Regional DNA Methylation and Gene Expression

by

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Summary

The detailed mechanisms of inhibition of transcription by DNA methylation are still unknown but it has become obvious that the formation of chromatin plays an important role in this process.

Methylation of a plasmid containing the SV40 promoter linked to the chloramphenicol acetyltransferase (CAT) gene results in a significant reduction in the expression of the reporter gene after transfection into cultured cells. Transcriptional inhibition of methylated DNA is apparent only 24 to 48 hours after transfection. This coincides with a decrease in *Msp*I accessibility of methylated DNA, *in vivo*, suggesting a role of chromatin formation and/or the involvement of methylated-DNA binding proteins in the inactivation process.

A new method was developed to methylate, *in vitro*, chosen sequences of this plasmid. This localised methylation of SV40 promoter, CAT gene or vector sequences reveals a reduced CAT activity in transient transfection assays. Transcriptional inactivation appears to be proportional to the length or the CpG density of the methylated region, but independent of its localisation.

Digestion with *Msp*I of nuclei, transfected with these regionally methylated plasmids showed a reduced accessibility of *Msp*I to both, methylated and unmethylated sequences of the plasmid. The results suggest, that upon methylation and transfection into cells, plasmid DNA forms inactive chromatin, this inactive chromatin spreads to unmethylated regions within the plasmid and can thereby inhibit gene expression.

In *in vitro* transcription assays, no differences were observed in the level of transcription from unmethylated or methylated templates and, furthermore, histone H1 did not preferentially inhibit transcription *in vitro* from methylated reporter gene constructs.

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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.* (1993) **289**, 1-15) with the following additions:

aprt	adenine phosphoribosyltransferase
CAT	chloramphenicol acetyltransferase
dsDNA	double-stranded DNA
5-aza-dC	5-aza-2´-deoxycytidine
5-mC	5-methylcytosine
g6pd	glucose-6-phosphate dehydrogenase
hprt	hypoxanthine-guanine phosphoribosyltransferase
Igf2	insulin like growth factor
Igf2r	insulin like growth factor receptor
MDBP	methylated DNA binding protein
MeCP	methylated CpG binding protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pgk	phosphoglycerate kinase
PMSF	phenylmethylsulphonylflouride
SAM	S-adenosyl-L-methionine
ssDNA	single-stranded DNA
sssDNA	sonicated salmon sperm DNA
SV40	simian virus 40
TCA	tricloroacetic acid
tk	thymidine kinase
X^a	active X chromosome
X^i	inactive X chromosome

Overview

CHAPTER ONE

Introduction

The main modification of higher eukaryotic DNA is methylation of position 5 of the cytosine ring in the dinucleotide CpG. The resulting base, 5-methylcytosine (5-mC) is highly unstable and readily converted to thymine by oxidative deamination (Coulondre *et al.*, 1978). Thus, methylcytosines in DNA represent hot spots of mutation for the genomes of "methylating" organisms. The advantages of methylation of higher eukaryotic DNA must therefore outweigh the mutational burden these genomes are carrying. The different aspects and functions of these "advantages" of DNA methylation are discussed in this introduction.

1.1 Methods to study DNA methylation

In 1948, at a time when the actual structure of DNA was still a mystery, Rollin Hotchkiss was the first to discover the presence of 5-methylcytosine in DNA from calf thymus using paper chromatography techniques (Hotchkiss, 1948). Since then, considerable progress has been made in the methodologies for analysis of nucleic acids in general, and for the characterization of modified bases in particular. Some of the latter are described in the following sections.

1.1.1 Sequence unspecific methods

Several methods have been employed to analyse the 5-methylcytosine content of DNA, which usually involves the hydrolysis of DNA followed by the fractionation of the hydrolysis products. Chemical cleavage of DNA can easily be achieved by treatment with acid, although deamination of cytosine and 5-methylcytosine can be associated with this method (Eick *et al.*, 1983). Enzymic hydrolysis of DNA produces little deamination (Ford *et al.*, 1980) and produces mononucleotides with 5′ phosphates (e.g. in the case of DNaseI) or 3′ phosphates (e.g. in the case of micrococcal nuclease) (Linn and Roberts, 1982). Fractionation of the cleavage products of DNA can be achieved by thin layer-chromatography (TLC) or high performance liquid chromatography (HPLC), and the method of choice depends largely on the amount and the method of preparation of the DNA to be analysed (Adams and Burdon, 1985). Pollack *et al.* (1984) reported an improved method, whereby nicks were introduced into the DNA by means of DNaseI and the nucleotide 3′ to the nick was labelled using α^{32}P-labeled 5′ triphosphate. On hydrolysis to the 3′ monophosphates, and separation via TLC, this modified nearest neighbour analysis indicated the proportion of the four CpN nucleotides that are methylated.

