this supression is found in CpG islands which have the expected frequency of CpG dinucleotides and this is proposed to occur because they are never methylated in the germ-line (Bird *et al.*, 1986).

Honess *et al.*, (1989) investigated and compared the frequencies of CpG dinucleotides in all three herpesvirus subfamilies. The α herpesviruses investigated (HSV and varicella-zoster virus) have frequencies close to those expected for their mononucleotide composition as did human cytomegalovirus, a β herpesvirus. In contrast, the members of the γ hepesvirus subfamily (Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS)) were both CpG supressed with an excess of TpG and CpA dinucleotides, indicative of sequences which have been subject to methylation.

Since virion DNA from productively infected cells is not detectably methylated, Honess *et al.*, concluded that any methylation mediated effects on dinucleotide composition must be due to methylation events occurring whilst the virus is in the latent state. The lack of CpG supression in HSV suggests that this virus is not methylated whilst latent and this is in accordance with the findings of Dressler *et al.*, (1987) who analysed the methylation status of HSV-1 DNA in the central nervous system of latently infected mice. However, in an *in vitro* model latency system Youssoufian *et al.*, (1982) found the HSV genome was extensively methylated but it may be that this apparent contradiction simply reflects the methylating capacity of the cells used in this experiment as *in vivo* HSV is normaly latent in non-dividing nervous tissue which has limited DNA methylase activity.

The γ herpesviruses (EBV and HVS) investigated are both maintained as multicopy episomes within proliferating lymphoblastoid cells and hence it is feasible that these could become methylated by these cells active DNA methylation

machinery and indeed, episomal EBV DNA is extensively methylated (Kinter and Sugden, 1981). Initial evidence for the involvement of DNA methylation in the EBV lytic cycle came from studying the effect of 5 azacytidine on latently infected EBV carrying cell lines. Treatment with this drug activated the lytic cycle in these latenly infected cell-lines (Ben-Sasson and Klein 1981) and Szyf *et al.*, (1985) demonstrated that genome wide hypomethylation precedes the expression of the virus. Different types of EBV carrying cell lines show different expression of a set of virus encoded latency associated genes and in more recent studies these cell-type specific expression patterns have also been correlated to the methylation status of the particular gene (Minarovits *et al.*, 1991).

1.3 Aims of Project

DNA methylation is now widely accepted to be involved, at least at some level, in the control of gene expression and in general terms, lack of methylation of a gene has been correlated with its expression.

Infection of RE cells with HSV-2 inhibits the methylation of newly synthesised cellular DNA and various HSV transformed cell strains also show reduced levels of methylation in comparison with the parent RE cells (Macnab *et al.*, 1988). It has been postulated that this widespread hypomethylation may be a factor in the expression of a set of tumour-specific cellular proteins which accumulate on HSV infection. Although the functions of DNA methylation are beginning to be understood, little is known about how DNA methylation patterns are altered. The aim of this project, therefore, was to investigate the mechanism whereby HSV infection causes the hypomethylation of newly synthesised cellular DNA in RE cells.

The *Bgl* II n fragment of HSV-2 has been shown to independently induce transformation when transfected into RE cells although this DNA is not necessarily retained in transformed cells. An additional aim of the project was, therefore, to investigate if this DNA fragment causes this "hit-and-run" type of transformation by directly altering the level of cellular DNA methylation.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Fine chemicals

Acrylamide, EDTA, glycerol, N-2-hydroxyethylpiperazine-*N'*-2-ethane-sulphonic acid (HEPES), *NN'*-methylene bis acrylamide, phenol, perchloric acid (PCA), SDS, and trichloroacetic acid (TCA), were supplied by FSA Laboratory Supplies (Fisons), Loughborough, England, U.K.

4-aminosalicylic acid, ammonium persulphate, bromophenol blue, 98% formic acid, 8-hydroxy quinoline, mercaptoethanol, and *NNN'N'*-tetramethyl-ethylenediamine (TEMED) were supplied by BDH Chemicals, Poole, Dorset, England, U.K.

Ampicillin, bovine serum albumin, butyryl-CoA, calcium ionophore (A23187), chloramphenicol, coomassie brilliant blue G and R, ethidium bromide, monensin, ouabain, piperazine-*NN'*-bis (2-ethane-sulfonic acid) (PIPES), phenylmethylsulphonylfluoride (PMSF), poly [d(I-C).d(I-C)], Tween 20 (polyoxyethylene sorbitan monolaurate), salmon testis DNA and valinomycin were supplied by Sigma Chemical Co., Poole, Dorset, England, U.K.

m-cresol, dimethyl sulphoxide (DMSO), and Triton X-100 were supplied by Koch-Light Laboratories Ltd, Colnbrook, England, U.K.

Tetramethylpentadecane (TMPD) was supplied by Aldrich Chemical Co., Gillingham, Dorset, England, U.K.

Dithiothreitol (DTT) was supplied by Boehringer Mannheim, East Sussex, U.K.

Xylenes was supplied by May and Baker, Manchester, England, U.K

Ecoscint A was supplied by National Diagnostics, New Jersey, USA.

Agarose (ultra pure, electrophoresis grade), was obtained from BRL,Life technologies Inc, USA.

All other chemicals were of the highest grade available.All solutions were prepared, where appropriate, with deionised distilled water (dH_2O).

2.1.2 Cell Culture Growth Media

BHK21 medium (Glasgow modification of Eagle's minimal essential medium), 10x concentrate, and Dulbecco's modification of Eagle's minimal essential medium (DMEM), 1x concentrate, were supplied by Gibco BRL Ltd, Paisley, Scotland, U.K.

Newborn calf serum, foetal calf serum, sodium bicarbonate (7.5%), L-glutamine (200 mM), penicillin (10,000 U/ml)/streptomycin (10,000 mg/ml), non-essential amino acids (100X), and gentimycin (10 mg/ml) were also obtained from Gibco.

Mycostatin sterile powder (500,000 U made up in 10 ml distilled water) was obtained from E.R. Squibb & sons Ltd., Hounslow, Middlesex, England,.U.K.

Tryptose phosphate broth was obtained from Difco Laboratories, Detroit, U.S.A.

2.1.3 Bacterial Growth Media

Bactotryptone, yeast extract and bactoagar were obtained from Difco, Detroit, USA.

2.1.4 Chromatographic Media

Amberlite CG50 (chromatographic grade type II) was obtained from BDH Chemicals, Poole, Dorset, England, U.K. Aminex A6 resin was obtained from BIORAD Laboratories Ltd, Watford, England, U.K. P-11 phosphocellulose was obtained from Whatman Biosystems Ltd., Maidstone, Kent, U.K.

2.1.5 Enzymes

Ascites DNA methylase (purified according to Turnbull and Adams, 1976), was kindly provided by Dr R.L.P. Adams. S1 nuclease and Proteinase K were obtained from Boehringer Mannheim East Sussex, U.K. Pronase was obtained from Calbiochem Novabiochem, Nottingham, England U.K.

2.1.6 Antibodies

Fluorescein-isothiocyanate conjugated (FITC) goat anti-mouse IgG was obtained from Sigma Chemical Co., Poole, Dorset, U.K. FITC conjugated rabbit anti-mouse IgG was obtained from ICN Flow Biomedicals Ltd., High Wicombe, Bucks., England, U.K. Mouse monoclonal anti-5'-bromo-2'-deoxyuridine (IgG), was obtained from Amersham International plc, Amersham, England, U.K. as part of the cell proliferation kit (RPN 20) which also contained the nuclease solution,

antibody diluent and the BrdU labelling reagent (1:10, 5'-bromo-2'-deoxyuridine:5'-fluoro-2'-deoxyuridine).

2.1.7 Radiochemicals

S-adenosyl-L-[methyl-^{3}H]-methionine	(500 mCi/mmol)
" "	(15 Ci/mmol)
" "	(87 Ci/mmol)
[6-^{3}H]-uridine	(23 Ci/mmol)
^{125}I-protein A	(30 mCi/mg)
chloramphenicol-D-threo-[dichloroacetyl-1-2-^{14}C]	(55 mCi/mmol)
L-^{35}S-methionine	(800 mCi/mmol)

The above isotopes were obtained from Amersham International plc, Amersham, England, U.K.

2.1.8 Continuous Cell Lines

Baby Hamster Kidney 21, clone 13 cells (BHK21/C13), established by Macpherson and Stoker (1962) and maintained in the Institute of Virology, were routinely used to prepare and titrate stocks of virus.

Bn5T is a tumour cell line derived from a focus of RE cells transformed by the *Bgl* II n fragment of HSV-2 strain HG52. These were established by Cameron *et al.*, (1985) and were used to study hypomethylation and transformation.

2.1.9 Viruses

HSV-2 strain HG52 (Timbury, 1971) and the *ts* mutant of HSV-1 (G), *ts*K, (Marsden *et al.*, 1976) were provided by Mrs M. Murphy, Institute of Virology, Glasgow University.

2.1.10 Cell Culture Solutions

BHK 21 medium (Glasgow modification of Eagle's minimal essential medium) was stored as a 10X concentrated solution. For use, 40 ml of the concentrate was added to 300 ml of sterile distilled water. This was supplemented with 40 ml of tryptose phosphate broth, 14 ml of sodium bicarbonate (7.5%), 4 ml of penicillin (10,000 U/ml)/streptomycin (10,000 μg/ml) and 4 ml of L-glutamine (200 mM) to give a final concentration of 1X BHK 21 medium.This medium was then further supplemented with 5% (v/v) or 10% (v/v) newborn calf serum and designated ETC_5 or ETC_{10} respectively.

Dulbecco's modification of Eagle's minimal essential medium (DMEM) was stored as a 1X concentrate. For use it was supplemented with 5 ml of L-glutamine (200 mM), 5 ml of non-essential amino acids (100X), 5 ml of penicillin (10,000 U/ml)/streptomycin (10,000 μg/ml), 0.3 ml mycostatin (50,000 U/ml), 0.5 ml of gentimycin (10 mg/ml) and 5% (v/v) foetal calf serum.

Versene was prepared as a stock solution in PBSA (0.2 g/l) containing 1% phenyl red. This was mixed 1:4 with trypsin immediately prior to use.

The complete compositions of GMEM and DMEM are given in table 2.1. The constituents of non-essential amino acids are given in table 2.2.

Table 2.1 Eagle's Media Formulations (amino acid and vitamin components).

	GMEM	DMEM
Amino acids (mg/l)		
L-arginine-HCl	42.0	84.0
L-cystine	24.0	48.0
L-glutamine	292.0	584.0
L-glycine		30.0
L-histidine HCl.H_20	21.0	42.0
L-isoleucine	52.4	105.0
L-leucine	52.4	105.0
L-lysine-HCl	73.1	146.0
L-methionine	15.0	30.0
L-phenylalanine	33.0	66.0
L-serine		42.0
L-threonine	47.6	95.0
L-tryptophan	8.0	16.0
L-tyrosine	36.2	72.0
L-valine	46.8	94.0
Vitamins (mg/l)		
D-Ca pantothenate	1.0	4.0
choline chloride	1.0	4.0
folic acid	1.0	4.0
i-inositol	2.0	7.2
nicotinamide	1.0	4.0
pyridoxyl-HCl	1.0	4.0
riboflavin	0.1	0.4
thiamin-HCl	1.0	4.0

Table 2.2 Eagle's Non-Essential Amino Acids

Eagle's non-essential amino acids were supplied as a 100x concentrate which contained:

	g/l
L-Alanine	0.89
L-Asparagine-H_20	1.50
L-Aspartic acid	1.33
L-Glutamic acid	1.47
Glycine	0.75
L-Proline	1.15
L-Serine	1.05

2.1.11 Bacterial Growth Solutions

L-broth	g/l
bactotryptone	10
NaCl	10
yeast extract	5

Adjusted to pH 7.2 with NaOH and made up to 1 litre with dH_2O.

2.1.12 Solutions

During the course of this work a number of solutions were used repeatedly the compostion of which are given below.

Buffer M

Tris-HCl pH 7.8	50 mM
EDTA	1 mM
dithiothreitol	1 mM
NaN_3	0.01%
glycerol	10%

Buffer M^+ has PMSF (60 µg/ml) added just before use.

PBS

A:	Na_2HPO_4	10 mM
	KH_2PO_4	1.84 mM
	NaCl	0.17 M
	KCl	3.35 mM
B:	$CaCl_2.6H_2O$	6.8 mM
C:	$MgCl_2.6H_2O$	4.9 mM

A, B and C were autoclaved separately,when appropriate, and mixed in a ratio of 8:1:1 before use.

Stopper Solution

SDS	1%
EDTA	2 mM
butanol	5%
p-amino salicylate	3%
salmon testis DNA	0.25 mg/ml
NaCl	0.125 M

SDS-mix

SDS	2%
EDTA	4 mM
butanol	10 %

Diphenylamine reagent

diphenylamine	1.5 g
glacial acetic acid	100 ml
H_2SO_4 conc.	1.5 ml

Before use, 0.1 ml 1.7 % acetaldehyde was added per 20 ml of reagent.

T.E. buffer

Tris-HCl	10 mM
EDTA	1 mM

SDS Polyacrylamide Gel Buffers

Resolving gel buffer :

Tris-HCl pH8.8	1.5 M
SDS	0.4%

Stacking gel buffer :

Tris-HCl pH 6.8	0.5 M
SDS	0.4%

Running buffer :

Tris	25 mM
glycine	
SDS	0.1%

Sample buffer :

SDS	6%
glycerol	30%
mercaptoethanol	15%
bromophenol blue	0.1%
Tris-HCl pH 6.8	0.2 M

Immunoblotting Buffers

Nitrocellulose transfer buffer :

Tris-HCl pH8.3	25 mM
glycine	0.192 M
methanol	20%(v/v)
SDS	0.02%

Blotting buffer :

Tris-HCl pH7.2	20 mM
NaCl	0.15 M
Tween 20	0.1%(v/v)
NaN_3	0.5 mg/ml

2.2 Methods

2.2.1 Cell Culture Methods

2.2.1.1 Primary Cell Culture

Rat embryo (RE) cells were prepared from 18-day-old embryos of a highly inbred colony of Hooded Lister Rats. The pregnant rat to be used was killed by terminal general anaesthesia. The abdomen was opened and the uterus removed aseptically, using fresh forceps and scissors at every stage, into a large sterile Petri dish. After removing the embryos from their sacs into further sterile Petri dishes, the placentae were removed and the embryos eviscerated. Generally, each rat produced around 10-15 embryos. The embryos were then placed in sterile universals, washed twice with versene and minced using scissors. The minced embryos were transferred to a sterile 250 ml conical flask containing 100 ml of 0.25% trypsin and were stirred gently at 37°C for 30 minutes on a magnetic stirrer. After allowing the tissue to settle briefly, the trypsinised cell suspension i.e non-settled material, was removed and placed into two sterile plastic 50 ml centrifuge tubes containing 2 ml of foetal calf serum. A further 100 ml of 0.25% trypsin was added to the flask and the trypsinisation procedure repeated. To pellet the cells, the cell suspension was centrifuged at 1000 rpm for 5 minutes at 4°C. The cell pellets were then resuspended in a small amount of DMEM+5%(v/v) foetal calf serum and this was used to seed 4-6 plastic burrlers in total. After gassing with 5%CO_2 in air the cells were uncubated for 1-2 days at 37°C to allow the cells to attach. Fresh medium was then added to these cells and the old medium was decanted into two fresh burrlers and incubated for a further 2 days at 37°C at which time the medium in these cells was also changed. All the cells were incubated at 37°C until 60% confluent, usually 1-2 days after changing the medium. At this stage the cells were

then incubated at 31°C and were maintained for several weeks by replacing the medium twice weekly.

Prior to subculturing, the medium was replaced with fresh medium and the cells returned to 37°C for 2-6 hours. The cells were then subcultured as follows; the medium was first decanted and the monolayer washed twice with 20 ml of trypsin:versene. 10 ml of fresh trypsin:versene was then added to the cells. This was incubated at 37°C for 3-5 minutes during which time the cells became released into suspension. To inhibit the trypsin action, 10 ml of medium supplemented with serum was added to the cell suspension. The cells were then used to seed either; 850cm^2 plastic roller bottles at a density of 5x10^7 cells in 150 ml DMEM+5%(v/v) foetal calf serum; 90 mm dishes at a density of 5x10^6 cells in 10 ml of medium or 50 mm dishes at a density of 2x10^6 cells in 2 ml of medium. The cells were then incubated overnight at 37°C and used the following day. All RE cells used were, therefore, secondary cells.

2.2.1.2 Growth of Continuous Cell Lines

BHK/C13 cells were cultured as monolayers in ETC_{10} (Glasgow modification). Stocks of cells were maintained in the cytology unit in the Institute of Virology and were provided on request. Plastic 850cm^2 roller bottles were seeded at a density of 2x10^7 cells in 150 ml of medium and 50 mm plastic dishes were seeded at a density of 2x10^6 cells per dish in 4 ml of medium.

Bn5T cells were cultured in DMEM supplemented with 5%(v/v) foetal calf serum as monolayers in plastic 850cm^2 roller bottles. At confluency, the cells were subcultured in the same way as RE cells (see section 2.2.1.1). Generally the cells were split 1:4 every 3-4 days. For experimental purposes the cells were split 1:2 and incubated overnight at 37°C.

2.2.1.3 Virus Infection of RE Cells

RE cells were grown to approximately 80% confluence in the appropriate culture vessel. The medium was discarded and the appropriate amount of virus, sonicated briefly immediately prior to use, was addded to the cells in the minimum volume of medium (or Tris-saline) necessary to cover the monolayer. To mock-infect cells, exactly the same procedure was followed as for infection except that no virus was added to the medium (or Tris-saline) at this point. To adsorb the virus, the cells were incubated for 1 hour at 37°C with HSV-2 or at 38.5°C with the mutant virus *ts* K. Following adsorption, the innoculate was removed, fresh medium was added to the cells and the incubation continued at the appropriate temperature. Infection was carried out, unless otherwise stated, at a multiplicity of infection of 20 PFU:cell. To harvest, the monolayer was washed 2-3 times with PBS and the cells scraped into a minimum amount of the same buffer. The cells were pelleted by centrifugation in a microfuge at 2,000 rpm for 5 minutes at 4°C and were stored, if necessary, at -20°C.

2.2.1.4 Production of HSV-2

HSV-2 stocks were prepared by infecting 20, just subconfluent, 850 cm^2 plastic roller bottles of BHK C13 cells with 1 PFU of virus per 300 cells in a total of 20 ml of ETC_{10}. The infected cells were incubated at 31°C for 4–7 days until the cells were begining to dislodge. The cells were then harvested by shaking to remove all the cells into the medium. This cell suspension was decanted into two sterile glass 250 ml MSE bottles and spun at 2,000 rpm for 10 minutes. The supernatants were discarded except for 2 x 5 ml which were retained to resuspend the infected cell pellets. These suspensions were transferred to sterile 25 ml glass universals and sonicated for 5 minutes at 4°C. The cells were pelleted by

centrifugation at 2,000 rpm for 10 minutes and the supernatants combined and stored at -70°C. The cell pellets were then resuspended in 5ml of fresh medium, sonicated and centrifuged as above. The second supernatants were also stored at 70°C. This procedure produced 20 ml of virus of between $1x10^8$–$8x10^8$ PFU:ml (for titration see the next section, 2.2.1.6).

The sterility of the virus was checked by streaking the virus onto blood agar plates and incubating at 37°C and 31°C for 7 days.

2.2.1.5 Titration of HSV-2

Serial 10-fold dilutions of virus were made in ETC_5. 200 µl of each dilution (in the range $1x10^{-4}$ to $1x10^{-9}$) was added to duplicate 50 mm dishes of almost confluent monolayers of BHK 21/C13 cells. The virus was then adsorbed for 1 hour at 37°C. Following the adsorption, 5 ml of overlay medium (1:1, ETC5:3% (w/v) methylcellulose) was added to the cells and the incubation continued at 37°C for a further 2 days. The overlay medium was discarded and the monolayers stained with Giemsa stain for 30 minutes at room temperature. Excess Giemsa stain was removed by carefully washing the plates in water. The plates were then dried and the plaques counted using a plate microscope. Titres were expressed as the number of plaque forming units per ml (PFU:ml). The titre obtained for HSV-2 was in the range $1x10^8$ to $8x10^8$ PFU:ml.

2.2.2 Estimation of Protein Concentration

Protein concentration was estimated by the method of Bradford, (1976) using 1 mg/ml bovine serum albumin (BSA) as the standard protein solution.

2.2.3 Estimation of DNA Concentration

2.2.3.1 Optical Density Measurement

The absorption at 260 nm of the appropriate dilution of the sample was measured using a quartz 1 ml cuvette in a Cecil linear readout spectrophotometer. The concentration was estimated assuming that an A_{260} of 1 is equivalent to 50 μg/ml of doubled stranded DNA or 40 μg/ml of denatured DNA.

2.2.3.2 Burton DNA Assay

The DNA concentration in cell extracts was measured according to the method of Burton, (1956). 0.9 ml of 0.5 N PCA was added to 0.1 ml of the sample or standard to be assayed and this was incubated at 70°C for 30 minutes. The samples were then cooled and 2 ml of diphenylamine reagent (see section 2.2.12) was added. The samples were left overnight in the dark to allow the colour to develop and the E_{600} was then determined.

2.2.4 Fractionation of RE Cells

RE cells were mock-infected or infected with HSV-2 for the appropriate length of time and washed and pelleted as described in section 2.2.1.3. All following steps were carried out at 0–4°C.

The cells were swollen by washing 1-2 times in buffer M^+ (1-2 ml per $5x10^6$ cells), the cells pelleted each time by centrifugation at 2,000 rpm for 5 minutes. The swollen cell pellet was resuspended in buffer M^+ containing TritonX-100 again in 1-2 ml per $5x10^6$ cells. The cells were then lysed by either incubating on ice for 15

minutes; passing through a large bore needle or by homogenisation (3-4 strokes). The cell lysis was checked microscopically. If required, an aliquot of sample was saved at this point and was termed cell lysate.

To prepare nuclei, the remaining sample was centrifuged at 3,000 rpm for 5 minutes. The supernatant was discarded and the nuclei resuspended in a minimum volume of buffer M^+. Again, if required, an aliquot of sample was saved at this point.

To prepare nuclear extract and residue, the nuclei were pelleted at 3,000 rpm for 5 minutes as above and were resuspended in a minimum volume of buffer M^+. Added to this was an equal volume of buffer M^+ containing 0.4 M NaCl to give a final concentration of 0.2 M NaCl. The cells were incubated on ice for 15 minutes and were centrifuged at 2,000 rpm for 5 minutes. The supernatant was removed to a clean tube and was termed 0.2 M NaCl nuclear extract. The pellet was resuspended in a minimum volume of buffer M^+ and was termed nuclear residue.

When DNA methylase activity was being assayed the samples were generally used immediately. For immunoblotting, the samples were mixed with 0.5 volumes of SDS-polyacrylamide gel sample buffer, boiled for 5 minutes and stored at -20°C until required.

2.2.5 DNA Methylase Assay

DNA methylase activity was assayed in a reaction mixture containing 5 µl poly[d(I-C).d(I-C)] (0.1 mg/ml) and 3 µl S-adenosyl-L-(methyl-^{3}H) methionine (87 Ci/mmol, 1 µCi/µl). The sample to be assayed was made up to 15 µl with buffer M^+ and hence the final volume was restricted to 23 µl.

After incubation at 37°C for 1 hour the reaction was stopped by the addition of 500 µl of stopper solution. DNA was then purified for scintillation counting as follows:

To obtain complete recovery of the DNA, 50 µg of pronase was added and the incubation continued for a further 15 minutes at 37°C. Protein was removed by extraction with 300 µl of phenol mix (88% phenol, 12% *m*-cresol, 0.1% 8-hydroxyquinoline). After centrifugation in a microfuge for 10 minutes, the upper aqueous layer was removed to a fresh tube and the DNA precipitated by mixing with 2 volumes of ethanol. The DNA was pelleted by centrifugation in a microfuge for 5 minutes, redissolved in 50 µl of 0.3 M NaOH and incubated for 1 hour at 37°C to degrade RNA. The solution was spotted onto filter paper squares (Whatman 3MM, 2.5 cm^2). The DNA was precipitated and acid soluble removed by washing 5 times with 5% TCA at 4°C. The DNA was then further washed with ethanol and ether and dried under a stream of air. The filters were transferred to scintillation vials and the DNA solubilised by heating at 60°C for 30 minutes in 300 µl of 0.5 M perchloric acid (PCA). Ecoscint scintillator was added and the radioactivity counted using a liquid scintillation counter.

2.2.6 Base Composition Analysis of DNA

2.2.6.1 Purification of DNA

RE cells were incubated for the appropriate length of time with [6-^{3}H]-uridine which results in the incorporation of tritium into cytosine, methyl cytosine and thymine. The cell monolayer was then washed 5 times with PBS to remove excess label and the cells scraped into a small volume of the same buffer. For purification of the DNA from 850 cm^2 plastic roller bottles for C_0t analysis see section 2.2.11. For purification of DNA from 50 mm or 90 mm dishes the cells were firstly pelleted by centrifugation in a microfuge for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 100-500 µl of SDS or stopper mix to lyse the cells. If

at this stage, the solution was extremely viscous 0.1 mg/ml Pronase was added and the cells incubated for 30 minutes at 37°C.

Protein was removed by adding 0.7 volumes of phenol mix and vortexing thoroughly. After centrifugation in a microfuge for 5 minutes, the upper aqueous layer was removed to a fresh tube and 2 volumes of ethanol were added to precipitate the DNA. The DNA was pelleted by centrifugation in a microfuge for 5 minutes, redissolved in 0.3 M NaOH and incubated at 37°C for 1 hour to degrade RNA. At this stage, the samples were transferred to thick walled Pyrex tubes. The DNA was precipated by the addition of 3 ml of 5% TCA and pelleted at 3,000 rpm for 15 minutes at 4°C. The pellet was washed a further twice with TCA and once with ethanol, each time pelleting by centrifugation at 4°C as before. The pellet was then left to dry before being pyrolysed.

2.2.6.2 Pyrolysis and Fractionation of the Bases

The base composition of DNA was assayed by a modification of Adams *et al.*, (1979b).

Radiolabelled DNA samples were dissolved in 50 µl of 98% formic acid and sealed in thick walled Pyrex tubes. The DNA was pyrolysed by heating at 175°C for 90 minutes. The tubes were cooled before opening and the formic acid was evaporated. The pyrolysed material was then dissolved in 50 µl of 20 mM ammonium carbonate buffer (adjusted to pH 10.2 with ammonia) and applied under pressure to an Aminex A6 column (27 cm x 12 cm) equilibrated at 50°C. The column was eluted with the same buffer at 50°C at a flow rate of 1 ml/minute. 1 ml samples were collected. To these were added 5 ml of Ecoscint scintillator and the radioactivity counted using a liquid scintillation counter. The elution of the bases was followed by measuring the absorbance at 260 nm and figure 2.1 shows a typical elution profile and the corresponding cpm.

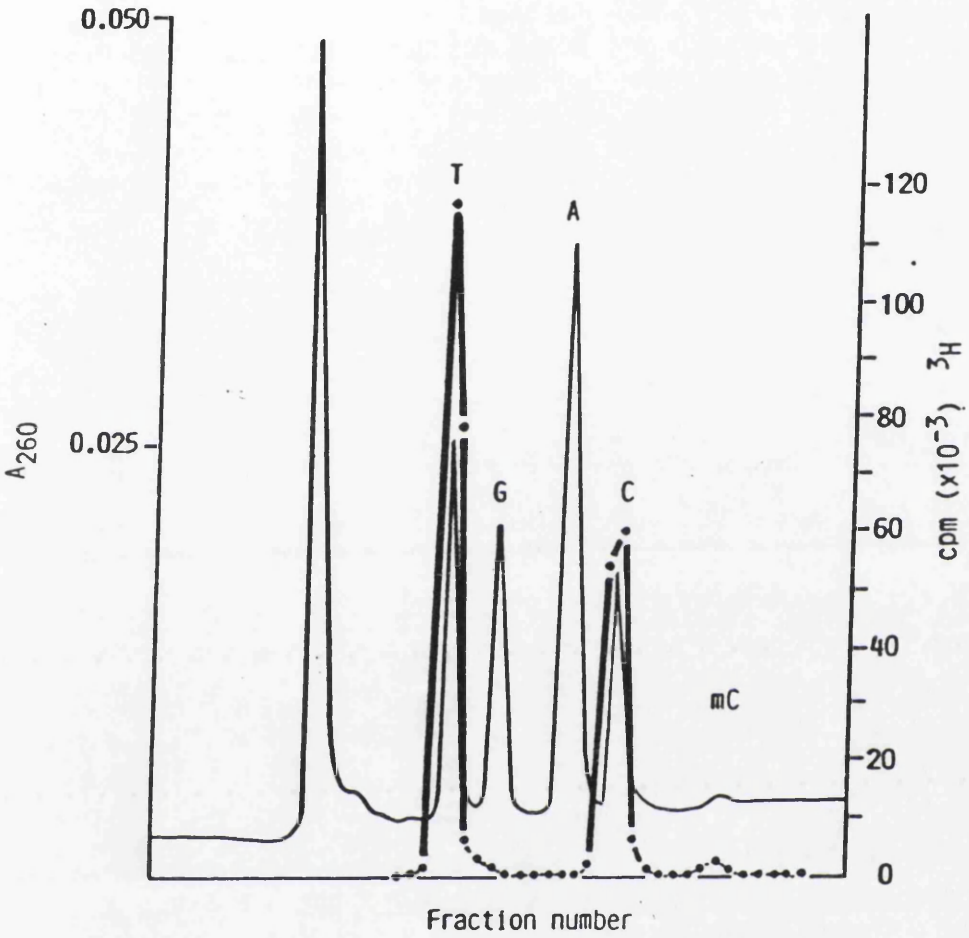


Figure 2.1 HPLC Base Analysis

DNA, labelled with [6-^{3}H]-uridine, was purified as in section 2.2.6.1. The DNA was then pyrolysed and the bases fractionated on an Aminex A6 column as described in section 2.2.6.2. The solid line shows the absorption profile at 260 nm. The broken line shows the elution of tritium incorporated into thymine, cytosine, methyl cytosine.

2.2.7 Analysis of SAM and SAH Levels

Intracellular pool sizes of SAM and SAH were measured by a modification of Arnaud *et al.*, (1985).

2.2.7.1. Preparation of Cell Samples

Intracellular pools of SAM and SAH in RE cells were radioactively labelled by the addition of ^{35}S-L-methionine (800 mCi/mmol) to the external medium to a final concentration of 20 μCi/ml. After the appropriate incubation period at 37°C the cells were washed 3 times with PBS containing 600 mg/litre of methionine and were scraped in to 1 ml of this buffer. The cells were pelleted in a microfuge for 5 minutes at 4°C and the DNA and protein precipitated by the addition of 500 μl of 7.5% TCA. The precipitate was pelleted by centrifugation in a microfuge for 10 minutes at 4°C and the supernatant removed to a fresh tube and stored at -20°C until use. Before use, the supernatant was thawed and extracted 3 times with an equal volume of ether saturated with water in order to remove TCA.

The precipitate was washed twice with 7.5% TCA and was resuspended in 500 μl of 0.3 M NaOH by boiling for 10 minutes. When required, this was used for protein determination.

2.2.7.2 P-11 Phosphocellulose Column Chromatography

The appropriate volume of cell sample, and controls where applicable, were loaded onto a 10 ml column of P-11 phosphocellulose equilibrated with 10 mM HCl at 4°C. The sample was then eluted with a stepwise gradient of HCl (10 mM, 100 mM, 500 mM) at a rate of 70 ml/hour. 10 ml samples were collected, 5 ml of each were removed to scintillation vials containing 5 ml of Ecoscint scintillator and

the radioactivity measured using a liquid scintillation counter. The remainder of each of the samples was used to determine the absorbance at 260 nm.

2.2.8 SAM'ase Assay

The rate of breakdown of S-adenosyl-L-methionine was measured by a modification of the method of Gefter *et al.*, (1966).

RE cells were mock-infected or infected for 4 hours with HSV-2 and harvested as described in section 2.2.1.3.

The cells were swollen in 1ml buffer M^+ and pelleted by centrifugation at 3,000 rpm for 5 minutes at 4°C. To lyse the cells, the cell pellet was resuspended in 300 µl buffer M^+ containing 1% Triton X-100 and left on ice for 15 minutes. 250 µl of this cell lysate was then incubated with 10 µCi S-adenosyl-L-[methyl-^{3}H] methionine (15 Ci/mol) at 37°C for 30 minutes. The reaction was stopped by quick freezing the samples. Prior to use, the samples were thawed and the cell debris removed by centrifugation in a microfuge for 2 minutes. 1 ml of Amberlite CG50 (100 mg/ml in 0.02 M phosphate buffer pH 7.0) was added to the supernatant in a fresh tube and vortexed thoroughly. The Amberlite was then sedimented by centrifugation in a microfuge for 1 minute and the supernatant removed to a scintillation vial. Ecoscint scintillator was added and the radioactivity counted using a liquid scintillation counter.

2.2.9 Immunoblotting

2.2.9.1 SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli, (1970). An 8.75% separating gel was prepared using the following components:

10 ml resolving gel buffer

11.7 ml acrylamide stock

(30% w/v acrylamide:0.8% w/v *NN'*-methylene bis acrylamide)

18 ml dH_2O

200µl 10% ammonium persulphate

50 µl TEMED

TEMED was added last and the gel poured immediately. 0.1% SDS was layered over the gel to exclude oxygen. A 3% stacking gel was then prepared using the following components:

2.5 ml stacking gel buffer

2 ml acrylamide stock (as above)

5.5 ml dH_2O

200 µl 10% ammonium persulphate

75µl TEMED (added immediately prior to use)

When the resolving gel was set, the SDS was removed, the stacking gel poured on top and the comb inserted. 0.5 volumes of the sample buffer was added to the samples and these were then denatured by boiling for 5 minutes. Electrophoresis was carried out in running buffer for 3-4 hours at 40 mA. When appropriate, the protein bands were visualised by staining with coomassie blue (0.1% coomassie brilliant blue R in 50% (v/v) methanol:10% (v/v) acetic acid) for 1 hour at 40°C and

destaining in 50% (v/v) methanol:10% (v/v) acetic acid for the appropriate length of time.

2.2.9.2 Western Blotting

Western blots were performed in a Biorad transblot apparatus. Proteins were transferred onto Hybond C^+ at 400mA for 4-5 hours or at 50mA overnight in nitrocellulose transfer buffer according to manufacturers instructions. To ensure transfer had occurred the membrane was stained breifly with Ponceau S stain (0.2 % (w/v) Ponceau S, 3% (w/v) trichloroacetic acid, 3% (w/v) sulfosalicylic acid) and destained with dH_2O. The blot was then transferred to blotting buffer and either stored overnight at 4°C or incubated for 1 hour at room temperature.

2.2.9.3 Detection of Proteins

Following incubation in blotting buffer, as described above in section 2.2.9.2, the membranes were incubated for 90 minutes at room temperature with blotting buffer, minus Tween, supplemented with 5% (v/v) heat inactivated goat serum (inactivated by heating to 65°C for 10 minutes), 3% Marvel (non-fat milk powder) and the appropriate volume of primary antiserum. 10 ml of buffer was sufficient to just cover one membrane. The membrane was then washed for 5 minutes at room temperature in blotting buffer, minus Tween, supplemented with 3% Marvel and a further 4 times in blotting buffer. To decorate the primary antibodies, the membrane was incubated for 1-2 hours with 1-2 μCi of ^{125}I-protein A (30 mCi/mg) in 10 ml of blotting buffer. Enough ^{125}I-protein A was added to give a minimum of $1x10^6$ cpm/10 ml of buffer. Finally, the membrane was washed 5 times in blotting buffer

at room temperature, (for a minimum of 5 minutes per wash), dried, and autoradiographed as below (2.2.9.4).

2.2.9.4 Autoradiography

Dried Hybond C^+ membrane were exposed to a sheet of Fuji RX medical X-ray film with an intensifying screen at -70°C for 1-10 days.

2.2.10 Immunofluorescence Microscopy

Sterile glass coverslips were placed in 50 mm plastic culture dishes (7-8 coverslips per dish) and were seeded with RE cells at a density of $1x10^6$ cells/dish. The cells were incubated overnight at 37°C and were infected or mock-infected for 4 hours at 10 PFU: cell with the appropriate virus (as described in section 2.2.1.3). For labelling with bromodeoxyuridine (BrdU), the labelling reagent (10:1 5-bromo-2'-deoxyuridine:5-fluoro-2'-deoxyuridine) was diluted 1:1000 with cell growth medium and this was added to the cells 1 hour post infection. Following the infection, the cells were washed 3x with PBS and were then fixed by immersing the coverslips in a large volume of 3:1 methanol:acetone at -20°C for 10 minutes. The coverslips were then dried and stored at -20°C until required.

To rehydrate the cells, the coverslips were immersed in PBS for 5 minutes. They were then incubated for 10 minutes in PBS+2% foetal calf serum (FCS) to block non-specific binding sites. The primary antibody was diluted to the appropriate concentration with PBS+2% FCS with the exception of the monoclonal anti-BrdU antibody which was diluted in buffer containing a nuclease in order to denature the DNA and allow access to the BrdU epitopes. The coverslips were incubated in a humidified box with 100 µl of the primary antibody solution for 1

hour at 37°C (or at room temperature using the anti-BrdU antibody). The coverslips were then washed 3 times in PBS and twice in PBS+2% FCS over 5-10 minutes. 100 µl of the appropriate FITC conjugated 2nd antibody, again diluted in PBS+2% FCS, was added and the coverslips incubated in a humidified box for a further 1 hour at 37°C. The cells were then washed 3 times with PBS and mounted in glycerol.

Microscopy was performed with a Leitz Wetzler fluoresence microscope with a 40x or 100x NPL Fluotar objective lens.

2.2.11 C_0t Analysis

2.2.11.1 Preparation of DNA

To radioactively label DNA from RE and Bn5T cells, subconfluent 850 cm^3 plastic roller bottles of cells were labelled for 2 days with 200 µCi of [6-3H]-uridine (23 mCi/mmol), in 50 ml of growth medium. Following the incubation, the cell monolayer was washed 3 times with PBS and the cells scraped into approximately 40 ml of the same buffer. The cells were pelleted by centrifugation at 1,000 rpm for 5 minutes at 4°C. DNA was then prepared, free of protein, by extraction with phenol/chloroform and precipitation with ethanol. To prepare phenol/chloroform, solid phenol was firstly melted and saturated with T.E. buffer. It was then mixed with an equal volume of chloroform:isoamyl alcohol (24:1).

The cell pellets were resuspended in 10 ml of PBS and added dropwise to an equal volume of SDS-mix with constant stirring. Proteinase K was added to a final concentration of 50 µg/ml and the solution incubated at 37°C for 1 hour. An equal volume of phenol/chloroform was then added and mixed vigorously for 1 minute. After centrifugation at 3,000 rpm for 20 minutes at 4°C in a Beckman benchtop centrifuge, the aqueous layer was removed to a clean tube. This extraction was

repeated twice more with phenol/chloroform and once with chloroform. The DNA was then precipitated by the addition of 1/10 th volume of 3 M Na acetate pH 6 and 2.5 volumes of ethanol. This was vortexed and left overnight at -20°C. The DNA was pelleted by centrifugation at 10,000 rpm for 10 minutes. To remove the RNA, the pellet was resuspended in 400 µl NaOH and incubated for 1 hour at 37°C. Following the incubation, the DNA was precipitated with 5 % TCA and pelleted at 3,000 rpm for 15 minutes at 4°C. The pellet was washed a further twice with 5 % TCA and once with ethanol and dried. The pellet was resuspended in a small volume of T.E. buffer and left overnight at 4°C to dissolve. The DNA concentration was then measured spectrophotometrically.

2.2.11.2 Fractionation of DNA into Frequency Classes

The theoretical basis of the reassociation kinetics was from Britten *et. al.*, (1974).

In order to obtain controlled rates of reassociation, the DNA was firstly fragmented into shorter lengths (< 1kb). The DNA was sheared by passing through a small gauge needle and the size of fragments checked by agarose gel electrophoresis.

To denature the DNA, NaOH, at a final concentration of 0.175 M, was added to 40 µg of DNA. The samples were then heated to 60°C for 5 minutes.

The following C_0t values were chosen to represent the three main frequency classes of DNA:

1. Highly repetitive DNA $C_0t < 0.01$
2. Moderately repetitive C_0t 0.01-70
3. Unique $C_0t > 70$

C_0t is defined as the initial concentration (C_0) in moles nucleotide per litre, multiplied by the time (t) in seconds it takes for the DNA to reanneal. Reassociation

is also dependent on salt concentration and where a correction is applied for this the appropriate term is equivalent C_0t (EC_0t). However, at 0.175 M Na^+, the equivalent value is 1 and hence EC_0t is simply C_0t. Since the concentration of DNA in the reassociation mixture is known, the time of reassociation was determined to give the above frequency classes. To start the reassociation, HCl was added to a final concentenration of 0.175 M to neutralise the NaOH (the two solutions were previously titrated against each other). In addition, 4 µl of 10x PIPES buffer (piperazine-*NN'*-bis [2-ethane-sulphonic acid]) were added and the volume made up to 40 µl with dH_20. This was then incubated for the appropriate time at 60°C. To stop the reassociation, the samples were placed on ice. 20 µl of S1 nuclease reaction buffer (330 mM Na acetate, 0.15 M NaCl, 0.3 mM $ZnSO_4$) were added and the volume made up to 200 µl with dH_2O. To digest the single-stranded DNA, 10 units of S1 nuclease were added and the samples incubated at 37°C for 30 minutes. At this stage, 200 µg of calf thymus DNA were added as a carrier. The DNA was then precipitated with 5 % TCA and pelleted at 3,000 rpm for 15 minutes. The pellet was washed a further twice with 5 % TCA and once with ethanol. The supernatant from each of the steps was retained and counted by liquid scintillation spectrophotometry to determine the percentage of single-stranded DNA in each of the samples. The DNA pellets were then air dried, pyrolysed with formic acid and the bases separated by HPLC as described in section 2.2.6.

2.2.12 Large Scale Isolation of Plasmid DNA

The alkaline lysis method of Birmboin and Daly, (1979), was used to prepare milligram amounts of pure pLW4 and *Bgl* II n plasmid DNA for use in transfection assays.

E. coli bacterial stocks (stored in a 50 % (v/v) glycerol/L-Broth solution), of HB101 cells transformedwith pLW4 and DH5 cells transformed with the *Bgl* II n plasmid were kindly provided by Dr M. Bryans, formerely of our laboratory, and Dr J.C.M.Macnab, respectively. These were plated onto LB plates containing ampicillin.

10 ml of L-Broth containing ampicillin (100 µg/ml) was inoculated with a single colony of bacteria transformed with the appropriate plasmid and incubated overnight at 37°C. 5 ml of this culture were then used to inoculate 500 ml of L-Broth. The bacteria were grown at 37°C with vigorous shaking until they reached an optical density of 0.8 at 630nm. At this point, chloramphenicol was added to a final concentration of 165 µg/ml and the culture was then further incubated at 37°C overnight. Chloramphenicol allows the plasmid copy number per cell to increase by preventing the bacteria multiplying while still allowing plasmid replication to occur.

To pellet the bacteria, the culture was centrifuged at 5,000 rpm for 10 minutes at 4°C in a Beckman J2-21 centrifuge. The supernatant was discarded and the pellet resuspended in a total volume of 3 ml of alkaline lysis buffer I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8) at 4°C. The bacterial suspension was then transferred to a Ti60 centrifuge tube and 150 µl of lysosyme (40 mg/ml in alkaline lysis buffer I) added. The suspension was mixed and incubated on ice for 30 minutes before adding 6 ml of alkaline lysis buffer II (0.2 M NaOH, 1 % SDS) and continuing the incubation for a further 5 minutes. 3.75 ml of alkaline lysis buffer III (3 M Na acetate, pH 4) was added and incubation on ice continued for a further 60 minutes. The samples were then centrifuged in a Ti60 rotor in a Beckman ultracentrifuge at 30,000 rpm for 30 minutes at 0°C. The supernatant, containing

the plasmid was retained and decanted into a sterile 30 ml Corex tube. To this was added 0.6 volumes of isopropanol and the DNA was left to precipitate by incubating at room temperature for 10 minutes. To pellet the DNA, the solution was centrifuged at 8,000 rpm for 15 minutes at room temperature in a Sorvall SS-34 rotor. The pellet was then dissolved in 10 ml of T.E. buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and was subjected to CsCl/EtBr centrifugation as follows;

CsCl (9.6 g) and EtBr (0.6 ml of a 10 mg/ml stock) were added and the solution clarified by centrifugation at 1,500 rpm for 30 minutes in a Beckman benchtop centrifuge. After removing the supernatant to a clean tube, the density of the solution was checked using a refractometer and adjusted to a density of 1.59 g/ml by the addition of solid CsCl as necessary. The solution was then transferred to VTi50 heat-sealable 5 ml tubes and centrifuged at 200,000 g at 20°C for 16 hours in a Beckman LH-8 ultracentrifuge. Long wave U.V. light was used to visualise the plasmid DNA which migrates ahead of the host chromosomal DNA in the caesium gradient. After removing the tops of the tubes, plasmid DNA was removed by side puncture of the tube using a 21 guage needle and a 5 ml syringe to withdraw the band. To remove the EtBr the band was extracted four times with an equal volume of isoamylalcohol. The lower aqueous was then transferred to a 15 ml Corex tube containg 4 volumes of T.E. buffer and to this was added a further two times the total volume of ethanol. The DNA was left to precipitate overnight at -20°C. To pellet the DNA the sample was placed on dry ice for 15 minutes and then centrifuged at 10,000 rpm in a Sorvall HB4 centrifuge for 30 minutes at 0°C. The supernatant was discarded and the pellet resuspended in 200 µl T.E buffer and transferred to an Eppendorf tube. The DNA was then further precipitated by the addition of 0.1 volumes of 3M Na acetate pH 6 and 2.5 volumes of ethanol at -20°C. This was left on dry ice for 10 minutes and spun for 15 minutes at 4°C in a microfuge. The supernatant was discarded and the pellet washed with 500 µl of ethanol. After a brief spin, the pellet was drained and redissolved in a minimum

volume of T.E. buffer. The DNA concentration was determined spectrophotometrically and the purity examined by agarose gel electrophoresis.