In addition to these methods, antibodies directed against 5-methylcytosine have been used to detect the presence of the base in intact chromosomes (Schreck *et al.*, 1977) or in DNA immobilized on filter membranes (Sano *et al.*,1980).

1.1.2 Sequence specific methods

Much information regarding the methylation pattern of DNA has been obtained by the use of bacterial restriction enzymes. There are several isoschizomeric restriction nucleases available, which digest either only the unmethylated (e.g. *Hpa*II), or the methylated and unmethylated recognition site (e.g. *Msp*I) and thus, reveal the

methylation status of the DNA by comparison of the digestion patterns (reviewed by Adams and Burdon, 1985; Saluz and Jost, 1993). An improvement in sensitivity of this method has been achieved by performing a PCR assay in such a way that only undigested (i.e. methylated or hemimethylated DNA) is amplified after the digest with methylation-sensitive restriction enzymes (Singer-Sam *et al.*, 1990).

However, using methylation-sensitive restriction enzymes limits the detection of methylcytosines to those contained within the recognition site of the nuclease. This is not a problem of the method introduced by Church and Gilbert (1984). Genomic DNA is digested with restriction enzymes defining the length of the fragment with the target sequence. The DNA is then subjected to chemical sequencing reactions (Maxam and Gilbert, 1980) and separated on a sequencing gel. The separated sequencing products are then transferred to a nylon membrane and the target sequence visualized by hybridization with the appropriate, labelled probe. Methylation of cytosine will appear as a gap in the C-ladder, as hydrazine does not react with 5-methylcytosine in the C-specific chemical modification reaction. Several improvements have been made to this method (Saluz and Jost, 1987; Saluz and Jost, 1989) and most notably, the introduction of the ligation-mediated polymerase chain reaction (LMPCR) method by Pfeifer *et al.* (1989) yielded enhanced sensitivity over the original protocols (recently reviewed by Hornstra and Yang, 1993). A new method for genomic sequencing has recently been introduced by Frommer *et al.* (1992), where bisulphite is used to specifically deaminate cytosine (but not 5-methylcytosine) to uracil. The target sequence is then amplified by PCR and upon sequencing of the PCR product 5-methylcytosine is detected as a band in the C-ladder, whereas unmodified cytosine becomes detectable as thymine.

1.1.3 The use of methylase inhibitors to study the effects of DNA methylation *in vivo*

Inhibition of DNA methylation is a commonly used approach to investigate the effect of demethylation of a given gene and its implications for the biological role of DNA methylation. Although several agents have been described to inhibit DNA methylation *in vivo* and *in vitro* (reviewed by Adams and Burdon, 1985), the most widely used drugs are the base analogues 5-azacytidine and 5-azadeoxycytidine. On incorporation into DNA, 5-azacytosine brings about an inhibition of DNA methylation (Jones and Taylor, 1980). The triazine ring is not able to accept methyl groups, however, the extent of inhibition is much greater than the level of incorporation would lead one to expect (Adams *et al.*, 1982; Creusot *et al.*, 1982). In addition, heavily substituted DNA inhibits methylation of unsubstituted DNA present in *in vitro* methylase assays (Adams *et al.*, 1984). These findings suggest that DNA methyltransferase binds irreversibly to DNA containing 5-azacytosine. Thus, treatment of cultured cells with 5-azacytidine can be used to efficiently demethylate cellular DNA (Veniola *et al.*, 1982; Wolf and Migeon, 1985; Pfeifer *et al.*, 1990a; Sasaki *et al.*, 1992a). However, 5-azacytidine is a toxic agent at concentrations higher than 1 μM, and leads to a marked change in the metabolism and development of exposed cells (Jones, 1984). Therefore, the significance of data obtained from experiments involving this drug must be assessed critically (Bird, 1992).

1.2 The presence of 5-methylcytosine in DNA

1.2.1 Distribution of 5-methylcytosine

Distribution of the fifth base, 5-methylcytosine (5-mC), varies widely among eukaryotes, being (unfortunately) virtually absent from organisms such as

Saccharomyces cerevisiae (Proffitt *et al.*, 1984) and *Drosophila melanogaster* (Urieli-Shoval *et al.*, 1981, Pollack *et al.*, 1984), but reaching levels of over 30% of all cytosines in higher plants (Adams and Burdon, 1985). Levels in higher vertebrate DNA vary between 4 and 6% of all cytosines (Ehrlich *et al.*, 1982; Gama-Sosa *et al.*, 1983).