2.2.13 Agarose Gel Electrophoresis

Electrophoresis on 1% (w/v) agarose gels was used to assess the purity of plasmid DNA and the size of genomic DNA. The agarose was dissolved in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 25 mM EDTA, pH 8.3) by boiling for 5 minutes. The agarose was left to cool to approximately 50°C and EtBr was then added to a final concentration of 0.5 μg/ml before pouring. One tenth volume of bromophenol-blue (BPB) containing sample buffer (15 % Ficoll, 100 mM EDTA, 0.1 %BPB) was added to the samples before applying to the gel. Electrophoresis was then carried out at room temperature at 40 mA for 1-2 hours on a BRL Horizon 58 mini gel kit in TBE buffer containing 0.5 μg/ml EtBr. The DNA was visualised using a UV transilluminator.

2.2.14 Transfection of Cells with Plasmid DNA

All the following procedures were carried out aseptically.
10 μg of the control plasmid, pLW4, and 10 μg of the appropriate test plasmid were mixed with 125 μl of 2 M $CaCl_2$ and 20 μg of salmon testis DNA. This was made up to 1 ml with sterile dH_20 and mixed thoroughly by inversion or pipetting. The mixture was then added dropwise, with constant mixing to ensure the formation of a fine precipitate, to a tube containing 1 ml of 2x HBS buffer (50 mM Hepes, 280 mM NaCl, 1,5 mM Na_2HPO_4, pH 7.12). The mixture was then incubated at room temperature for 30 minutes to allow the formation of the $Ca_3(PO_4)$:DNA precipitate.1 ml of this precipitate was added directly to duplicate,

subconfluent 50 mm dishes of RE cells. The cells were incubated at 37°C for 45 minutes at which time 4 ml of growth medium were added and the incubation continued at 37°C. 4 hours after the addition of the precipitate, 2 ml of a boost solution, (20% glucose and 10% DMSO in 1xHBS buffer), were added for 4-5 minutes. This was removed and the cells washed twice with growth medium. 4 ml of fresh growth medium were then added and the incubaton continued at 37°C for a further 20 hours. To allow the determination of % m^5C levels, 100 µl [6-^{3}H]-uridine (23 Ci/mmol) was added at this point to the medium. In addition, where appropriate, 100 mM $ZnSO_4$ was also added to the medium at this point and the incubation was continued for a further 24 hours. The cells were then harvested as follows:

The medium was discarded and the cell monolayer washed 3 times with PBS. 1 ml of TEN buffer (0.04 M Tris-HCl, pH 7.8, 0.01 M EDTA, 0.12 M NaCl) was then added and the cells incubated for 5 minutes at room temperature before being scraped off the dish with a rubber policeman. The cells were pelleted by centrifugation at 1,000 rpm for 5 minutes in a microfuge at 4°C and resuspended by pipetting in 90 µl of 0.25 M Tris-HCl, pH 7.8 on ice. The cells were then lysed by three cycles of freeze/thawing consisting of 5 minutes incubation on dry ice (-70°C) followed by 5 minutes at 37°C. The cell debris was pelleted by centrifugation at 5,000 rpm for 4 minutes in a microfuge. The supernatant was removed to a fresh tube and aliquots were used for protein estimation and for assaying CAT activity. The cell debris pellet was resuspended by pipetting in 100 µl of SDS-mix (2% SDS, 4 mM EDTA, 10% butanol). The tritium labelled DNA was then extracted from the cell debris and analysed as in section 2.2.6.1.

2.2.15 Chloramphenicol Acyl Transferase Assay

Chloramphenicol acyl transferase activity was assayed by the method of Seed and Sheen, (1988).

60 µl of cell extract were added to an Eppendorf tube containing 100 mM Tris-HCl, pH 7.8, 0.2 µCi [^{14}C]-chloramphenicol (55 mCi/mmol) and 250 µM butryl CoA (freshly prepared) in a total volume of 100 µl . After incubation at 37°C for 1 hour the reaction was terminated by the addition of 2 volumes of a 2:1 mixture of TMPD (tetramethyl pentadecane):xylenes and mixed vigorously by vortexing. After centrifugation for 1 minute in a microfuge, 90% of the upper organic phase was removed to a scintillation vial and the radioactivity counted using a liquid scintillation counter.

Chapter 3 The Mechanism of HSV-2 Infection Induced Hypomethylation of Newly Synthesised DNA in RE cells.

3.1 Introduction

Macnab *et al.*, (1988) demonstrated that infection of RE cells with HSV-2 results in the hypomethylation of host cell DNA synthesised during infection. In order to verify these results, RE cells were infected for 4 hours with HSV-2 at 20 PFU/cell as described in section 2.2.1.3. The cells were labelled between 1 and 4 hours post infection with [6-^{3}H] uridine (50 μCi per 50 mm dish) in order to incorporate tritium into cytosine, methylcytosine and thymine. DNA was then isolated, pyrolysed and the bases fractionated by HPLC as described in section 2.2.6.

The results are presented in table 3.1 and show that HSV-2 infection causes a significant reduction in the methylation of DNA synthesised during the infection. Macnab *et al.*, (1988) could not detect any appreciable amounts of viral DNA at 4 hours post-infection and hence the results reflect the level of methylation in newly synthesised cellular DNA. Both the levels of methylation and the C/T ratios correlate well with those reported by Macnab *et al.*, (1988).

	C/T ratio=(C+m^5C/T)	%m^5C=[(m^5Cx100)/(C+m^5C)]
Mock-Infected	0.49 ± 0.02 (4)	3.38 ± 0.14 (4)
Infected	4.38 ± 1.18 (4)	0.46 ± 0.12 (4)

Table 3.1 The Effect of HSV-2 Infection on %m^5C

The results show the mean values from 3-4 separate experiments ± standard deviation.

This inhibition of DNA methylation that occurs following infection of RE cells with HSV-2 could, theoretically, occur in a number of ways. For example, HSV-2 infection might.

(i) Directly inhibit DNA methylase activity *in vivo*.

This could be achieved by altering the structure of the enzyme, for example by proteolysis or by modification of certain amino acids by phosphorylation or glycosylation. Alternatively, infection may induce inhibitory cofactors which bind to DNA methylase and inhibit its action in this way.

(ii) Alter the metabolism of the cell.

This may result in rate limiting concentrations of the methyl group donor, S-adenosyl methionine or high concentrations of the feedback inhibitor, S-adenosyl homocysteine.

(iii) Alter the cellular environment.

Changes in the pH or ionic concentration of the cell might indirectly inhibit DNA methylase activity *in vivo*.

(iv) Alter the accessibility of DNA methylase to DNA.

DNA binding proteins may be induced which physically block the access of DNA methylase to its substrates sites on DNA either by binding to the specific substrate sites or by altering DNA conformation. Alternatively, there may be a movement of DNA methylase or replicating DNA within the cell, distancing the enzyme from its substrate.

The aim of the experiments described in this chapter is to address the above hypotheses and investigate the mechanism of HSV-2 induced hypomethylation of newly synthesised DNA in RE cells.

3.2 The Intracellular Distribution of DNA Methylase Activity

DNA methylase in mammalian cells, with the exception of bovine thymus (Sano *et al.*, 1983), is found associated with the nucleus. 70-80% of the enzyme, however, is only loosely associated with the nucleus and can be removed by extraction with low salt treatment (Turnbull and Adams, 1976). The remaining activity remains firmly associated with 2 M NaCl resistant nuclear matrix-like structures (Burdon *et al.*, 1985). There is evidence to suggest that this "bound" form of the enzyme is responsible for maintenance methylation *in vivo* (Davis *et al.*, 1985, Burdon *et al.*, 1985). Additional evidence for the importance of the "bound" enzyme in methylating newly synthesised DNA comes from findings that DNA methylase activity is associated with a high molecular weight "replitase" complex which contains a number of enzymes required for DNA replication (Noguchi *et al.*, 1983). More recent reports confirm the existence of such complexes which are found associated with the nuclear framework (reviewed by Huberman, 1987).

Macnab *et al.*, (1988) reported that the activity of DNA methylase in 0.2 M NaCl nuclear extracts was unaltered following infection of RE cells with HSV-2 despite the dramatic inhibition of methylation of newly synthesised DNA. It is possible, however, that infection may cause a dissociation of methylase from the matrix and a redistribution of methylase within the cell which would result in the loss of the ability to methylate newly synthesised DNA *in vivo*.

The aim of the following set of experiments was to investigate if HSV-2 infection alters the distribution of DNA methylase activity in the cell and nucleus of RE cells.

3.2.1 The DNA Methylase Microassay

The original standard assay for DNA methylase was developed by Turnbull and Adams, (1976) to assay partially purified enzyme fractions. However, in order to analyse DNA methylase activity in small numbers of tissue cultured cells it was necessary to devise a more sensitive microassay. Both assays involve measuring the incorporation of radioactivity from S-adenosyl-L-(methyl-^{3}H)-methionine into DNA. Methylated proteins and RNA are excluded by phenol extraction and alkali digestion, respectively.

In the microassay, to ensure the complete recovery of DNA, an incubation with protease is included after the initial incubation to facilitate the isolation of DNA that may be tightly associated with protein. In this section, the parameters investigated were the concentration of the DNA substrate and the concentration of S-adenosyl-L-(methyl ^{3}H)-methionine. Other parameters which have been previously investigated by Dr Adams in the development of the microassay are (i) the nature of the DNA substrate and (ii) the linnearity of the reaction (Adams *et al.*, 1991).

3.2.1.1 Poly [d(I-C).d(I-C)] Concentration Curve

The best substrate for DNA methylase *in vitro* is hemi-methylated DNA and this is thought to be the normal substrate *in vivo* (Turnbull and Adams, 1976; Bestor and Ingram, 1983; Pfiefer *et al.*, 1983). Mammalian enzymes also prefer denatured DNA to native indicating that the enzyme probably requires single-stranded regions with which to interact (Drahovsky and Morris, 1971; Adams *et al.*, 1979a). Hemi-methylated DNA can be prepared in a number of ways e.g. from primed single stranded M13 replicated in the presence of methyl dCTP (Adams, 1990). These methods, however, are time consuming and it is difficult to obtain reproducible samples of such DNA. In the standard assay, denatured *M. luteus* DNA is used as

the substrate in the presence of 100 mM NaCl, which stimulates the reaction. Although this DNA is unmethylated, it has a high proportion of C+G (72 mole percent) and hence a high CpG content. Poly [d(G-C).d(G-C)] is, theoretically, an even better unmethylated substrate than *M. luteus* DNA. This, however, produces a very stable structure which is less likely to "breathe" to form the single-stranded regions necessary for DNA methylase to bind to and this reduces its capacity to be methylated. A more suitable substrate, and the one chosen for use in the microassay, is poly [d(I-C).d(I-C)]. Replacing guanosine with inosine improves the ability to accept methyl groups (Pfiefer and Drahovsky, 1986) presumably because this reduces the stability of the molecule and allows DNA methylase to interact.

To optimise the concentration of poly [d(I-C).d(I-C)] in the assay, 5 µg of a crude fraction of DNA methylase, prepared from Krebs II ascites by Dr R.L.P.Adams, were incubated at 37°C for 1 hour with 1.5 µM S-adenosyl-L-(methyl-^{3}H)-methionine (87 Ci/mmol) and varying amounts of poly [d(I-C).d(I-C)] in a total volume of 23 µl in buffer M^{+}. The incorporation of ^{3}H into DNA was then assayed as described in section 2.2.5.

Figure 3.1. shows that a concentration of 40 µg/ml (0.5 µg in 23 µl) was saturating in the microassay and this was the concentration used in subsequent assays.

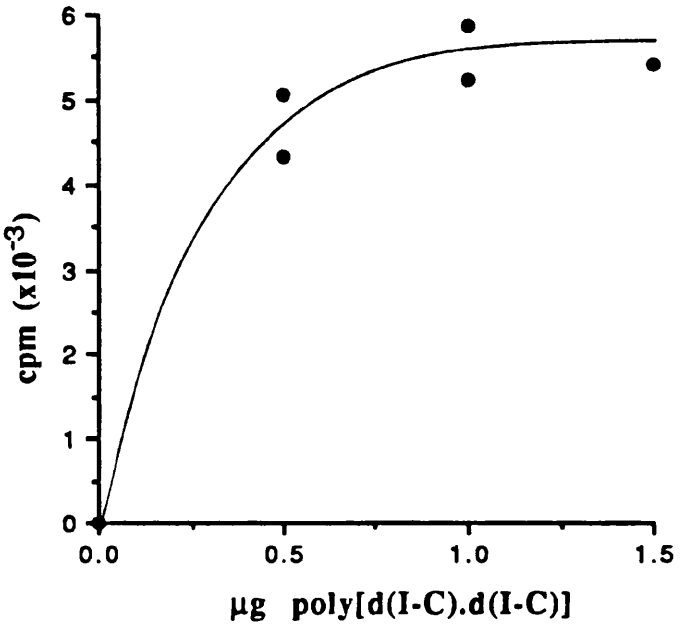


Figure 3.1 DNA Methylase Microassay:
Poly [d(I-C).d(I-C)] Concentration Curve

5 µg of a crude fraction of DNA methylase and 1.5 µM S-adenosyl-L-(methyl-^{3}H) methionine (87 Ci/mmol) were used to methylate 0, 0.5, 1.0, 1.5, µg of poly[d(I-C).d(I-C)] in a DNA methylase microassay (see section 2.2.5). The level of methylation is expressed as cpm of ^{3}H incorporated into DNA.

3.2.1.2 S-Adenosyl-L-(methyl-^{3}H)-methionine Concentration Curve

To optimise the concentration of S-adenosyl-L-(methyl-^{3}H)-methionine in the microassay, 5 µg of a crude fraction of DNA methylase was incubated with 0.5 µg poly [d(I-C).d(I-C)] and varying amounts of S-adenosyl-L-(methyl-^{3}H)-methionine in a total volume of 23 µl in buffer M^+ at 37°C for 1 hour. The incorporation of ^{3}H into DNA was then assayed as described in section 2.2.5.

Figure 3.2. shows that a concentration of 1.5 µM was saturating in the assay. This is a similar concentration to that used in the standard assay and this was then used in subsequent assays. To obtain the maximum amount of counts incorporated into DNA, a new high specific activity form of S-adenosyl-L-(methyl-^{3}H)-methionine (TRK 581, 87 Ci/mM), recently made available from Amersham International was used in the assaay. To obtain a concentration of 1.5 µM requires the use of 3 µCi/23 µl and it was partly to economise on this expensive reagent that the assay volume was limited to 23 µl.

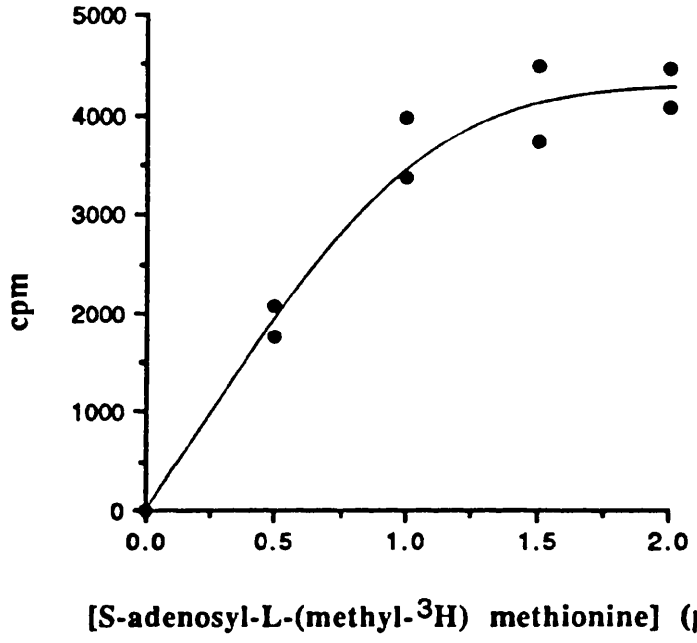


Figure 3.2 DNA Methylase Microassay: SAM Concentration Curve

5 μg of a crude fraction of DNA methylase and 0, 0.5, 1.0, or 1.5 μM S-adenosyl-L-(methyl-^{3}H) methionine (87 Ci/mmol) were used to methylate 0.5 μg poly[d(I-C).d(I-C)] in a DNA methylase microassay (see section 2.2.5). The level of methylation is expressed as cpm of ^{3}H incorporated into DNA.

3.2.2 The Effect of HSV-2 Infection on the Intracellular Distribution of DNA Methylase Activity

Subconfluent 50 mm dishes of RE cells were infected or mock-infected for 4 hours with HSV-2 as described in section 2.2.1.3. Following infection, the cells were fractionated into cell lysate, nuclei, 0.2 M nuclear extract and a fraction termed nuclear residue (as described in section 2.2.4.).

Figure 3.3. shows that the distribution of methylase activity is not significantly altered following infection with HSV-2.

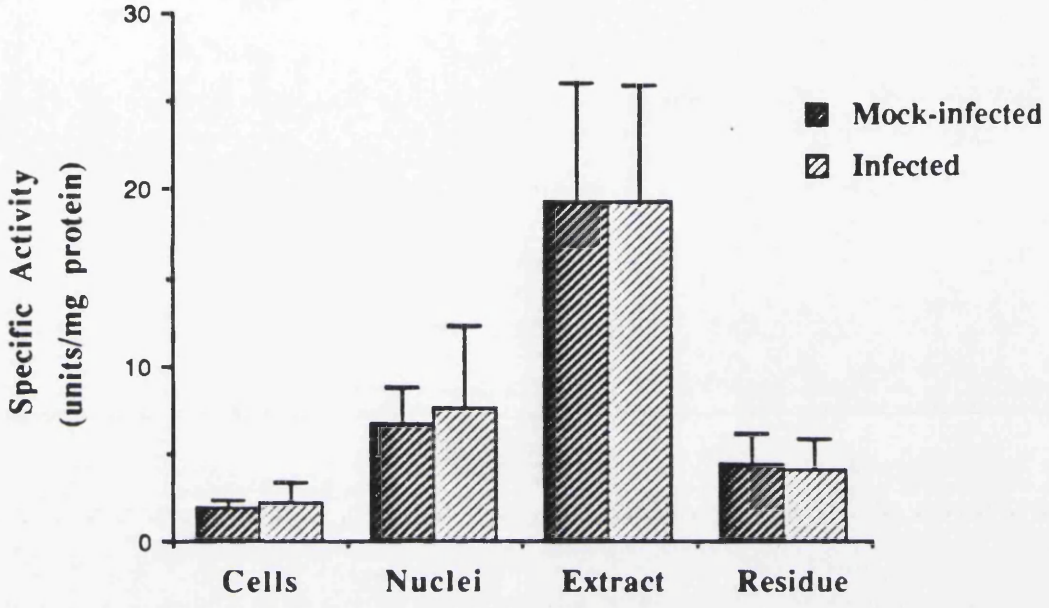


Figure 3.3 The Effect of HSV-2 Infection on the Intracellular Distribution of DNA Methylase

1-40 μg of protein from the appropriate cell fraction of RE cells, mock-infected or infected for 4 hours with HSV-2 were assayed for DNA methylase activity (see section 2.2.4, 2.2.5). This activity is expressed as specific activity (units/mg protein) and the results are mean values of 8-20 estimations from 2 separate preparations ± standard deviation.

(1 unit = 1 pmole methyl groups incorporated into DNA/hour)

3.3. The Effect of HSV-2 Infection on the Metabolism of S-Adenosylmethionine and S-Adenosylhomocysteine

In section 3.2. DNA methylase activity was assayed *in vitro* in the prescence of excess DNA and SAM and the results showed no difference in the enzymic activity following infection with HSV-2. *In vivo,* however, HSV-2 infection may alter the metabolism of SAM, causing it to be a limiting factor in the reaction and leading to an inhibition of DNA methylation. Alternatively, HSV-2 infection could alter the metabolism of one of the products of the DNA methylase reaction, S-adenosyl homocysteine (SAH), a potent inhibitor of methyltransferase catalysed reactions (Uleland, 1982). SAH can be degraded by the enzyme, SAH hydrolase (EC 3.3.13) to L-homocysteine and adenosine, see figure 3.4.

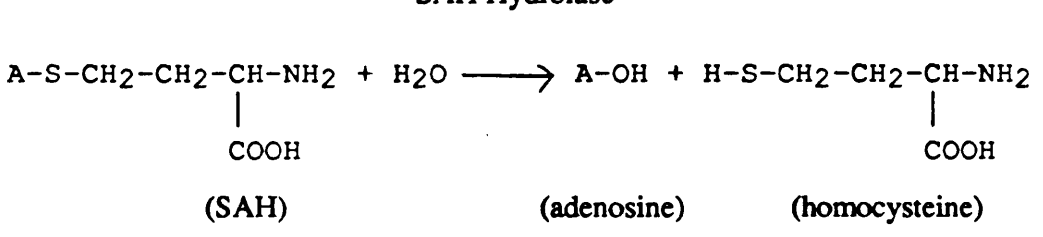


Figure 3.4 **SAH Hydrolysis** (A represents the adenosyl group)

Although the reaction equilibrium lies in the direction of condensation, adenine and homocysteine are continuously utilized in metabolic pathways within the cell and thus *in vivo* the enzymatic reaction is towards the hydrolysis of SAH. If the balance of adenine or homocysteine is disrupted in the cell, however, SAH levels can rise and lead to an inhibition of methylation of DNA and other compounds such as proteins, lipids or polysaccharides. For example, Kredich and Martin, (1977) showed that the treatment of cultured S49 mouse lymphoma cells with a specific

inhibitor of adenosine deaminase resulted in high levels of adenosine and consequently high levels of SAH which lead to an inhibition of DNA methylation. It is possible, therefore, that HSV-2 infection could lead to metabolic alterations in the cell which could result in the accumulation of SAH and cause an inhibition in DNA methylation.

To study these two hypotheses the rate of SAM breakdown and the ratio of SAM/SAH was measured in RE cells mock-infected and infected with HSV-2.

3.3.1. The Rate of S-Adenosylmethionine Breakdown

T3 bacteriophage encodes an enzyme SAM hydrolase (SAM'ase) which catalyses the breakdown of SAM to 5'-deoxy-5-(methyl thio)-adenosine (MTA) and homoserine see figure 3.5. This enzyme is the product of the first T3 gene transcribed and it allows the phage to overcome the restriction system of its host *E. coli* B or *E. coli* K 12 (Spoerel *et al.*, 1979). The expression of this protein also results in an inhibition of the methylation of newly synthesised *E. coli* DNA (Gefter *et al.*, 1966).

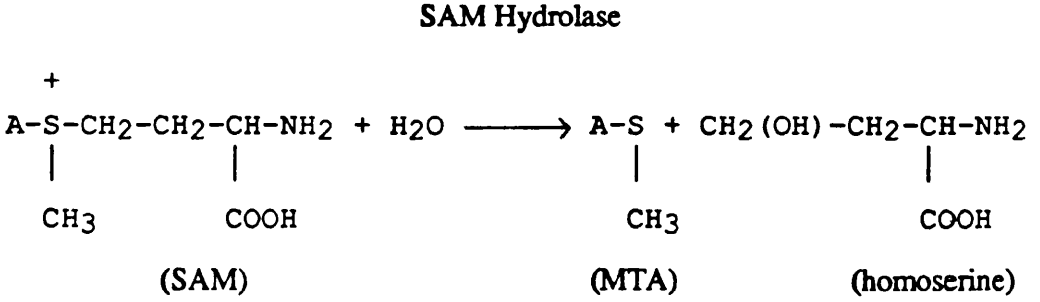


Figure 3.5 **SAM Hydrolysis (A represents the adenosyl group)**

The aim of the following experiment was to investigate if infection with HSV-2 induces a similar SAM'ase type activity in RE cells. A modification of the assay developed by Gefter *et al.*, (1966) was used to monitor SAM breakdown. In this assay the formation of 5´-deoxy-[5 methyl-^{3}H-thio]-adenosine from S-adenosyl-L-[methyl-^{3}H]-methionine was determined. The former does not bind to an Amberlite CG50 column (equilibrated with 0.02 M potassium phosphate pH 7.0) whereas SAM does bind under these conditions. Control samples containing 10 µCi S-adenosyl-L-[methyl-^{3}H] methionine (15 Ci/mmol) in 250 µl buffer M^+ were applied to a 1 ml column of Amberlite CG50 equilibrated with 0.02 M potassium phosphate buffer. The sample was then eluted in a total of 5 ml of 0.02 M potassium phosphate buffer and the radioactivity counted in a scintillation counter. 4-5% of the total counts applied were eluted from the column indicating very little degradation of the stock solution. Similar values, however, could be obtained by simply mixing the above with 1 ml of a 1 mg/ml Amberlite suspension (in 0.02 M potassium phosphate pH 7.0), sedimenting the Amberlite by centrifugation and counting the radioactivity present in the supernatant in a liquid scintillation conter. A concentration curve of Amberlite versus cpm unbound was carried out (results not shown) to ensure a saturating concentration of Amberlite.

Subconfluent 50 mm dishes of RE cells were mock-infected or infected for 4 hours with HSV-2 as described in section 2.2.1.3. Cell lysates were prepared as described in section 2.2.8 and 250 µl of this lysate was incubated at 37°C for 30 minutes with 10 µCi S-adenosyl-L-[methyl ^{3}H]-methionine (15 Ci/mmol). The formation of tritiated MTA was then assayed as in section 2.2.8.

The results in table 3.2.show that HSV-2 infection does not significantly alter the rate of breakdown of SAM.

	Specific Activity (pmoles SAM cleaved/30 minute/mg protein)
Mock-infected	83.0 ± 17.4 (5)
Infected	83.6 ± 22.4 (5)

Table 3.2 SAM breakdown in mock-infected and infected RE cells

The results are expressed as a mean value of 5 observations ± standard deviation.

3.3.2 Measurement of the Intracellular Ratio SAM/SAH

Intracellular pool sizes of SAM and SAH in RE cells were measured according to Arnaud *et al.*, (1985) (for details of this procedure see section 2.2.7.). Briefly, ^{35}S-L-methionine (800 mCi/mmol) was added to the extracellular medium of the cells at a final concentration of 20 µCi/ml for the appropriate time. The cells were then harvested and treated with 7.5% TCA and the acid-soluble material analysed by ion-exchange chromatography on a P-11 phosphocellulose column.

3.3.2.1 Separation of Standards

Initial experiments were carried out to determine the optimal [HCl] gradient required to effectively separate SAM, SAH and methionine.

A mixture of 500 µl of unlabelled cell extract (prepared as in section 2.2.7.2.), 100 µl SAH (10 mM in 5 mM HCl), 0.5 µCi S-adenosyl-L-(methyl ^{3}H)-methionine and 15 µCi ^{35}S-L-methionine (800 mCi/mmol) was applied to a 10 ml P-11 phosphocelluiose column equilibrated with 10 mM HCl.The column was

eluted with a stepwise HCl gradient at a rate of 70 ml/hour. 10 ml samples were collected, a fraction of each was used to measure the absorbance at 260 nm and a second aliquot of each was used to measure the radioactivity by liquid scintillation counting. Figure 3.6. shows the separation of the three standards.

Separation of a mixture of cell extract (unlabelled), SAM and methionine (as above) gave no detectable absorbance at 260 nm (results not shown) hence, the absorbance values in figure 3.6B correspond only to the cold SAH added. The gradient used is a slight modification of that used by Eloranta *et al.*, (1976) in the original separation procedure. They used a stepwise gradient of 1, 10, 50 and 500 mM HCl but in our hands SAH failed to elute in the 50 mM HCl fraction as expected. Instead, both SAH and SAM eluted in the 500 mM HCl fraction. The modified gradient (10,100 and 500 mM HCl), however, is clearly able to separate the three standards.

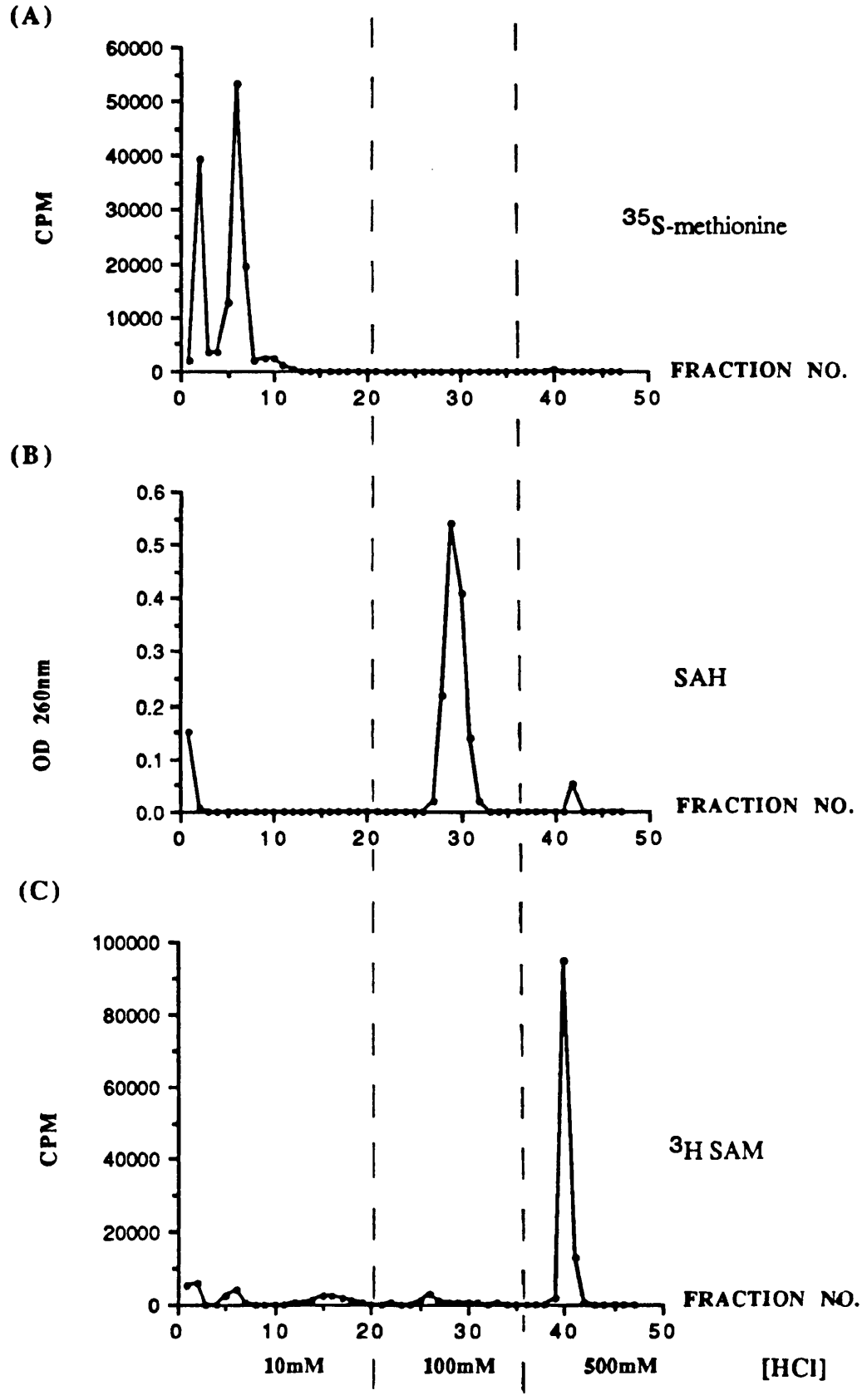
Figure 3.6 Separation of Standards on a P-11 Phosphocellulose Column

500 µl of unlabelled cell extract, 100 µl SAH (10 mM in 5 mM HCl), 0.5 µCi S-adenosyl-L-(methyl ^{3}H) methionine and 15 µCi of ^{35}S-L-methionine (800 mCi/mmol) were separated on a P-11 phosphocellulose column with a stepwise [HCL] gradient as described in section 3.2.2.1. The results are shown on three separate graphs.

(A) cpm derived from ^{35}S-methionine.

(B) Absorbance at 260 nm derived from SAH.

(C) cpm derived from S-adenosyl-L-(methyl ^{3}H) methionine.


(A)
CPM
60000
50000
40000
30000
20000
10000
0
0 10 20 30 40 50
FRACTION NO.
35S-methionine
(B)
OD 260nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 10 20 30 40 50
FRACTION NO.
SAH
(C)
CPM
100000
80000
60000
40000
20000
0
0 10 20 30 40 50
FRACTION NO.
3H SAM
10mM
100mM
500mM
[HCl]

3.3.2.2 Timecourse of Extracellular Methionine Equilibration with Intracellular SAM

Pool sizes of SAM and SAH were measured by first equilibrating cells with ^{35}S-L-methionine and then isolating and separating the SAM and SAH as described in section 2.2.7. In order to obtain reliable results on the pool sizes of SAM and SAH it was necessary to ensure equilibration of the extracellular ^{35}S-L-methionine with the internal SAM pool.

At time zero, 80 μCi aliquots of ^{35}S-L-methionine (800 mCi/mmol) were added to the medium of duplicate plates of RE cells to give a final concentration of 20 μCi/ml. This does not affect the methionine concentration in the medium which remains at 30 mg/l. Cells were harvested at 15 minutes, 30 minutes, 1 hour, 2 hours and 4 hours after the addition of the 35S-L-methionine. In addition, at 4 hours, the medium from one set of plates was replaced with unlabelled medium and the cells harvested after a further 2 hours incubation at 37°C. All of the samples were then separated on a P-11 phosphocellulose column as in section 2.2.7. to determine the amount of ^{35}S labelled SAM present in the cell (SAM elutes in the 500 mM HCl fraction).

Figure 3.7. shows the result of the time course. Equilibration of extracellular methionine with intracellular SAM has occurred by around 2 hours and hence this was the labelling time used in future experiments. This is in agreement with the results obtained German *et al.*, (1983). They reported that the time required for equilibration of extracellular methionine with intracellular SAM did not exceed 90 minutes in exponentially growing WI-L2 lymphoblasts. The graph also shows that nearly all of the labelled SAM is metabolised within 2 hours of the removal of ^{35}S-L-methionine from the extracellular medium demonstrating the rapid turnover of SAM within the cell.

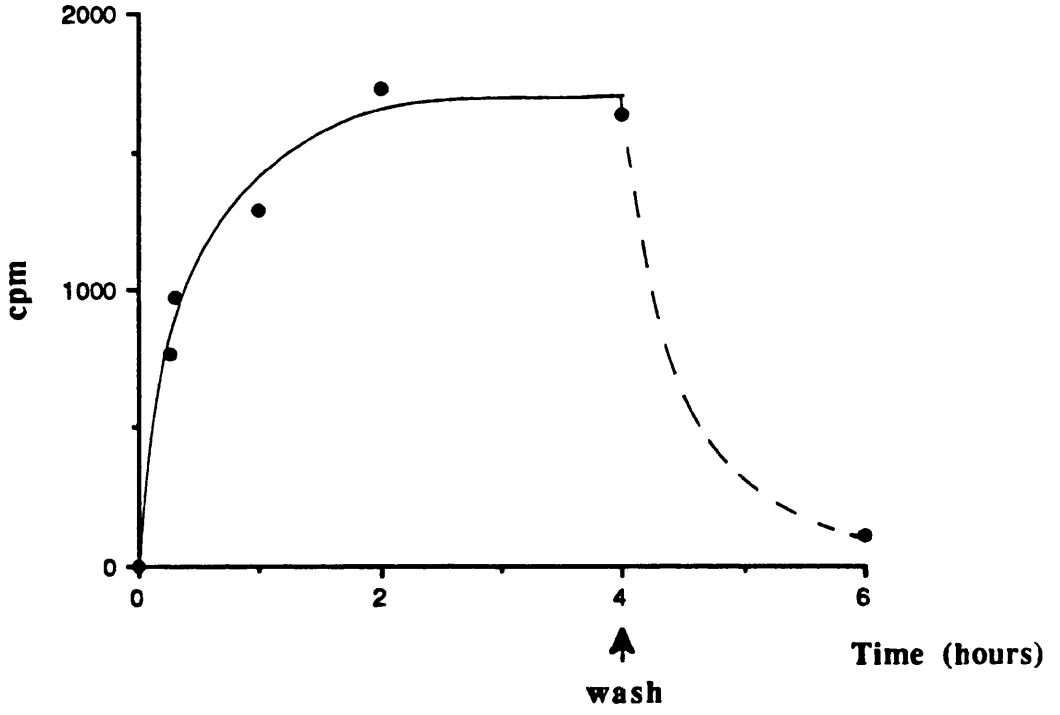


Figure 3.7 Timecourse of Equilibration of Extracellular Methionine with Intracellular SAM

Subconfluent RE cells were incubated for 0.5, 1, 2, 4 or 6 hours with normal medium supplemented with ^{35}S-L-methionine (800 mCi/mmol) at a final concentration of 20 μCi/ml. At 4 hours, the labelled medium was removed and replaced with normal medium. The samples were then fractionated on a P-11 phosphocellulose column (as in section 2.2.7.2.) to determine the amount of ^{35}S labelled SAM present in the cells and this is expressed as cpm.

3.3.2.3 Comparison of the Ratio of SAM/SAH in HSV-2 Infected and Mock-Infected RE cells

90 mm plates of subconfluent RE cells were infected at 20 PFU/cell as described in section 2.2.1.3. 2 hours post-infection, 80 μCi of ^{35}S-L-methionine (800 mCi/mmol) was added to the extracellular medium at a final concentration of 20 μCi/ml. 4 hours post-infection, acid-soluble material was prepared as in section 2.2.7.1. and analysed on a P-11 phosphocellulose column as described in 3.2.2.1. 100 μl of SAH (10 mM in 5 mM HCl) was added along with the extract as an internal standard.

Figure 3.8. shows a typical separation profile of a ^{35}S-L-methionine labelled cell extract. The peak of absorbance at 260nm reflects the elution profile of the added SAH.

Table 3.3. shows the amounts of SAM and SAH and the ratios of SAM/SAH in HSV-2 mock-infected and infected RE cells. These results demonstrate that infection of RE cells with HSV-2 does not alter the ratio SAM/SAH.

	SAM (pmoles/mg protein)	SAH (pmoles/mg protein)	SAM/SAH
Mock-infected	216.3	30.6	7.1
	185.8	20.8	8.9
Infected	213.5	23.7	9.0
	155.9	15.5	10.1

Table 3.3 SAM/SAH ratio in mock-infected and infected RE cells

The results shown are duplicate observations.

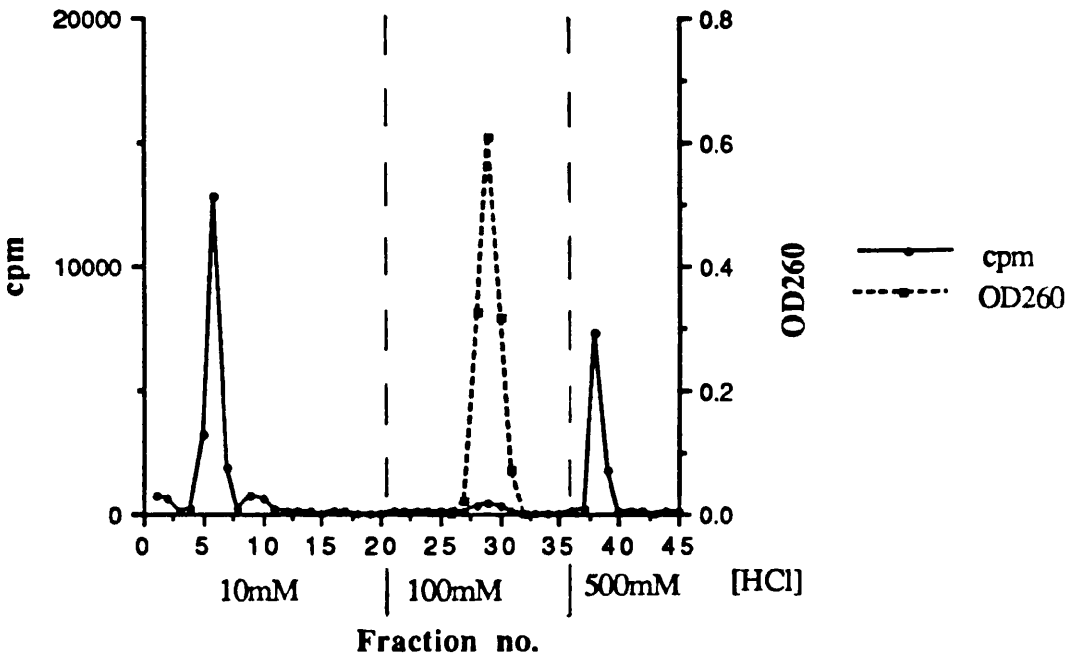


Figure 3.8 Separation Profile of SAM and SAH on a P-11 Phosphocellulose Column

RE cells were labelled for 2 hours with ^{35}S-L-methionine at a concentration of 20 µCi/ml. 500 µl of the acid soluble material was separated on a P-11 phosphocellulose column as with a stepwise [HCl] gradient as described in section 2.2.7.2.

3.4 Endogenous DNA as a Methyl Group Acceptor

In section 3.2. DNA methylase activity was assayed *in vitro* by its ability to add methyl groups to an excess of exogenous poly[d(I-C).d(I-C)]. This activity was found to be unaltered following infection with HSV-2 despite the hypomethylation which is seen to occur in the cell. *In vivo*, however, HSV-2 may cause an alteration in the suitability of endogenous DNA to act as a substrate for DNA methylase. HSV-2 is known to encode a number proteins with the ability to bind to DNA (Bayliss *et al.*, 1975; Purifoy and Powell, 1976) and these may inhibit the DNA methylation, for example, by directly blocking the access of DNA methylase to its substrate sites or by indirectly inhibiting the access of DNA methylase by altering DNA chromatin structure.

The aim of the following experiment was, therefore, to assay the ability of endogenous DNA in the nuclei of HSV-2 mock-infected or infected RE cells to accept methyl groups from S-adenosyl methionine.

Nuclei were prepared from RE cells mock-infected or infected with HSV-2 for 4 hours as described in sections 2.2.1.3 and 2.2.4. The appropriate volume of nuclei containing 2-20 μg of DNA was incubated with 3 μCi S-adenosyl-L-(methyl ^{3}H)-methionine (87 μCi/mmol) in a total volume of 23 μl in buffer M^{+}. The incorporation of methyl groups into DNA was then assayed as in section 2.2.5.

Figure 3.9 shows that DNA in infected cell nuclei has a greater ability to accept methyl groups than that in mock-infected nuclei. Both mock-infected and infected nuclear preparations had a similar protein:DNA ratio as well as the same specific activity of DNA methylase as measured using exogenous poly [d(I-C).d(I-C)] as a substrate (results not shown).

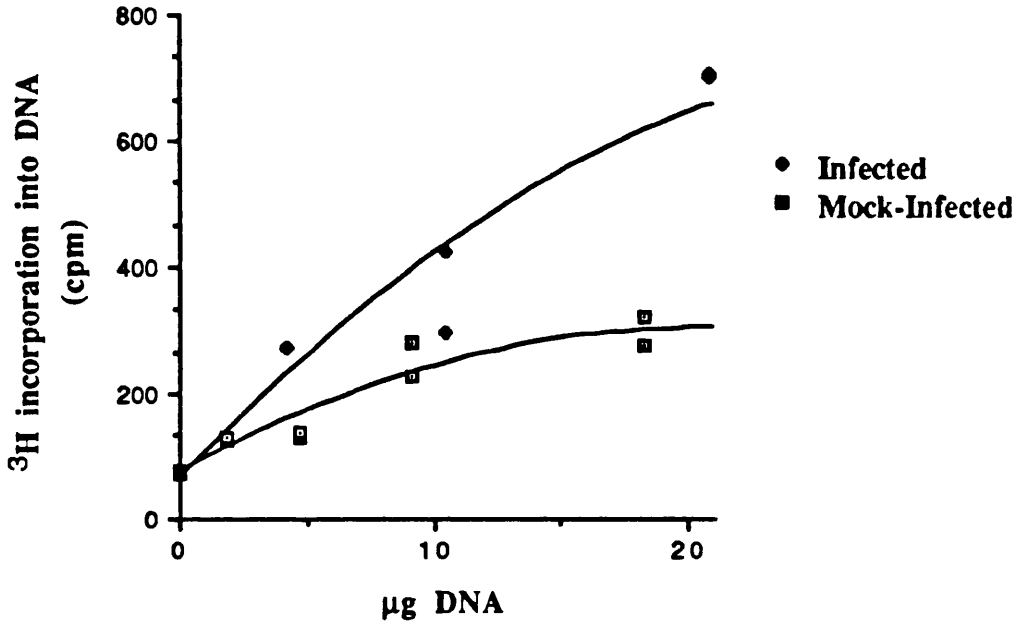


Figure 3.9 Endogenous DNA as a Methyl Group Acceptor

Nuclei were prepared from RE cells infected of mock-infected with HSV-2 for 4 hours (see sections 2.2.1.3 and 2.2.4). The appropriate volume of nuclei was then incubated with 3 µCi of S-adenosyl-L-(methyl ^{3}H)-methionine (87 µCi/mmol) for 1 hour and then assayed for the incorporation of ^{3}H methyl groups into DNA (section 2.2.5).

3.5 Characterisation of the Molecular Weight of DNA Methylase Following Infection with HSV-2

The molecular weight of eukaryotic DNA methylase, which has now been purified from a number of plants and animals, has been variously estimated between 50-200 kDa (Adams, 1990). This variation in size may be the result of proteolysis as the enzyme is sensitive to proteolytic degradation both *in vivo* and *in vitro* (Adams *et al.*, 1986, 1989).

Although DNA methylase in cultured rat cells has not previously been characterised, the mouse DNA methylase has been extensively investigated. It consists of a single subunit of 185-190 kDa (Turnbull and Adams, 1976) and this observation has been confirmed by the cloning and sequencing of the cDNA encoding DNA methylase from cultured murine erythroleukaemia cells (Bestor *et al.*, 1988). When the enzyme is subjected to trypsin it is degraded through a series of intermediates to yield a 100 kDa form. Degradation to this form of the enzyme results in an increase in *de novo* enzyme activity and this activity only decreases as the 100 kDa form is degraded (Adams *et al.*, 1986, 1989). In somatic cells *in vivo*, there is little evidence for *de novo* methylation suggesting DNA methylase requires to recognise a methyl cytosine on one strand of hemimethylated DNA before catalysing the transfer of a methyl group to the opposite strand. It has been postulated by Adams *et al.*, (1986) that proteolysis removes the domain which recognises hemimethylated sites and hence removes a constraint and allows *de novo* methylation to occur.

In the following experiments, polyclonal antisera raised against purified DNA methylase and against peptides synthesised from the predicted amino acid sequence of murine DNA methylase were used to probe western blots of cell extracts from HSV-2 mock-infected and infected RE cells. Hence, the effect of HSV-2 infection on the size of DNA methylase was investigated.

3.5.1 Polyclonal Antiserum

Antiserum 173 is a polyclonal antiserum raised in a rabbit against DNA methylase purified from mouse Krebs II ascites cells. This antiserum has a titre of greater than 1 in 12,000 as measured in an ELISA assay and can inhibit transmethylation when added to a methylase assay (Adams *et al.*, 1986).

Bestor *et al.*, (1988) have cloned and sequenced the cDNA encoding mouse DNA methylase . The inferred protein sequence has 1573 amino acids and appears to be organised into two structural domains. The 1000 amino acid N-terminal domain contains a putative zinc DNA-binding motif and the C-terminal domain of 570 amino acids shares strong sequence specificities with bacterial type II DNA methyltransferases. The C-terminal domain is, therefore, thought to contain the catalytic site and the N-terminal domain may be important in the regulation of activity.

Antisera 34, 35 and 36 are polyclonal antisera raised against synthetic peptides corresponding to the predicted amino acid sequence. Figure 3.10 shows the position of these peptides in the predicted amino acid sequence of DNA methylase. Antiserum 34 is raised against a peptide at the N-terminus, the region thought to be most sensitive to proteolytic degradation. Antiserum 35 is raised against a synthetic peptide corresponding to a region in the N-terminal domain adjacent to the Cys-rich region which has the characteristics of a metal-binding region, the region thought to be important in regulating the enzyme activity. Antiserum 36 is raised against a synthetic peptide corresponding to a region in the C-terminal domain between the Pro-Cys motif, thought to be located at the catalytic centre, and a 31 amino acid sequence which is highly homologous to bacterial type II methylases. In all of the above the synthetic peptides were prepared by Biomac in the department of Biochemistry. These were coupled to KLH and were injected subcutaneously into rabbits.

All 4 antisera were prepared by Dr R.L.P.Adams.

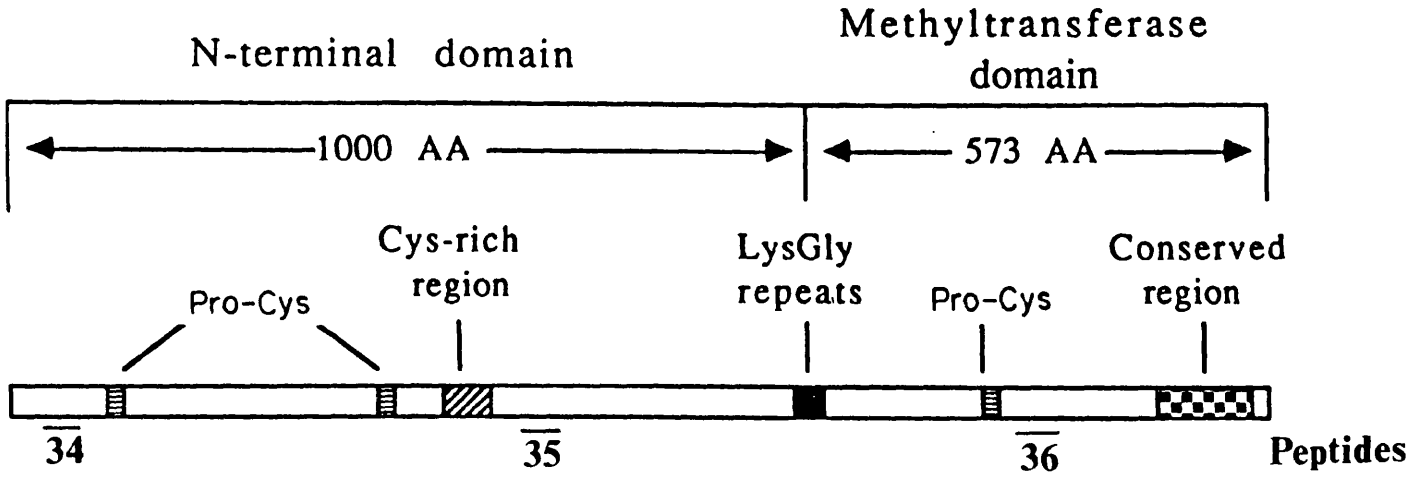


Figure 3.10 Position of Peptides on Predicted Amino Acid Sequence of DNA Methylase.

3.5.2 Immunoblots of HSV-2 Infected and Mock-Infected RE Cells

Subconfluent RE cells were mock-infected or infected with HSV-2 for 4 hours (as in section 2.2.1.3.). 50 μg of 0.2 M NaCl nuclear extract and nuclear residue fractions, prepared as in section 2.2.4., were electrophoresed on an 8.75% SDS-polyacrylamide gel (as described in section 2.2.9.1.). This was transferred onto a nylon membrane (Hybond C+) by western blotting as in section 2.2.9.2. The blots were then probed with the appropriate antibody and were illuminated using protein A labelled with ^{125}I as described in section 2.2.9.3.

Mouse DNA methylase is a single subunit enzyme of 185-190 kDa but because of its sensitivity to proteolysis it is often observed by SDS-polyacrylamide gel electrophoresis or immunoblotting as a group of 3 or 4 high molecular weight bands of 150-190 kDa. Bestor and Ingram, (1985) reported the purification of three closely related forms of DNA methylase from murine erythroleukaemia cells of 190,175 and 150 kDa and these are speculated to be generated by proteolysis. When Adams *et al.*, (1989) carried out a time course of trypsinisation of purified DNA methylase they observed 16 different sized bands during the course of the experiment.

Figure 3.11.shows, with all 4 antisera, the highest molecular weight DNA methylase present in the nuclear residue fraction. This is in accordance with a number of findings that suggests that it is the nuclear matrix bound form of the enzyme that is responsible *in vivo* for the maintenance methylation reaction.

Antiserum 173 (figure 3.11a), detects a high molecular weight band in the nuclear residue fraction of 190 kDa and bands of approximately 185 kDa and 160 kDa in the nuclear extract. There are also a number of faint lower molecular weight bands but there are no major differences between the samples from mock-infecteced and infected cells.

Antiserum 34 and 35 (figure 3.11 b&c), detect some high molecular weight material but strongly react with a group of proteins of 50-70 kDa. These antisera are raised against peptides in the N-terminal domain, the region thought to be sensitive to proteolysis, and hence they may correspond to highly antigenic cleavage products of DNA methylase. Again, there are no major differences in the pattern of bands obtained in mock-infected or infected cells.

Antiserum 36 (figure 3.11d), detects the characteristic high molecular weight bands in the nuclear residue fraction along with a number of lower molecular weight species. There is one major difference, however, in the pattern of bands in the mock-infected and infected cells. A 40 kDa band appears to be present in both the nuclear extract and residue fractions but only from HSV-2 infected cells. The 40 kDa band present in the 0.2 M nuclear extract was not seen, however, in subsequent experiments when an additional wash with buffer M^+ was added before the 0.02 M NaCl extraction although it was still present in the nuclear residue fraction. This indicates that the 40 kDa band seen in the 0.2 M NaCl nuclear extract is probably a protein that can be readily leached from the nuclei. The appearance of the 40 kDa band in the nuclear residue fraction does not appear to correspond to any major degradation of the other bands. It may be that only very limited proteolysis is occurring but which produces a highly antigenic protein. Alternatively, it may be a viral protein which cross reacts with the antipeptide antiserum 36 and this possibilty is discussed further in the following section.

Figure 3.11 The Size of DNA Methylase in HSV-2 Infected and Mock-Infected RE Cells

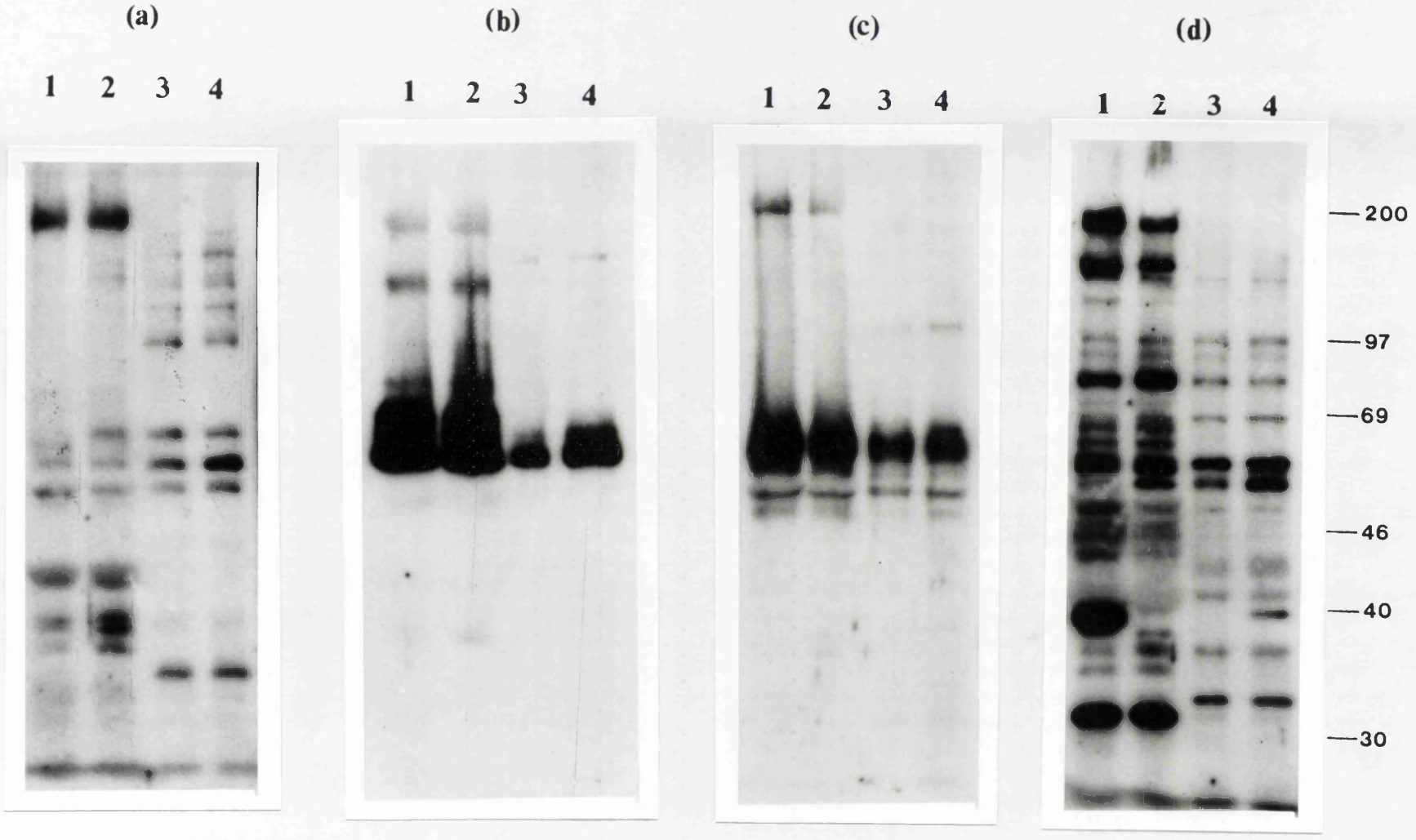
RE cells were infected or mock-infected with HSV-2 for 4 hours. 50 μg of 0.2 M nuclear extract or 50 μg of nuclear matrix were electrophoresed on an 8.75% SDS-polyacrylamide gel and western blotted. The blots were then reacted with either (a) a 1:500 dilution of antibody 173 , (b) a 1:25 dilution of antibody 34, (c) a 1:100 dilution of antibody 35 or (d) a 1:25 dilution of antibody 36. All 4 blots were then reacted with ^{125}I-labelled protein A and visualised by autoradiography (see section 2.2.9).

Lane 1 : 50 μg nuclear matrix (infected).

Lane 2 : 50μg nuclear matrix (mock-infected).

Lane 3 : 50 μg 0.2 M NaCl nuclear extract (mock-infected).

Lane 4 : 50μg 0.2 M NaCl nuclear extract (infected).


(a)
1 2 3 4
(b)
1 2 3 4
(c)
1 2 3 4
(d)
1 2 3 4
200
97
69
46
40
30

3.5.3 Timecourse of 40 kDa Protein Accumulation

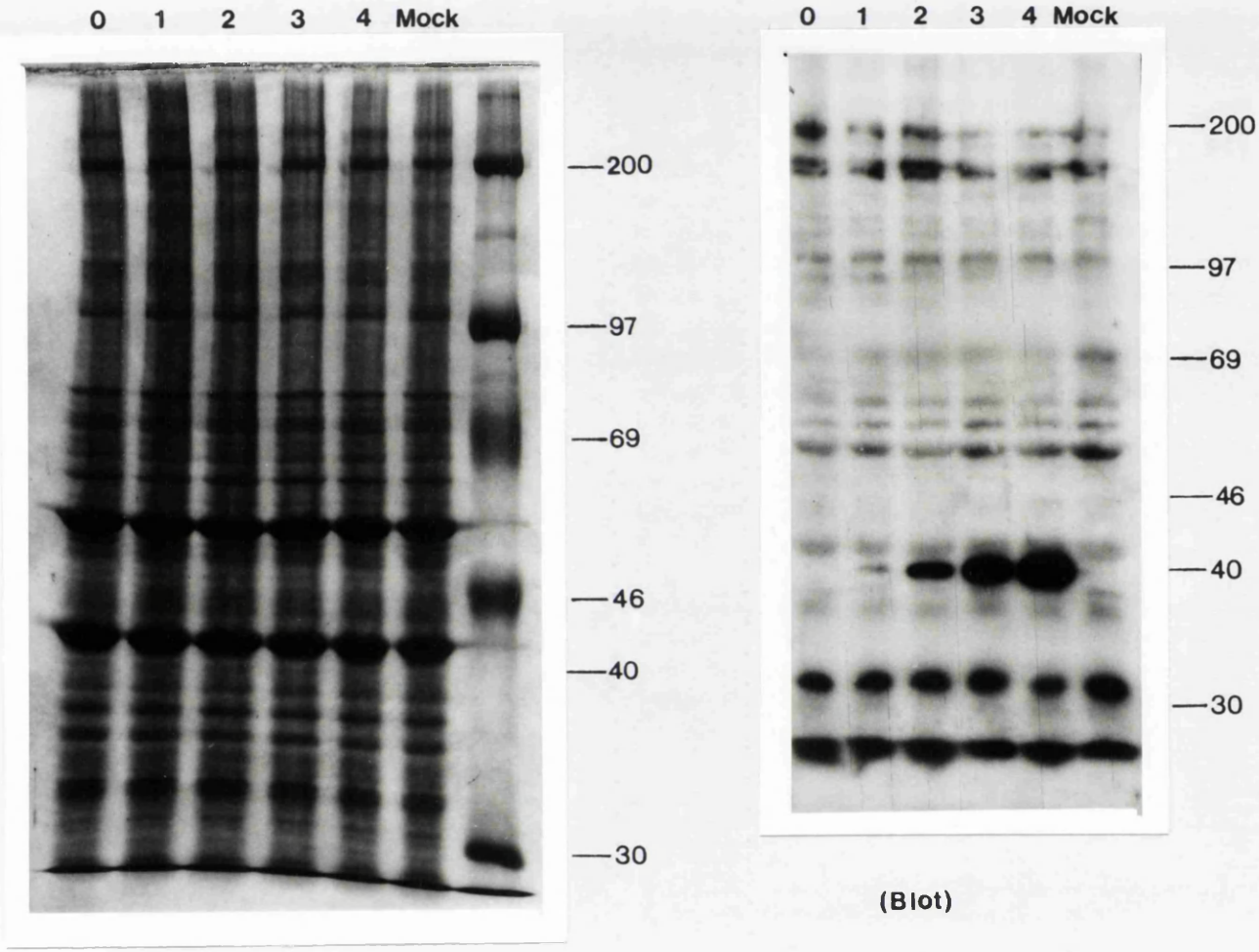
Subconfluent RE cells were infected for 1, 2, 3 or 4 hours or mock-infected for 4 hours with HSV-2 as described in section 2.2.1.3. 50 µg samples of nuclear residue prepared as in section 2.2.4.were electrophoresed on an 8.75% SDS-polyacrylamide gel and western blotted onto hybond C^+ membrane (see sections 2.2.9.1.and 2.2.9.3.). The membrane was then probed with a 1:25 dilution of Ab 36 and illuminated with ^{125}I labelled protein A as in section 2.2.9.4.

Figure 3.12 shows that the 40 kDa protein is visible as early as 1 hour post-infection and it accumulates with time up to the last time point of 4 hours post-infection. Again, this does not appear to coincide with the dissappearance of any of the other bands. If the band is of viral origin, the pattern is most characteristic of an immediate early protein, the first set of viral proteins which are maximally expressed at 3-5 h post-infection. This subset of viral proteins, however, are well characterised (see section 1.2.2) and none are of the appropriate size. The possible origin of the 40 kDa polypeptide is discussed further in section 3.8.

Figure 3.12 Timecourse of 40 kDa Protein Accumulation

RE cells were infected with HSV-2 for 1, 2, 3, or 4 hours or mock-infected for 4 hours. 50 μg samles of nuclear matrix were electrophoresed on an 8.75% SDS-polyacrylamide gel and western blotted. The blot was reated with a 1:25 dilution of antibody 36 followed by ^{125}I-protein A and visualised by autoradiography.

Time (hours)


0 1 2 3 4 Mock
200
97
69
46
40
30
(Gel)
0 1 2 3 4 Mock
200
97
69
46
40
30
(Blot)

3.6 Alterations in the Intracellular Ionic Environment as a Possible Mechanism for the Inhibition of DNA Methylation

HSV induced DNA polymerase has a number of biochemical properties different from its cellular counterpart. The HSV induced enzyme has a requirement for high salt concentrations (150 mM) for maximal activity and these high salt concentrations correspondingly inhibit the cellular enzyme by 90-100% (Keir *et al.*, 1966; Weissbach *et al.*, 1973). In addition, the optimal Mg^{2+} concentrations are not the same. The viral enzyme shows maximal activity at 3 mM whereas the cellular enzyme requires a concentration of 8 mM for maximal activity. Mao *et al.*, (1975) further demonstrated that 0.2 mM $MnCl_2$ completely inhibited the HSV DNA polymerase but only inhibited the cellular enzyme by 35%.

The HSV induced ribonucleotide reductase also shows different biochemical properties to its cellular counterpart. Langelier and Buttin, (1981) demonstrated that at a concentration of 50 mM NH_4SO_4 only 5% of the cellular activity remained whereas the HSV induced enzyme still retained 60% of its activity at this concentration.

These findings suggest that the intracellular ionic conditions may be altered on infection of cells with HSV-2. In the following two sections attempts were made to mimic these possible alterations in the intracellular environment to investigate if this was a possible mechanism whereby DNA methylase could be reversibly inhibited *in vivo*.

3.6.1 The Effect of Increasing External [NaCl] on DNA Methylation

When native DNA is used as a substrate to measure DNA methylase activity in vitro the reaction is strongly inhibited by salt. Adams *et al.*, (1979a), reported that 50 mM NaCl brings about a 70% inhibition in activity. The probable reason for this being that the enzyme requires single-stranded regions with which to interact and these are available when the enzyme breathes, a situation favoured by low salt and high temperature. If denatured DNA is used in the assay, however, salt is stimulatory up to 100 mM NaCl.

To manipulate the internal NaCl concentration, the appropriate amount of NaCl was added to the external medium of subconfluent RE cells growing in 50 mm dishes to give a final concentration of 0-100 mM NaCl above the 120 mM already present in the cell medium. 100 µCi [6-^{3}H]-uridine (23 Ci/mmol) was added along with the NaCl to a final concentration of 50 µCi/ml and the cells were labelled for 6 hours at 37°C. Following the incubation, DNA was prepared, pyrolysed and the bases separated on an Aminex A6 column as described in section 2.2.6. As the DNA is labelled using [6-^{3}H]-uridine, the bases thymine, cytosine and methylcytosine are all labelled with tritium (see figure 2.1). The percent m^5C is then calculated as below.

$$\%m^5C = [(m^5C \times 100)/(C + m^5C)]$$

The cpm in thymine, cytosine and methylcytosine were summed to give a measure of the extent of DNA synthesis.

Figure 3.13 shows the effect of increasing the external concentration of NaCl on DNA synthesis and $\%m^5C$. When the external NaCl concentration is raised to 220 mM, DNA synthesis is inhibited by 75% (figure 3.13a). This is in accordance

with Saborio *et al.*, (1974) who reported that these conditions lead to a dissociation of polysomes with concomitant inhibition of protein synthesis and cell growth.

The %m^5C however, does not show the same inhibition (figure 3.13b) hence the limited DNA made under these conditions of high salt is fully methylated. DNA methylase *in vivo* therefore does not appear to be as sensitive to alterations in the NaCl concentration as it is *in vitro*. As was discussed above, high salt *in vitro* probably probably inhibits the breathing of DNA but *in vivo*, DNA helicases and single-stranded DNA-binding proteins generate and stabilise the single-stranded regions thought necessary for DNA methylase to interact with DNA.

The above results suggest, therefore, that any possible increase in salt concentration following HSV-2 infection is not responsible for the inhibition of methylation of newly synthesised cellular DNA in infected cells.

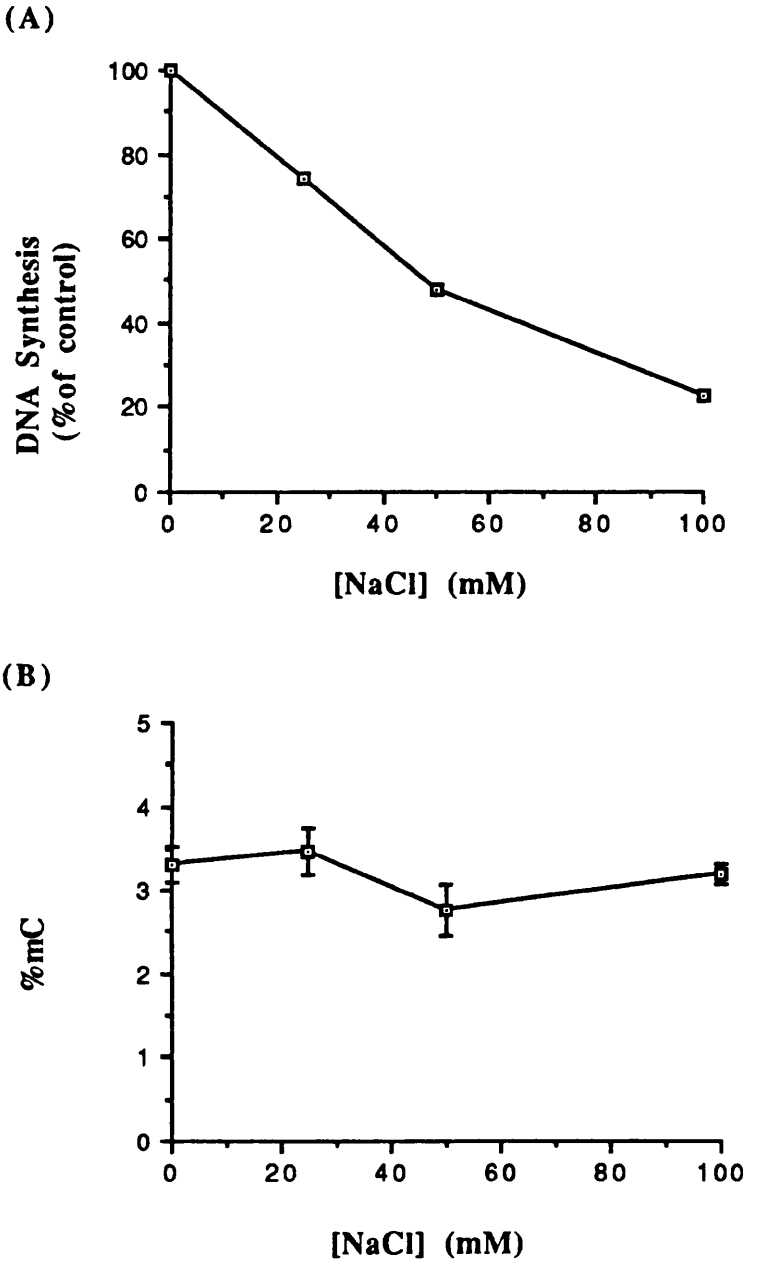


Figure 3.13 **The Effect of NaCl Concentration on DNA Methylation and DNA Synthesis**

RE cells were incubated in the presence of 0, 25, 50, and 100 mM excess NaCl and 100 μCi [6-^{3}H] uridine for 6 hours. %m^{5}C was determined according to section 2.2.6. DNA synthesis is expressed as cpm in thymine plus cytosine plus methylcytosine (see section 3.6.1).

3.6.2 The Effect of Various Ionophores and Ouabain on DNA Methylation

Ionophores are small, hydrophobic molecules that dissolve in lipid bilayers and increase ion permeability of the bilayer either by acting as mobile carriers or as channel formers. Certain ionophores show high discrimination between different ions and thus are useful tools to specifically alter the ionic milieu of a cell. In this experiment, cells were treated with a number of ionophores and the effect on DNA methylation levels were investigated. The ionophores used were as follows:

(i) Valinomycin	This transports K^+ down its electrochemical gradient and hence depletes intracellular K^+ levels.
(ii) A23187	This catalyses the electroneutral exchange of divalent cations such as Ca^{2+} and Mg^{2+} with two H^+ and is widely used to increase the concentration of free Ca^{2+} in the cytosol.
(iii) Monensin	This catalyses the electroneutral exchange of Na^+ and H^+. This results in increased intracellular levels of Na^+.

In addition, ouabain, a potent inhibitor of the Na^+/K^+ ATP'ase was used. This is not an ionophore but inhibition of the Na^+/K^+ ATPase prevents the uptake of K^+ and the active extrusion of Na^+ and thus upsets the cells ionic balance.

Subconfluent RE cells were incubated with the appropriate concentration of ionophore in a total volume of 2 ml for 2 hours at 37°C. 100 μCi [6-^{3}H] uridine (23 Ci/mmol) was then added and the cells were incubated for a further 6 hours. Following the incubation, DNA was prepared, pyrolysed and the bases separated on an Aminex A6 column (section 2.2.6).

Figure 3.14 shows that none of the treatments significantly altered the level of $\%m^5C$ hence DNA methylase activity *in vivo* does not appear to be affected by alterations in the concentration of Na^+, K^+ or Ca^{2+} in the cell.

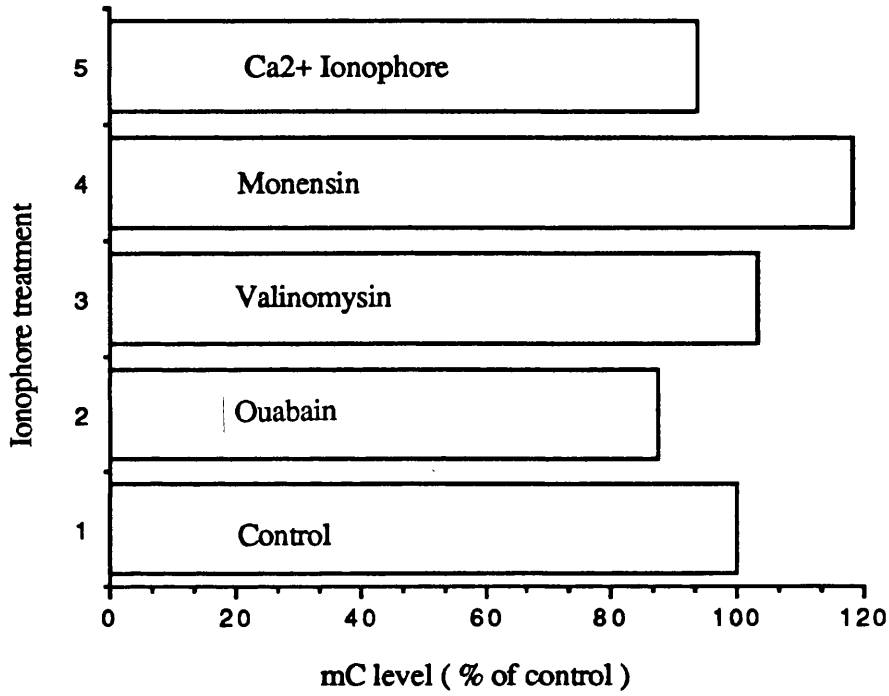


Figure 3.14 The Effect of Ionophores and Oubain on DNA Methylation Levels

RE cells were incubated in the presence of 1 mM ouabain, 0.25 mM valinomycin, 100 μM monensin and 7 μM A23187 (Ca^{2+} ionophore) for 2 hours. The cells were then labelled with 100 μCi [6-^{3}H] uridine for a further 6 hours. $\%m^5C$ was determined as in section 2.2.6 and is expressed as % of the control value i.e. RE cells not treated with ionophore.

3.7 Immunofluorescence Microscopy

DNA replication occurs at numerous discrete sites in eukaryotic cell nuclei, each site containing a cluster of replication forks (Nakamura *et al.*, 1986; Mills *et al.*, 1989; Nakayasu and Berezney, 1989), closely associated with the nucleoskeleton (Jackson and Cook, 1986; Van der Velden and Wanka, 1987). There is evidence to suggest that the enzymes involved in DNA replication are clustered into functional complexes, termed replicases, also associated with the nuclear matrix (reviewed by Huberman, 1987). Further evidence for the localisation of replication proteins at these sites has come from the use of antibodies against some of these replication proteins such as DNA polymerase α and proliferating cell nuclear antigen (PCNA) (an accessory factor to DNA polymerase ∂). Immunofluorescence microscopy studies have shown that these proteins colocalise with the sites of DNA replication which are visualised using antbodies against bromo-deoxyuridine (BrdU) incorporated into newly synthesised DNA (Nakamura *et al.*, 1984; Celis and Celis, 1985; Bravo and Macdonald-Bravo, 1987; Hutchison and Kill, 1989). Eukaryotic DNA replication, therefore, appears to be a highly structured and complex process.

The DNA methylase responsible for maintenance methylation *in vivo*, is thought to be that which remains firmly associated with the 2 M NaCl resistant, nuclear-matrix structures (Burdon *et al.*, 1985). There is still debate, however, as to its localisation within this salt-resistant nuclear fraction and its association with the replication apparatus. Noguchi *et al.*, (1983) reported that DNA methylase co-purified with a high molecular weight complex of proteins released from nuclei by sonication. This complex is composed of proteins involved in DNA replication, contains both template and newly synthesised (nascent) DNA and is capable of catalysing the incorporation of ^{14}C-rCDP into DNA suggesting it is a functional complex *in vivo*. Ciafa *et al.*, (1988), however, have demonstrated that the so-called "matrix-bound" form of DNA methylase is not attached to the matrix itself, as previosly suggested by several groups (Burdon *et al.*, 1985; Drahovsky *et al.*,

1985; Tubo and Berezney, 1987) but is localised in the DNA loops of such nuclear preparations. Digestion of the nuclear matrix fraction with DNAse 1 to leave only the "DNA attachment" sites showed no DNA methylase activity remained in this fraction. Such DNA attachment sites have been suggested to be the sites of replication complexes (Gross and Garrard, 1987) and therefore the above findings suggest that DNA methylase may not be as intimately asssociated with these complexes as was previously suggested.

The replication of HSV also occurs in ordered structures termed replication compartments containing all the elements necessary for viral DNA replication (reviewed by Knipe, 1990). De Bruyn-Kops and Knipe, (1988) demonstrated using anti-BrdU immunofluoresence, that cellular replication complexes relocate to the sites of viral DNA replication. Wilcock and Lane, (1991) further demonstrated using antibodies against several cellular proteins that various proteins involved in cellular DNA replication e.g DNA polymerase α and single stranded DNA binding protein, also relocate to these viral replication sites whereas the non-replication proteins tested did not.

Although the exact location of DNA methylase in replicating cells is not known it is essential that it has access to the newly synthesised DNA. It was discussed above that HSV infection alters the localisation of both replicating cellular DNA and certain cellular proteins. The inhibition of cellular methylation following HSV-2 infection may, therefore, be due to a disruption in the association of cellular replication and DNA methylase which would distance the enzyme from its substrate.

To investigate this hypothesis, immunofluorescence microscopy was used to compare the localisation of DNA methylase and the sites of replication in cells mock-infected or infected with HSV-2.

3.7.1 The Effect of HSV-2 Infection on the Intracellular Distribution of DNA Methylase

RE cells were grown directly onto glass coverslips at a density of $1x10^6$ cells per 50 mm dish. Subconfluent cells were then infected or mock-infected for 4 hours as in section 2.2.1.3. Following infection the cells were fixed with 3:1 methanol:acetone and stored at -70°C until required.

To locate the DNA methylase, the cells were incubated with 100 µl of a 1:10 dilution of either Antibody 34, 35 or 36. These are all polyclonal antisera raised against synthetic peptides corresponding to the predicted amino-acid sequence of murine DNA methylase (see section 3.5.1). In addition, as a control, cells were incubated with 100 µl of a 1:10 dilution of PR-6, a monoclonal antibody raised against GRP-94 (a glucose regulated protein), kindly donated by Dr J.C.M. Macnab, followed by an incubation with a 1:20 dilution of rabbit anti-rat IgG. The cells were all then incubated with 100 µl of a 1:80 dilution of FITC-conjugated goat anti-rabbit IgG. For details of the immunofluorescence staining and microscopy procedures see section 2.2.10.

Figure 3.15 shows that the staining pattern with all 3 anti-methylase antibodies differs in mock-infected and infected cells. Antibodies 34 and 35 show a faint nuclear staining in mock-infected cells (figures 3.15a, c) which appears to be evenly distributed. With antibody 36 in figure 3.15e however, it is difficult to distinguish the staining from that of the background staining. Various methods were tried to reduce this background which included;

(i) Increasing the concentration of the blocking agent, foetal calf serum, from 2% to 5%.

(ii) Including 0.2% Tween 20 in all buffers.

(iii) Titrating both the first and second antibodies.

(iv) Increasing the length of washes.

These approaches, however, did not abolish the background staining (results not shown) but as time was limited no further parameters were investigated. It is clear, however, that the pattern of staining is altered on infection. With all 3 anti-methylase antibodies the infected cells show distinctive perinuclear staining (figures 3.15b, d, f).

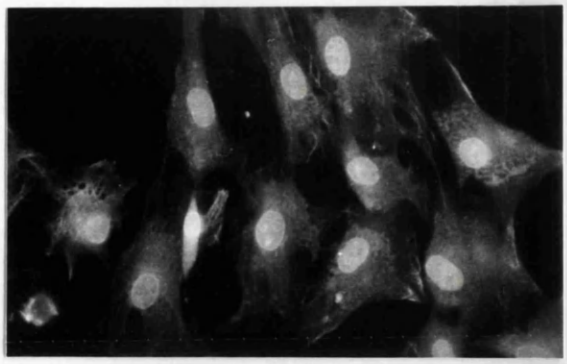
The control cells (figures 3.15g, h) show the same granular cytoplasmic staining in both infected and mock-infected cells.

The increase in intensity of immunofluorescence staining seen with the anti-methylase antibodies following infection may be the result of a more concentrated localisation of DNA methylase or it may be a result of increased immunogenicity as the results in section 3.2.2 indicate that there is no increase in nuclear DNA methylase levels following infection with HSV-2.

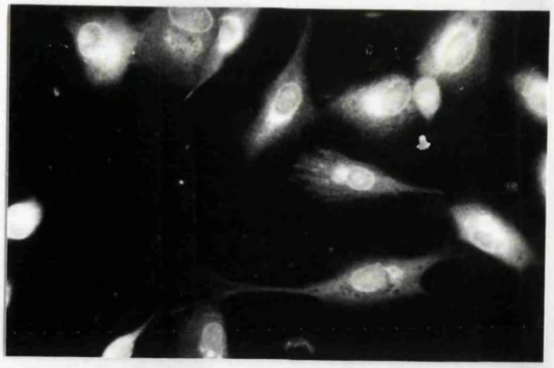
Figure 3.15 The Intracellular Distribution of DNA Methylase

RE cells were mock-infected (a, c, e, g) or infected (b, d, f, h) with HSV-2 for 4 hours and were fixed with 3:1 methanol:acetone (see section 2.2.10). The cells were then incubated with antibody 34 (a, b), antibody 35 (c, d), antibody 36 (e, f) or PR-6 (g, h) followed by an incubation with the appropriate FITC-conjugate antibody (see section 3.7.1). Immunofluoresence micrographs were taken using a Leitz Wetzler fluorescence microscope equiped with a 40X NPL fluotar objective lens.

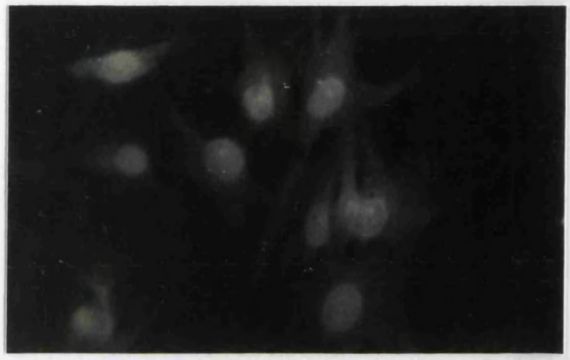
(a)



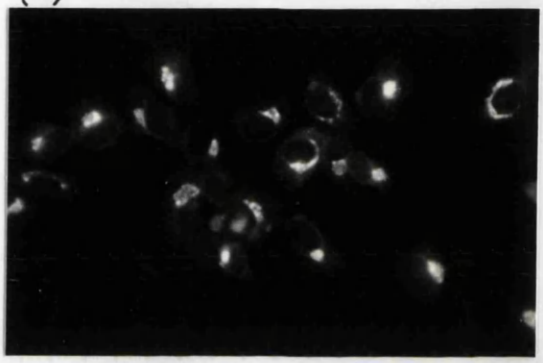
(b)



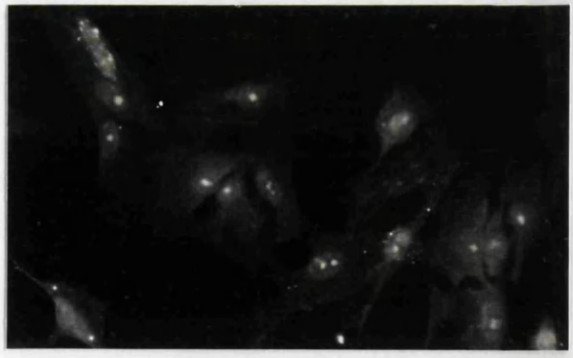
(c)



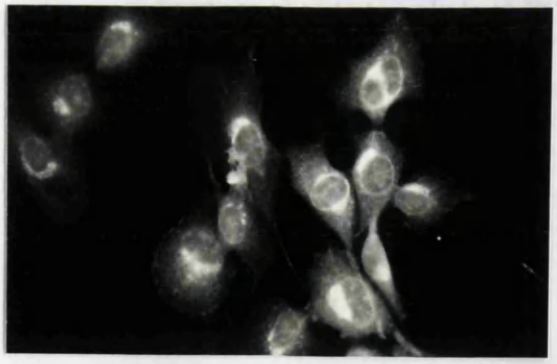
(d)



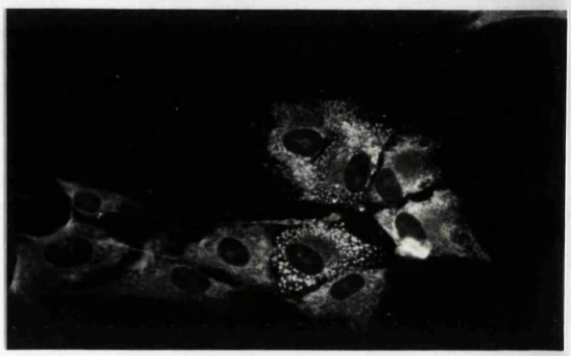
(e)



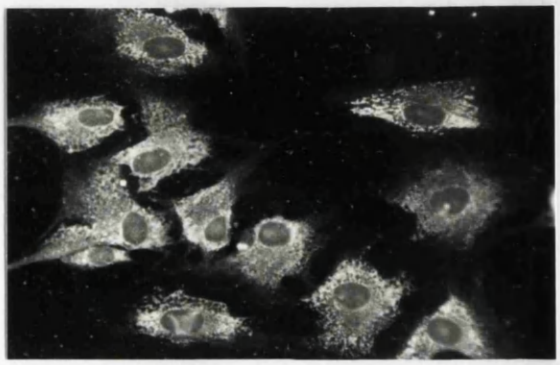
(f)



(g)



(h)



3.7.2 Localisation of the Sites of DNA Replication Following Infection with HSV-2

De Bruyn-Kops and Knipe, (1988) demonstrated that replication of both cellular and viral DNA in HSV infected cells takes place in the same replication compartments throughout the nucleus. In section 3.7.1 it was shown that DNA methylase concentrates around the nuclear periphery following infection with HSV-2. This suggests that in infected cells DNA methylase may not be present in these replication compartments and hence not have acess to its substrate DNA. It was therefore decided to localise the sites of DNA replication in HSV-2 infected cells and compare these to the localisation of DNA methylase.

Two different antibodies were used to localise these sites using immunofluoresence microscopy; an antibody against the major DNA binding protein of HSV-2 and an antibody against BrdU incorporated into newly synthesised DNA. Infected cell protein 8 (ICP 8), the major DNA binding protein, of HSV-1 is one of the seven viral proteins essential for viral DNA synthesis (see section 1.2.3). Prior to the induction of viral synthesis ICP8 is found at numerous discrete sites in the nucleus, associated with the nuclear framework, termed prereplicative sites. Following the onset of viral DNA synthesis ICP8 accumulates in large globular compartments associated with viral DNA. De Bruyn-Kops and Knipe, (1988) demonstrated that the cellular DNA replication sites relocate following infection and colocalise with ICP8 in these prereplicative and replicative compartments. Wilcock and Lane, (1991) also demonstrated, using immunofluorescence microscopy, that various cellular proteins involved in cellular DNA replication also relocate to viral replication sites. In the absence of ICP8, i.e using mutant strains of virus, relocation of cellular replication does not occur hence ICP8 is thought to be responsible for the organisation of the viral DNA replication machinery and for the relocalisation of both replicating cellular DNA and cellular replication proteins. ICP11/12 is the HSV-2 homolog to ICP8 in HSV-1 (Yeo *et*

al., 1981). Mutations in the gene encoding ICP11/12 also prevent HSV viral DNA replication suggesting a similar, important function for the HSV-2 major DNA binding protein. Hence, antibodies against ICP11/12, were used as one marker for viral replication sites.

To detect ICP11/12, HSV-2 mock-infected and infected cells (prepared as in section 2.2.10) were incubated in a humid chamber for 1 hour at 37°C with a 1:50 dilution of LP4 (a mouse monoclonal anti ICP11/12 kindly provided by Dr J.C.M. Macnab). This was then followed by a 1 hour incubation with a 1:250 dilution of FITC-conjugated goat anti-mouse IgG. DNA methylase was detected using antibody 34 as in section 3.7.1.

Figure 3.16b shows the distribution of ICP11/12 and hence sites of replication in cells infected for 4 hours with HSV-2. The staining covers most of the nucleus, with the possible exception of the nucleoli. This is in contrast to figure 3.16d where DNA methylase is localised around the nuclear periphery as was also shown in section 3.7.1. There appears again, however, to be a slight problem with background staining as seen in figure 3.16a which shows a faint reaction of uninfected cells with LP4.

BrdU immunofluorescence microscopy was also used as second alternative way to identify sites of DNA replication. Cells were labelled with the BrdU labelling reagent (1:10, 5′-bromo-2′-deoxyuridine:5′-fluoro-2′-deoxyuridine) from 1-3 hours post-infection. The BrdU epitopes were then detected using anti-BrdU monoclonal antibody followed by an FITC-conjugared second antibody as described in section 2.2.10. DNA methylase was detected in BrdU-labelled cells using antibody 36 (as well as 34 and 35, results not shown) as described in section 3.7.1.

Figure 3.17 shows that the sites of DNA replication again appear to be distributed throughout the nucleus (figure 17a, b)). DNA methylase is also seen to relocate to the nuclear periphery.(figure 3.17c, d) following infection.

In order to investigate the spatial relationship of ICP11/12, DNA replication and DNA methylase in closer detail it would be necessary to perform double labelling experiments along with confocal microscopy but time did not allow for this to be carried out. However, what is clear from this experiment is that DNA methylase is not associated with the bulk of the HSV major DNA binding protein or sites of DNA replication and therefore is excluded from most, if not all of the DNA replication compartments.

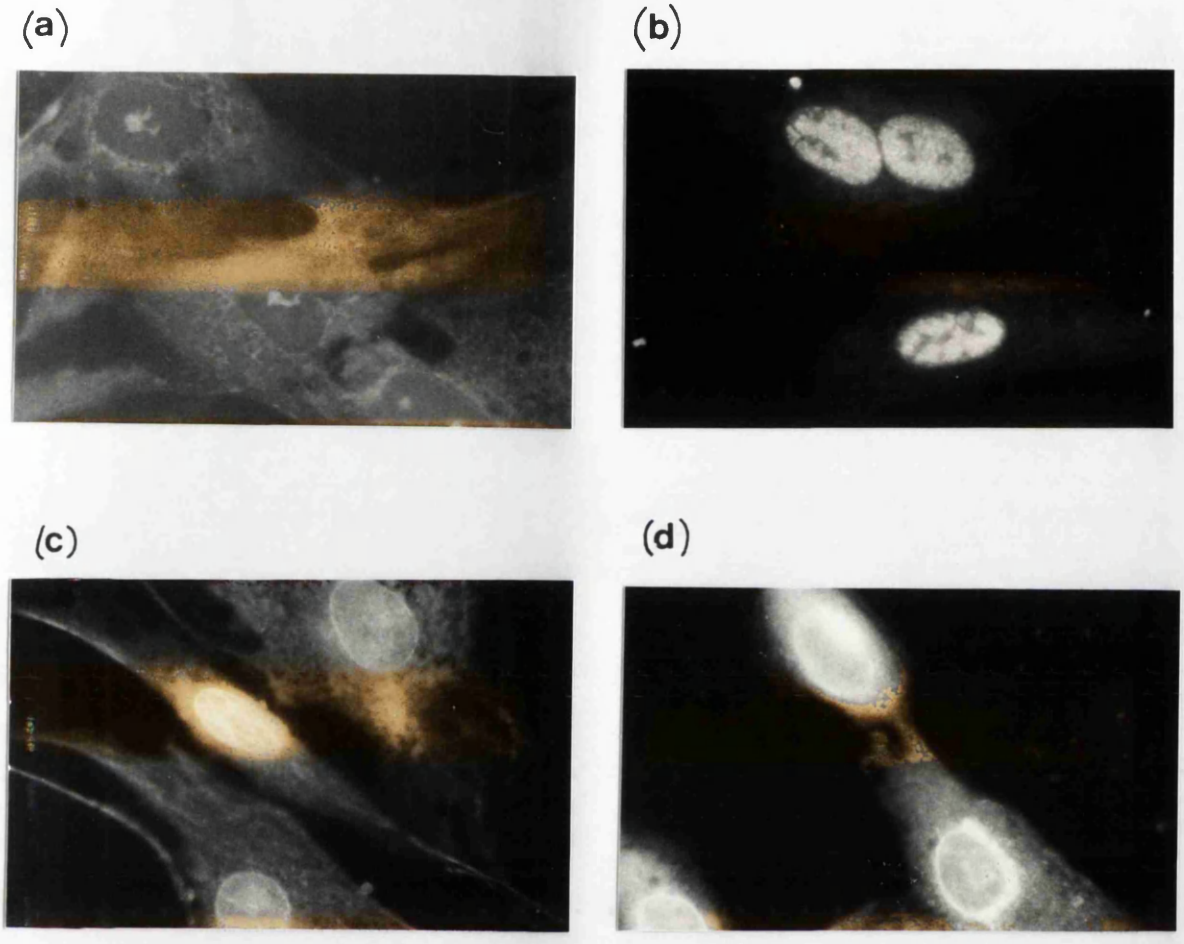


Figure 3.16 **The Cellular Localisation of DNA Methylase and ICP11/12**

RE cells were mock-infected (a, c) or infected (b, d) for 4 hours with HSV-2 and were fixed with 3:1 methanol:acetone (see section 2.2.10). The cells were then reacted for 1 hour with a 1:50 dilution of LP4 (a, b) or a 1:10 dilution of antibody 34 (c, d) followed by a 1 hour incubation with a 1:250 dilution of FITC conjugated anti-mouse (a, b) or a 1:80 dilution of FITC-conjugated anti-rabbit IgG(c, d) (see section 2.2.10).

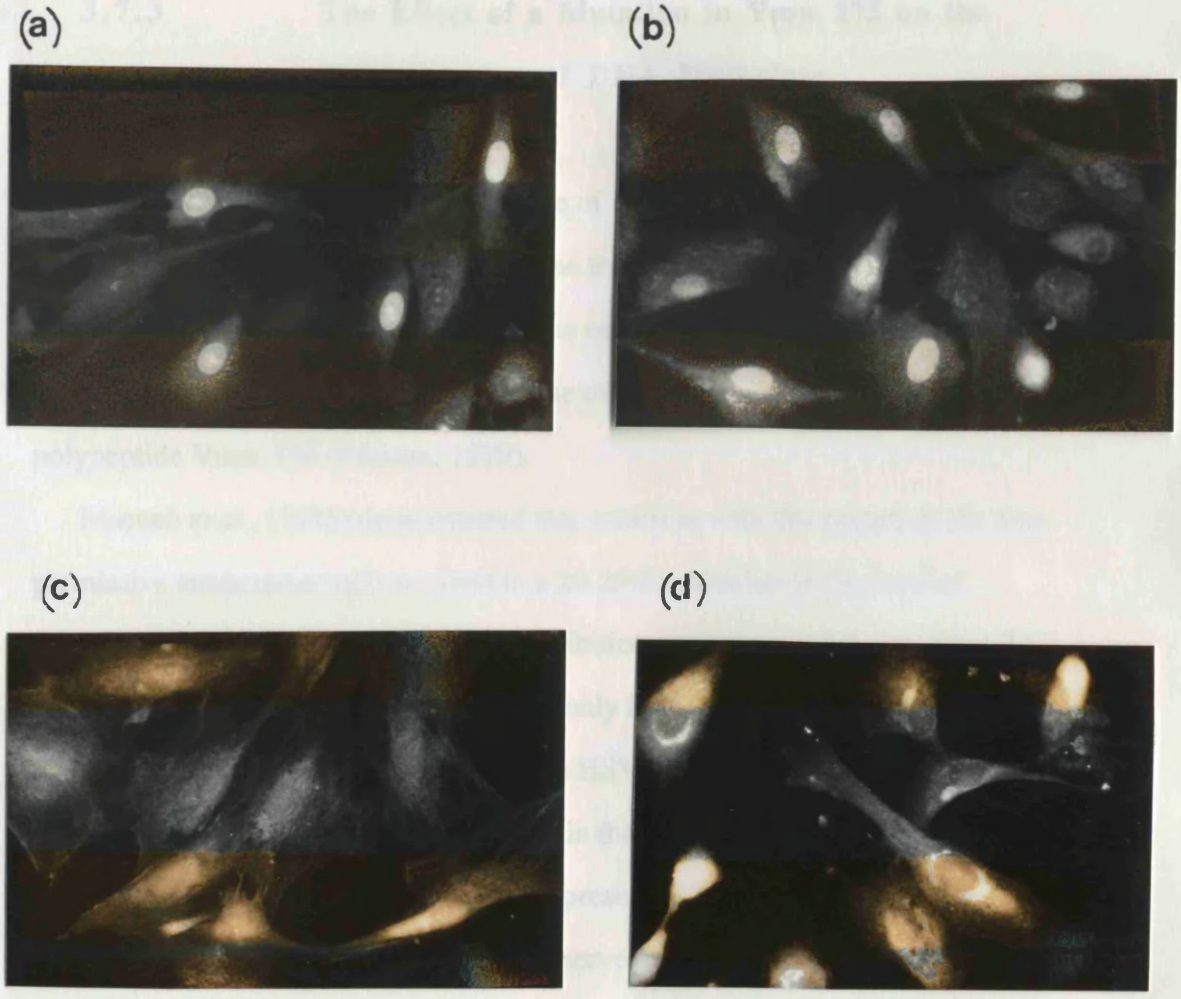


Figure 3.17 **The Cellular Localisation of DNA Methylase and Replicating DNA**

RE cells were mock-infected (a, c) or infected (b, d) with HSV-2 for 4 hours in the presence of BrdU (see section 2.2.10). The cells were then incubated for 1 hour with a 1:100 dilution of anti BrdU antibody (a, b) or a 1:10 dilution of antibody 36 (c, d) followed by a 1 hour incubation with 1:250 dilution of FITC conjugated anti-mouse (a, b) or a 1:80 dilution of FITC conjugated anti-rabbit (c d) antibodies.(see section 2.2.10).

3.7.3 The Effect of a Mutation in Vmw 175 on the Relocalisation of DNA Methylase

Vmw 175 (ICP4) is the product of one of the five IE genes expressed by HSV and is essential for viral early and late gene transcription (see section 1.2.2). *Tsk* is a temperature sensitive mutant which at the non-permissive temperature of 38.5°C makes a defective Vmw 175 along with the other IE early proteins and one early polypeptide Vmw 136 (Preston, 1979).

Macnab *et al.*, (1988) demonstrated that infection with this mutant at the non-permissive temperature only resulted in a 20-25% inhibition in the level of methylation as opposed to the 90-95% inhibition seen using wild-type virus. This may demonstrate that Vmw 175 is not the only factor involved in the inhibition of DNA methylation following infection with HSV. Alternatively, the mutant virus infection may have been "leaky" resulting in the expression of small amounts of the protein. Whichever the case however, the presence of a functional Vmw 175 appears to play an important role in the observed inhibition of DNA methylation.

The aim of this experiment was, therefore, to investigate if a functional Vmw 175 was also required to cause an alteration in the nuclear localisation of DNA methylase as seen following HSV infection in sections 3.7.1 and 3.7.2.

RE cells were grown directly onto glass coverslips and were infected with HSV-2 or *ts*K at the non-permissive temperature (38.5°C) for 4 hours after which time the cells were then fixed with 3:1 methanol:acetone (see section 2.10). To locate DNA methylase the cells were reacted with one of the two anti-methylase antisera 35 or 36 followed by an incubation with FITC-conjugated goat anti-rabbit IgG.

In an attempt to reduce the background, the primary antibody concentration used in this experiment was reduced to a 1:100 dilution. At this dilution, the characteristic perinuclear staining seen in infected cells in the previous experiments is clearly visible in the cells infected with wild-type virus with both antibodies 35

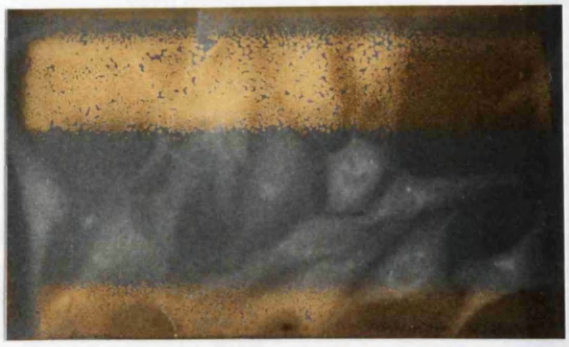
and 36 (figures 3.18e, f). This is not seen however, in the cells infected with *ts*K (figures 3.18c, d) and the faint staining throughout the cell is presumably background (which has been seen to be a slight problem in the previous two sections).This background staining also makes it difficult to distinguish the location of DNA methylase in mock-infected cells.

This experiment therefore shows that Vmw 175 expression is necessary for the redistribution of DNA methylase to the nuclear periphery following infection with HSV-2.

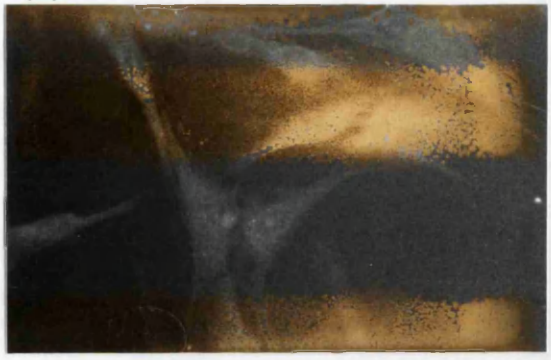
Figure 3.18 The Effect of a Mutation in the HSV Protein, Vmw 175 on the Localisation of DNA Methylase

RE cells were infected with HSV-2 (e, f), *ts*K (c, d) or mock-infected (a, b) for 6 hours at38.5°C and were fixed with 3:1 methanol:acetone (see section 2.2.10). The cells were then reacted with a 1:100 dilution of antibody 36 (a, c, e) or antibody 35 (b, d, f) for 1 hour followed by a 1 hour incubation with a 1:80 dilution of FITC conjugated goat anti-rabbit IgG.

(a)



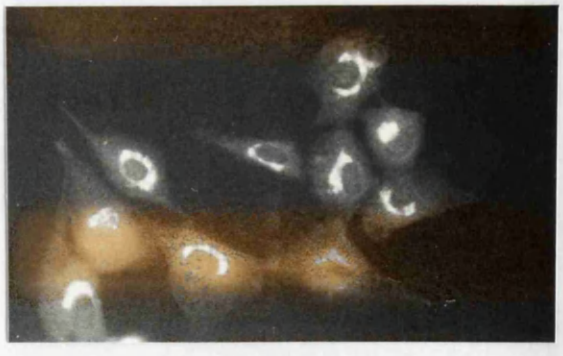
(b)



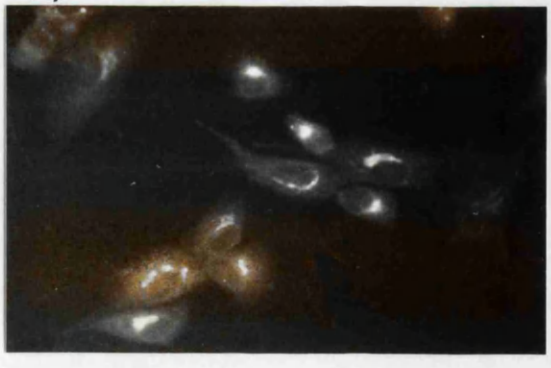
(c)

(d)

(e)



(f)



3.8 Discussion

The aim of the experiments presented in this chapter was to investigate the possible mechanisms by which HSV-2 infection could inhibit the methylation of newly synthesised cellular DNA in RE cells.

The Intracellular Distribution of DNA Methylase Activity

From the data obtained in section 3.2 it is unlikely that the reduction in methylation levels, following infection with HSV-2, occurs as a result of a loss of DNA methylase activity. Both the overall level of activity, as measured in cell lysate, and the activity of the "bound" form of the enzyme thought to be responsible for maintenance methylation *in vivo* (as measured in the nuclear residue fraction) were found to be unaltered following infection.

A number of groups have similarly reported that the reductions in methylation levels seen in transformed cells do not necessarily correspond with reductions in DNA methylase activity and indeed some groups have shown increases in DNA methylase activity in transformed cells (Lapeyre *et al.*, 1981; Arnaud *et al.*, 1985; Kautiainen and Jones, 1986). Szyf *et al.*, (1985, 1991) have demonstrated that the expression of DNA methylase is cell cycle-dependent and they speculate that imbalances in the cell cycle that occur in transformed cells may lead to these increases in DNA methylase activity. Hence, a reduction in DNA methylase activity is not a prerequisite for the inhibition of DNA methylation *in vivo*.

The Effect of HSV-2 Infection on SAM and SAH Metabolism.

The second hypothesis addressed in this chapter was that HSV-2 could cause an imbalance in the metabolites SAM and SAH which could indirectly lead to an inhibition of DNA methylation *in vivo* (as discussed in section 3.3). Measurement of the rate of SAM breakdown shows no differences between mock-infected and infected cells ruling out the possibility that infection with HSV-2 induces a

SAM'ase type enzyme. The amount of SAM degraded in this assay corresponds to a turnover of approximately 40% of the total amount of SAM (as measured in section 3.2.2.3) in 30 minutes. This is in accordance with the results obtained in section 3.2.2.2 where >90% of ^{35}S-labelled SAM is metabolised within 2 hours of removing the source of the label i.e. ^{35}S-methionine. This also correlates with the results of Guilidori *et al.*, (1984) who demonstrated that the half-life of SAM *in vivo* was approximately 30 minutes. SAH is a potent inhibitor of the DNA methylase reaction and alterations in its metabolism and therefore in the ratio of SAM:SAH have been shown to be sufficient to cause a decrease in the m^5C content of DNA in rats (Wilson *et al.*, 1984). Adams and Burdon, (1985) have shown that when SAM and SAH are present at equimolar concentrations *in vitro*, DNA methylase activity is inhibited by some 84%. *In vivo*, the ratio of SAM:SAH has been variously estimated between 3 and 25 (Wilson *et al.*, 1984; De Sanchez *et al.*, 1991) which is in accordance with the results obtained in this study however, no significant difference was seen in the SAM:SAH ratio in HSV-2 infected and mock-infected RE cells. Arnaud *et al.*, (1985) similarly found that the hypomethylation of rat kidney cells transformed by avian sarcoma virus (compared to normal control cells) was not related to alterations in SAM:SAH ratios which they calculated as being 10.94 and 12.32 respectively. The values obtained, in this study, for the amount of SAM and SAH relates to intracellular concentrations of approximately 30 μM and 3μM respectively and these values also correlate well with the reported K_m and K_i values for DNA methylase (Adams and Burdon, 1985). HSV infection therefore, does not appear to cause the hypomethylation of newly synthesised DNA by altering the metabolism of SAM or SAH.

Endogenous DNA as Methyl Group Acceptor

In experiment 3.4, DNA from HSV-2 infected cells accepts methyl groups more readily than DNA from mock-infected RE cells. As discussed in section 1.1.2.2 the preferred substrate of DNA methylase is hemi-methylated DNA. Following

infection of RE cells with HSV-2, only the newly synthesised strand of DNA fails to be methylated. The parent strand is not demethylated as the level of methylation in DNA synthesised prior to infection is not altered (Macnab *et al.*, 1988) and therefore the cellular DNA in infected cells must be largely hemi-methylated. This hemi-methylated DNA is theoretically an ideal substrate for DNA methylase and this correlates with the finding that DNA from infected cells accepts methyl groups more readily than DNA from mock-infected cells. The aim of the experiment was to investigate if DNA methylation was inhibited by DNA binding proteins blocking the access of DNA methylase to its substrate sites. However, although endogenous DNA from infected cells accepts methyl groups more readily than that from mock-infected cells this may be the net result of a balance between increased hemi-methylated sites and inhibitory proteins. From the results alone, therefore, it cannot be concluded that are no such inhibitory proteins bound to DNA in infected cell nuclei.

Characterisation of the Molecular Weight of DNA Methylase following Infection with HSV-2

The results obtained section 3.5 show that there is no significant degradation of the high molecular weight species of DNA methylase following infection with HSV-2. However, one of the antipeptide antibodies used (Ab 36) detects an HSV-2 induced 40 kDa polypeptide in the nuclear residue fraction which can be detected as early as one hour post-infection. This polypeptide is highly antigenic and it may be generated from only limited proteolysis of DNA methylase. Alternatively, the polypeptide cross-reacts with the anti-methylase antibody may not be part of DNA methylase. If the band is of viral origin the pattern of accumulation is most characteristic of an immediate early protein. However, none of the set of 5 IE proteins are of the appropriate size and hence, the 40 kDa polypeptide could only correspond to a breakdown product of one of these proteins. Computer analysis was used to compare the amino acid sequence of the

peptide 36 with the amino acid sequences in the EMBL databank. Some identity was found with the IE protein Vmw 175 (4 out of 10 amino acids are identical, see Fig. 3.19) although it is uncertain as to the degree of cross-reactivity that this identity would confer.

```
1     CKDMSPLVAA   10     Peptide 36
          || ||
695   QSLRPLLAA    703    Vmw 175
```

Figure 3.19 Identity Between Peptide 36 and Vmw 175

(The numbers represent the amino acid position)

In addition, there is no evidence to suggest that the Vmw175 protein is degraded in this way and no accumulation of a 175 kDa protein is seen in the timecourse assay in section 3.5.

A second alternative is that the 40 kDa polypeptide may be a cleavage product of DNA methylase. Antibody 36 was raised against a synthetic peptide corresponding to a region with the C-terminal of the predicted amino acid sequence of murine DNA methylase (see section 3.5). Since these experiments were performed, cDNA clones encoding the human DNA methylase have been isolated and sequenced and a sequence identical to peptide 36 is found within the homologous region of the predicted amino acid sequence of the human enzyme suggesting that this region may be functionally important (Yen *et al.*, 1992). Both of these mammalian DNA methylases also show strong sequence similarities to all of the approximately 30 known bacterial cytosine 5-methyltransferases. These comparisons also suggest that the carboxy terminal one-third of the enzyme is the catalytic domain (see section 3.5). However, peptide 36 does not lie within the predicted active site which is thought to be centered around the most carboxy-terminal Pro:Cys motif (Chen *et al.*, 1991). Cleavage of 400 amino acids from the carboxy terminus to give a polypeptide of approximately 40 kDa would not, theoretically, remove the active

site although it may reduce activity. The findings in section 3.2 show that DNA methylase activity measured *in vitro* is not inhibited at 4 hours post-infection at a time when the 40 kDa protein is present on immunoblots. It cannot be ruled out that this extreme carboxy-terminal region of DNA methylase is important in allowing the methylation of DNA *in vivo* but is not required for activity *in vitro*. However, the results in section 3.5 suggests that the bulk of the enzyme is unaltered following infection and therefore limited proteolysis of the enzyme may not result in any significant alteration in the overall DNA methylase activity.

Alterations in the Ionic Environment as a Possible Mechanism for the Inhibition of DNA Methylation

Manipulation of the internal ionic environment by increasing the external [NaCl] in experiment 3.6 had a dramatic effect on the synthesis of DNA however, the $\%m^5C$ in the limited amount of DNA synthesised was unaltered under these conditions of hypertonicity.

Robbins *et al.*, (1970) demonstrated that the inhibition of macromolecular synthesis following exposure of HeLa cells to hypertonic medium was readily reversible suggesting that the cells are not permanently damaged by this treatment. Saborio *et al.*, (1974) reported that hypertonicity inhibits protein synthesis by causing a dissociation of polysomes but the mechanism of inhibition of DNA synthesis is still unclear. Inhibition of DNA replication and/or an increase in the ionic concentrations caused by the increased osmolarity however had no effect on the level of DNA methylation. These results correlate to the results of Woodcock *et al.*, (1982) who similarly showed, using specific inhibitors, that inhibition of DNA replication has no effect on DNA methylase activity *in vivo*. In addition, more specific manipulations of the ionic environment using ionophores to alter the concentrations of specific ions also had no effect on the level of DNA methylation.

The results from these two experiments show that DNA methylase activity *in vivo* is relatively stable in a variety of ionic environments. Therefore, any

alterations in the ionic milieu of the cell following infection with HSV is unlikely to be the cause of viral induced inhibition of cellular DNA methylation.

Immunofluoresence microscopy

In section 3.7, HSV-2 infected cells showed strong perinuclear staining when reacted with all three anti-peptide, anti-methylase, antisera. This is in contrast to the staining pattern seen in mock-infected cells which showed only weak nuclear staining, in some cases indistinguishable from the background staining, when reacted with the same antiserum.

Previous immunofluoresence studies on the intracellular localisation of DNA methylase have shown that it is only visible during S-phase but at this time it appears to be evenly distribute throughout the nucleus (Vogel *et al.*, 1988). There have been no studies to date, however, investigating the colocalisation of DNA methylase and replication compartments and hence the spacial relationship between DNA replication and methylation is still unclear.

What is clear from the studies presented in section 3.7 is the dramatic alteration of DNA methylase localisation following infection with HSV-2 which appears to remove the DNA methylase from the proximity of the majority of sites of DNA replication (as demonstrated in section 3.7.2). From these results it is speculated that the inhibition of methylation of cellular DNA following infection with HSV-2 occurs as a consequence of this distancing of the enzyme from its substrate i.e. newly synthesised hemi-methylated DNA. This hypothesis also correlates with the other findings described in this project in that such a mechanism would not require any direct or indirect aletrations in DNA methylase activity *in vivo*.

The remaining question to be addressed is how DNA methylase is relocated following infection with HSV. Only one candidate HSV function, the IE protein Vmw 175, was investigated as time was limited. From the results obtained in section 3.7.3 it appears that the expression of Vmw 175 is a requirement for the relocalisation of DNA methylase. This correlates with the findings of Macnab *et al.*, (1988) who

demonstrated, using an HSV mutant, that a functional Vmw 175 is required to cause maximal inhibition of DNA methylation. However, Vmw 175 is a trans-acting factor (see section 1.2.2) and from these experiments alone it is impossible to determine if relocalisation is mediated by this protein or by proteins expressed as a consequence of Vmw 175 expression.

Chapter 4 DNA Hypomethylation and HSV Transformation.

4.1 The Effect of HSV Transforming Regions on DNA Methylation

Macnab *et al.*, (1988) demonstated that cell-lines derived from foci morphologically transformed by the *Bgl* II n fragment of HSV-2 were hypomethylated in comparison to the parent RE cells. Although the genes encoding the entire small subunit (RR_2) and part of the large subunit (RR_1) map within this fragment there is no requirement for retention of the fragment or for the continued expression of ribonucleotide reductase in the maintenance of the transformed state (Cameron *et al.*, 1985) (see section 1.2.5).

The aim of the experiment in this section was to investigate if the *Bgl* II n transforming region or regions of HSV-2 encoding ribonucleotide reductase could possibly initiate transformation by influencing the level of DNA methylation. To address this question, four different plasmids containing the HSV-2 sequences of interest were transfected into RE cells using the calcium phosphate DNA co-precipitation method (see section 2.2.14).

The *Bgl* II n plasmid contains the *Bgl* II n fragment of HSV-2 cloned into the the vector pAT 153 at the *Bam* H I site and was constructed by A.J. Davison of the Institute of Virology (see figure 4.1).

Plasmids containing sequences encoding either, or both, of the two subunits of ribonucleotide reductase were constructed by P. Clarke, S. Simpson, I. Nikas and J.B. Clements also in the Institute of Virology. The plasmid pOC_1 contains the gene encoding the ribonucleotide reductase large subunit, RR_1 and pOC_2 contains the gene encoding the small subunit, RR_2, both of which are under the control of the metallothionine promoter (MT-1). The plasmid pOC_3 contains the genes encoding both of the ribonucleotide reductase subunits, the small subunit gene being under control of its own promoter whilst the large subunit gene is under control of the metallothionine promoter (see figure 4.1). All three pOC plasmids have been demonstrated to be transcribed in transfection assays and this

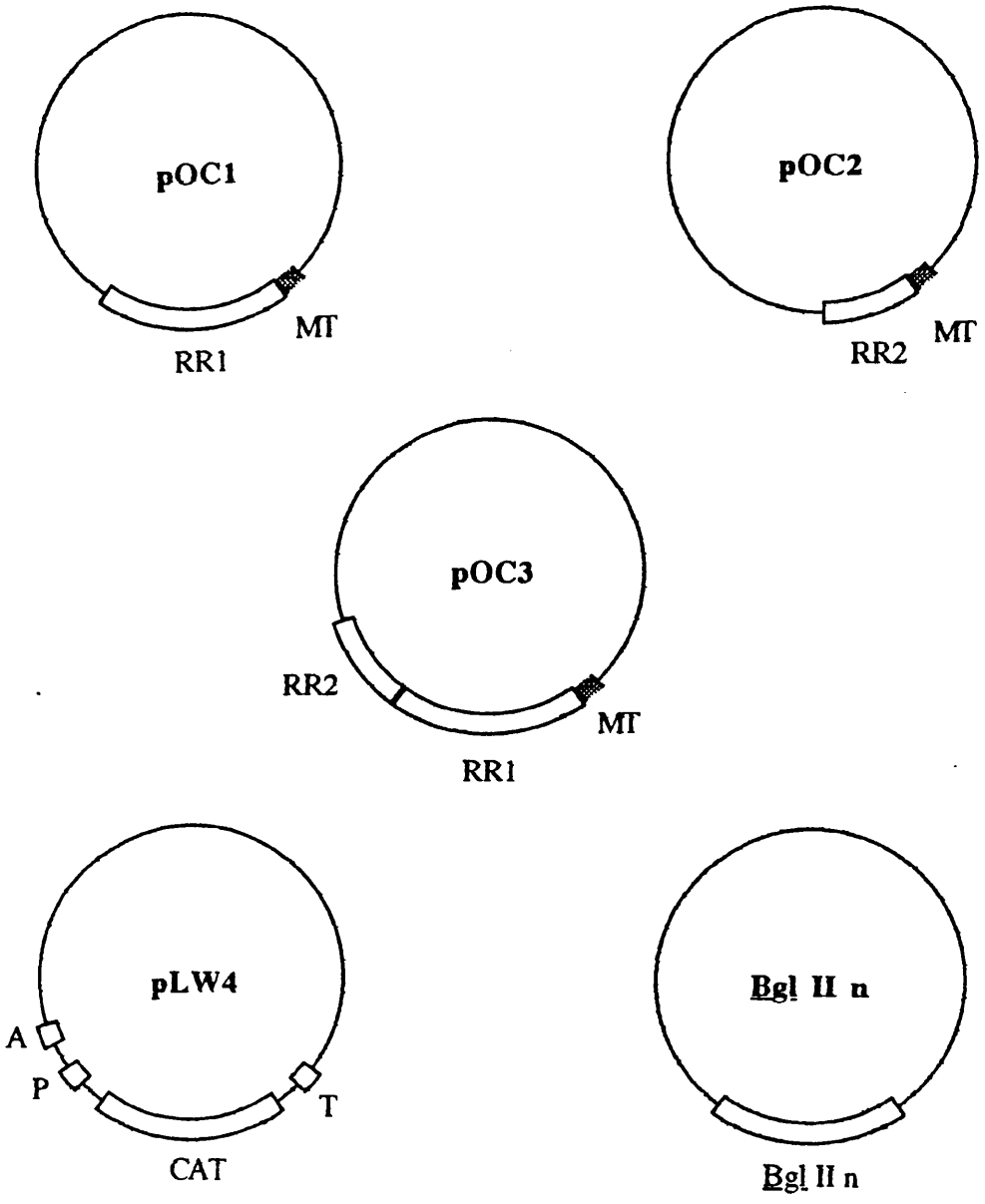


Figure 4.1 Plasmids used in Transfection Assays

Plasmids not shown to scale. The abbreviations used are as follows:

RR: Ribonucleotide Reductase

MT: Metallothionine Promoter

A: Activator Sequence

P: HSV IE Promoter

T: Terminator Sequence (for details see section 4.1)

transcription is optimally induced with 100 mM Zinc Sulphate (Clarke, 1990). However, only cells transfected with pOC_3 or co-transfected with pOC_1 and pOC_2 were shown to demonstrate viral ribonucleotide reductase activity as both of the functional subunits are required for activity.

To monitor the success of the transfection, the plasmid pLW4 was co-transfected with each of the plasmids described above. The plasmid pLW4 was constructed by Lindsay Whitton (Gaffney *et al.*, 1985) at the Institute of Virology, Glasgow and it contains the bacterial chloramphenicol acyl transferase (CAT) gene under the control of an HSV-2 IE promoter. In addition, it contains an activator sequence which has been demonstrated to increase the transcription of the CAT gene 2-3 fold in transcription assays (Gaffney *et al.*, 1985). Mammalian cells do not contain the gene encoding CAT and hence any CAT activity found in transfected cells gives a measure of the success of transfection.

Chloramphenicol acyl transferase transfers acyl groups to the hydroxyl groups on C_1 and C_3 of chloramphenicol and this activity is normally measured by quantifying the level of ^{14}C-chloramphenicol acylated using thin-layer chromatography. However, a new, simpler and more sensitive method introduced by Seed and Sheen, (1988) was used in this project. This assay uses butyryl-CoA as oppossed to acetyl-CoA as the acyl donor and the greater hydrophobicity of butyrylated chloramphenicol allows an effective discrimination between free and butyrylated chloramphenicol by a simple phase-extraction assay (see section 2.2.15).

RE cells were transfected with a suspension containing 5 µg of the test plasmid and/or 5 µg of the control plasmid pLW4 (as described in section 2.2.14) made up to a total of 20 µg with calf thymus DNA. 48 hours after transfection, the cells were harvested and the level of CAT activity (see section 2.2.15) and m^5C (see section 2.2.6.1) determined. Table 4.1 shows the results obtained. None of the plasmids used has a significant effect on the $\%m^5C$ when transfected into RE cells as

compared to the control values. The CAT activity in cells co-transfected with the pOC plasmids and pLW4 is lower than that in cells transfected with pLW4 alone.

However in each of the transfection assays, CAT activity was detected signifying that DNA was being taken up by the cells.

Plasmid	CAT Activity (% of control)	%m^5C
pLW4-Control	100	3.77 ± 0.69 (5)
Carrier Only	0.50 ± 0.17 (6)	3.68 ± 0.43 (5)
*Bgl*II n Plasmid	114, 102	3.39, 3.28
pOC_1:Zn Induced	59.7, 66.7	3.91
pOC_1:Uninduced	13, 18	4.28, 4.18
pOC_2:Zn Induced	65, 66	3.79
pOC_2:Uninduced	20, 19	3.63, 3.81
pOC_3:Zn Induced	38.5, 56	3.97
pOC_3:Uninduced	23, 33	3.82, 4.19

Table 4.1 The Effect of HSV-2 Transforming Regions on DNA Methylation.

The transfection assays using the pOC plasmids were carried out as two separate experiments. The first without added $ZnSO_4$ and the second with $ZnSO_4$ added 24 hours after transfection.

4.2 The Intragenomic Distribution of m^5C in Bn5T Cells

Hsiao *et al.*, (1985), demonstrated that treatment of mouse embryo fibroblasts with the demethylating agent, 5-azacytidine can lead to cell transformation and other groups have shown similar results in the same (Jones and Taylor, 1980) or in different (Darmon *et al.*, 1984; Reitz *et al.*, 1984) cell systems. In these cases, widespread demethylation is suggested to lead to the chance activation of cellular genes involved in cell transformation although this may, in fact, be only one factor in a multistage process (see section 1.1.4).

DNA methylation levels in Bn5T cells are less than half that of the parent RE cells. The aim of this experiment was to investigate if this hypomeythlation is widespread throughout the genome and hence could feasibly influence gene expression. It is possible, however, that reduced methylation levels *in toto* could result from a drastic reduction in, for example, the amount of methylation of satellite DNA with the same or even increased methylation in the other DNA fractions which encode proteins.

To study the intragenomic distribution of m^5C, subconfluent burrlers of RE and Bn5T cells were labelled for 2 days with 6-^{3}H uridine (200 µCi, 23 Ci/mmol) in a total of 50 ml of media. The tritium labelled DNA, prepared as in section 2.2.11.1, was then fractionated into different frequency classes (see section 2.2.11.2). Briefly , the DNA was denatured in 0.175 M NaOH at 60°C for 10 minutes. Renaturation was initiated with the addition of 0.175 M HCl and PIPES buffer and the reaction mixture incubated at 60°C for the appropriate length of time to achieve the desired C_0t value. C_0t values were chosen to represent the following three classes of eukaryotic DNA.

(i) Highly repetitive DNA : $C_0t < 0.01$

(ii) Moderately repetitive DNA : C_0t 0.01-70

(iii) Unique DNA : $C_0t > 70$

C_0t value is defined as the initial concentration (C_0) expressed as moles nucleotide per litre multiplied by the time (t) in seconds it takes for the DNA to reanneal.

Following the appropriate reannealing time, single-stranded DNA was digested with S1 nuclease. The duplex DNA was precipitated with 5% TCA and washed twice with 5% TCA and once with absolute alcohol. To determine the $\%m^5C$ in each of the frequency classes the DNA was pyrolysed with formic acid for 1.5 hours at 175°C and the bases separated on an Aminex A6 column as described in section 2.2.6.2.

Figure 4.2 shows that in both RE and Bn5T DNA samples, the satellite DNA fraction ($C_0t < 0.01$) has the highest proportion of its cytosines methylated. This is in accordance with a number of previous studies which have shown rapidly annealing DNA to be the most highly methylated DNA fraction in various tissues and cells (Drahovsky *et al.*, 1979, Ehrlich *et al.*, 1982).

In the unique, single copy fraction, the methylation levels in both Bn5T and RE are very similar to the total DNA levels. There is a slight difference in the relative methylation levels in the mildly repetitive fraction but the pattern overall suggests that there is a lower level of methylation in all classes of DNA in Bn5T cells compared to RE cells with HSV-2.

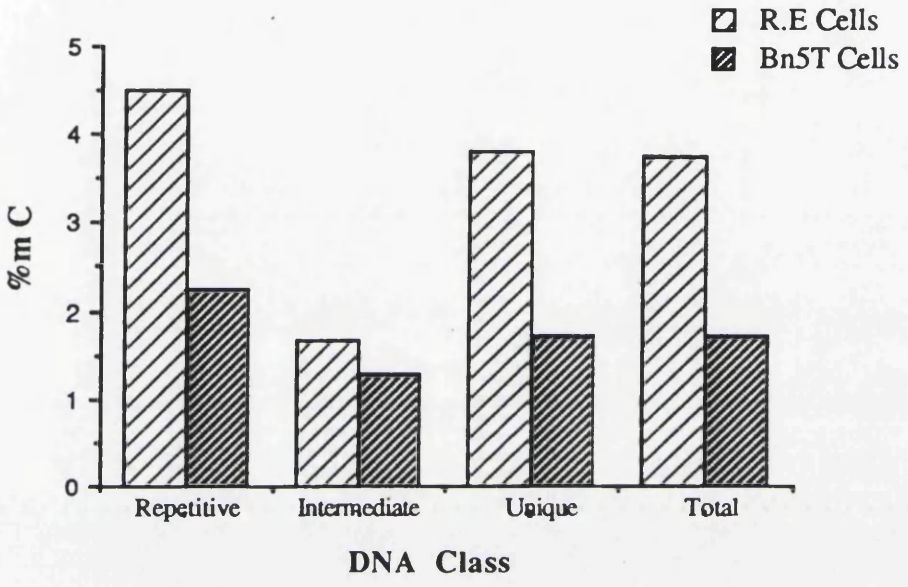


Figure 4.2 DNA Methylation Levels in Various C_0t fractions of RE and Bn5T Cells

Burrlers of RE and Bn5T cells were labelled with 6-^{3}H uridine (200 µCi, 23 Ci/mmol) for 2 days. The tritium labelled DNA was then purified and fractionated into the different frequency classes as described in section 4.2.(see section 2.2.11 for details). The %m^{5}C in each of these fractions was then determined according to section 2.2.6.

4.3 Discussion

The aim of the experiments in section 4.1 of this chapter was to assess the ability of known transforming regions of HSV-2 to directly influence DNA methylation levels when transfected into RE cells. In addition to the *Bgl* II n plasmid which contains the region of HSV-2 responsible for generating the Bn5T cell-line, three further plasmids containing overlapping sequences were also used (see Fig. 4.1 and Fig. 1.6).

The evidence accumulated to date from studies on the mechanism of HSV transformation suggests that, although the regions of the HSV genome that independently cause transformation centre around the genes encoding ribonucleotide reductase there is no requirement for the expression of a complete, functional enzyme either in the establishment or in the maintenance of the transformed state (discussed in section 1.2.5). Hence, although we carried out the transfection studies with the pOC plasmids $\pm ZnSO_4$ it was not essential that these subunits were expressed.

The transfection procedure itself appears to have been successful as all transfected cells showed levels of CAT activity significantly higher than control values. However, the CAT activity in cells co-transfected with pOC plasmids and pLW4 was reduced compared to those transfected only with pLW4. The reason for this is unclear, it may be that there is competition for transcription factors in the co-transfected cells and that using smaller amounts of DNA may have eliminated this problem.

What is clear from these experiments is that none of the sequences transfected into RE cells had any effect on the level of DNA methylation as measured 48 hours after transfection. These results suggests that the HSV-2 transforming region investigated does not exert its oncogenic effect by causing a widespread reduction in DNA methylation. However, it cannot be ruled out that DNA methylation levels are altered in a small proportion of cells as this experiment would not detect such

changes. It would appear, therefore, that the reduction in methylation is a secondary event in the transformation of primary RE cells by the *Bgl* II n fragment although these reduced methylation levels may be an important factor in maintaining the transformed state in these cell-lines.

Bovine sperm DNA has reduced levels of methylation in comparison to other bovine cells however, this reduction *in toto* is due almost entirely to the loss of methyl groups from satellite DNA only, which has been shown to have near zero levels of methylation (Adams *et al.*, 1983). It is unclear as to whether this satellite DNA is undermethylated because it is in some way protected from the de novo methylation that occurs in embryogenesis or if it occurs by a specific demethylation event such as selective DNA replication in the absence of methylation. The results in section 4.2, however, show that the reduced levels of methylation in Bn5T cells is due to a reduction in the methlation level of all classes of DNA. This suggests that the mechanism of demethylation is non-specific and not targeted to any particular subclass of DNA

Chapter 5 Discussion

5.1 Introduction

Infection of RE cells with HSV results in a reduction in the methylation levels of newly synthesised cellular DNA. The results obtained in chapter 3 demonstrate that, despite this inhibition of DNA methylation, RE cells infected with HSV-2 appear to retain all the factors necessary for DNA methylation to take place. Thus, (a) SAM and SAH levels are not significantly different from those measured in mock-infected cells and the levels correspond with the reported K_m and K_i values for DNA methylase (see section 3.3), (b) DNA methylase is present in cell nuclei as shown by activity in an *in vitro* methylase assay and (c) cellular DNA in these cells retains the ability to accept methyl groups.

However, upon infection, DNA methylase concentrates around the nuclear periphery. This is in contrast to the sites of DNA replication which are distributed throughout the nucleus and this differential distribution leads us to speculate that the inhibition of DNA methylation following infection occurs as a result of DNA methylase being distanced from its substrate, newly synthesised DNA.

5.2 Margination of Cellular Chromatin upon Infection with HSV

The first visible effect of HSV infection involves changes in the cell nucleus. These effects include the swelling and displacement of the nucleolus, chromosomal breakage, displacement of chromatin and a condensation of this displaced chromatin at the nuclear membrane. All of these cytopathic effects are widely documented and have been used as a diagnostic feature of HSV infection (Crouse *et al.*, 1950; Shwartz and Roizman, 1969; Dargan and Subak-Sharpe, 1983).

Margination of cellular chromatin is visible in HSV infected cells at 3-4 hours post-infection. The mechanism and the HSV functions involved in this chromatin margination, however, are still unclear. Dargan and Subak-Sharpe (1983), using a

variety of temperature sensitive mutants, suggest that the margination of chromatin is *initiated* very early in the HSV replication cycle at around 0-1 hour post-infection. Knipe, (1989) postulates that chromatin marginates as a result of the expansion of globular replication compartments in HSV infected cells and there is evidence to suggest that cellular chromatin and viral DNA replication compartments show complementary distributions (Randall and Dinwoodie, 1986). However, this hypothesis appears to condradict that of Dargan and Subak-Sharpe (1983). Formation of globular replication compartments require the expression of the viral protein ICP8 (discussed in section 1.2.3) and the mutant studies of Dargan and Subak-Sharpe suggest that expression of this protein is not required for chromatin margination to take place.

Following infection with HSV cellular DNA synthesis is rapidly inhibited by as much as 80% at 2.5 hours post-infection (Roizman, 1969). It is possible that this inhibition of cellular DNA replication occurs a result of chromatin margination which removes the majority of DNA from the nuclear sites of replication. The cellular DNA which continues to be synthesised in infected cells, which we have demonstrated to be hypomethylated, may correspond to DNA that has been "left behind" after margination or alternatively this DNA may have been actively retained within the interior of the nuclei. Wilcock and Lane, (1991) demonstrated that various cellular proteins involved in cellular DNA replication colocalise with the sites of viral DNA replication in cells infected with HSV. Again, it is not clear if these proteins play a role in HSV replication and hence are actively recruited into viral DNA replication complexes or are redistributed passively as a result of their association with replicating DNA.

The DNA methylase responsible for maintenance methylation is thought to be bound to DNA loops in a form that resists extraction with 2 M NaCl. Hence, DNA methylase may simply be marginating to the nuclear periphery in association with cellular chromatin.

In section 3.8 the immunofluorescence studies using the mutant *ts*K suggest that a functional Vmw 175 is required to allow the relocation of DNA methylase. This correlates with the results of Macnab *et al.*, (1988) who demonstrated that there is only a slight reduction of methylation levels following infection with *ts*K and suggests that the relocation of DNA methylase and the inhibition of DNA methylation are related. Dargan and Subak-Sharpe, (1983) however, demonstrated that cells infected with the *ts*K mutant show the characteristic margination of chromatin seen following infection with wild-type virus. This study differs from ours and that of Macnab *et al.*, (1988) in that the infection was left to proceed for a longer time period of 24 hours. It is possible that there is a delay in chromatin margination in cells infected with *ts*K or, alternatively, HSV functions other than, or in addition to, chromatin margination are required to displace DNA methylase to the nuclear periphery. For example, this displacement of DNA methylase to the nuclear periphery may require the formation of replication compartments which require the viral DNA-binding protein ICP8 for assembly.

5.3 Nuclear Localisation of DNA Methylase

DNA methylase activity measured in cell nuclei varies markedly during the cell-cycle. In resting cells DNA methylase activity is essentially absent. Entrance into S-phase correlates with an increase in activity which then falls off again in G_2/M (Adams, 1990; Szyf et al., 1985). This cell-cycle variation in activity corresponds to variations in mRNA levels suggesting that the DNA methylase gene is transcriptionally regulated (Szyf et al., 1991; Adams et al., 1992 in press). This cell-cycle variation also implies that DNA methylase must be transported into the nucleus at the commencement of S-phase. There is no evidence at present, however, to suggest whether this transport to the nucleus occurs by means of a

nuclear localisation signal within the enzyme or is mediated by means of a "carrier protein".

An alternative mechanism to explain the perinuclear localisation of DNA methylase, therefore, could be that HSV infection inhibits the entry of newly synthesised DNA methylase into the nuclear interior causing it to accumulate around the nuclear periphery. However, the results of the DNA methylase assays in section 3.1 showed that there is no alteration in DNA methylase activity in either the 0.2 M NaCl nuclear extract or in the nuclear residue fraction which suggests that the DNA methylase is still closely associated with the nucleus following HSV infection. Double labelling studies, for example using anti-DNA methylase antibodies in conjunction with a stain for DNA such as Hoescht or DAPI (4, 6-diamidino-2-phenylindole) at early times after infection, may help to clarify the relationship between DNA methylase and cellular chromatin in HSV infected cells. In addition, it would be desirable to carry out an extensive study using mutants to pin-point the virus functions involved in the inhibition of DNA methylation and the relationship of this with the nuclear localisation of DNA methylase.

5.4 Cellular Transformation with HSV-2

Transformation by HSV was first reported by Duff and Rapp in 1971 and 1973. As well as transforming cells in culture, HSV has been associated with cervical carcinoma for over 3 decades. Early links between HSV and cevical carcinomas were made on the bases of seroepidemiological studies and HSV sequences have been identified in human genital preneoplastic and neoplastic diseases. However, more recent evidence suggests that the human papilloma virus (HPV) is a more likely candidate for the causative viral agent (reviewed by Macnab, 1987; Galloway and McDougall, 1989)

The transfection assays performed in chapter 4 suggest that the transformation of RE cells is not directly caused by the inhibition of DNA methylation. This may not reflect the importance of the inhibition of methylation in oncogenesis *in vivo*. Carcinogenesis is thought to be a multistep, multifactorial, process involving both the activation of oncogenes and the inactivation of tumour supressor genes brought about by a combination of heritable genetic defects and environmental mutagenic factors. It is possible, therefore, that abortive infections which leave DNA methylation levels reduced may leave the cell more susceptible to other oncogenic agents. Zur Hausen (1982, 1986) hypothesises that genital cancer may result from a synergistic interaction between HPV infection and other initiating factors. These factors, which zur Hausen speculates to include smoking and herpes simplex infection, may act by modifying cellular genes that would otherwise exert control over the integrated HPV viral genes and thereby also control tumour progression. Therefore, although not essential to bring about tumorogenesis HSV may be a contributory factor to the establishment of the transformed state.

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