There seems to be a rough correlation between genome size and the degree of DNA methylation and it has been proposed that one role of DNA methylation in eukaryotes is the inactivation of untranscribed DNA. In invertebrates such as fungi (Rothnie *et al.*, 1991) or echinodermata (Bird *et al.*, 1979), only repetitive satellite sequences appear to be methylated. Some authors suggested models whereby DNA methylation serves as a signal to mark and diversify duplicated sequences and thereby protect them against recombination-mediated chromosome rearrangements, thus stabilizing the genome (Selker, 1990; Kricker *et al.*, 1992). Interestingly, satellite DNA in higher vertebrates is also enriched in 5-mC, where it can account for about 50% of all 5-methylcytosine in the mouse genome (Miller *et al.*, 1974).

However, as will be discussed later, DNA methylation is not restricted to these regions in vertebrate DNA and it seems that methylcytosine has evolved from being associated initially with repetitive DNA to become a component of non-repetitive DNA in vertebrates and higher plants. This evolutionary process has facilitated the involvement of DNA methylation in various biological functions which are discussed below.

1.2.2 The methylating tool: DNA methyltransferase

In principal, two ways can be thought of for generating methylated cytosines in DNA. The direct incorporation of 5-methyldeoxycytidine into the DNA during the replication process generates methylated DNA, and is in fact known to be the case for some bacteriophages (Adams and Burdon, 1985). However, the only known way to

generate 5-mC in eukaryotic DNA is by a post-synthetic, enzymic process, catalysed by the DNA methyltransferase (EC 2.1.1.37). The cDNA for the murine enzyme has recently been cloned (Bestor *et al.*, 1988) and the open reading frame indicates a primary translation product of M_r 170,000. However, on SDS-polyacrylamide gel electrophoresis several groups reported an apparent size of 190,000 (reviewed by Adams *et al.*, 1990), suggesting a possible post-translational modification of the enzyme. The enzyme uses S-adenosyl-L-methionine as the methyl group donor, and is thought to form an intermediate enzyme-substrate complex in which the pyrimidine is covalently bound to the cysteine of a conserved proline-cysteine dipeptide (Wu and Santi, 1987).

Current knowledge suggests the existence of only one type of DNA methyltransferase in mammals, being responsible for both *de novo* and maintenance methylation. Mammalian DNA methyltransferase is thought to act primarily as a maintenance methylase in methylating hemimethylated DNA, which is the product of semiconservative replication. This leads to the clonal inheritance of methylation patterns from one cell generation to the other (Holliday and Pugh, 1975, Stein *et al.*, 1982). In fact, Leonhardt *et al.* (1992) demonstrated that the enzyme is associated with replication foci during S phase, making coupling of replication and DNA methylation a likely scenario. *De novo* methylation is the ability of DNA methyltransferase to establish new methylation patterns on previously unmethylated DNA and is thought to occur primarily during early developmental stages and gametogenesis. The primary target site for mammalian DNA methylases is the cytosine in the dinucleotide CpG, although in plants methylation at CNG trinucleotides is observed (Gruenbaum *et al.*, 1981). The persistent speculation of the existence of more than one DNA methylase in plants has been confirmed for *Pisum sativum* (Pradhan and Adams, 1993).

In an experiment designed to help understand the importance of DNA methylation in mammals Li *et al.* (1992) reported the disruption of both alleles of the methylase gene in mice. The mutant embryos completed gastrulation but were stunted and developmentally retarded and died at midgestation. The inactivation of the gene

appeared to be incomplete, as about 30% of methyltransferase activity remained in the mutated mice. This finding shows that even a reduction of 60% of the methylcytosine content has a dramatic effect on the development of mice. The exact reasons for this effect are not known but an obvious hypothesis is the inappropriate activity of genes, which are normally repressed by DNA methylation.

Overexpression of the DNA methylase gene in mouse fibroblasts caused the tumorigenic transformation of the cells (Wu *et al.*, 1993), giving further evidence for the necessity of a balanced expression of the methylase gene in order to achieve normal growth and development.

1.3 CpG islands

As mentioned earlier, in vertebrate DNA, methylation is present only at the dinucleotide CpG, which can therefore be seen as a recognition sequence for the DNA methyltransferase. However, the sequence CpG occurs only at about 20% of the expected frequency in vertebrate DNA. Although an active repair mechanism exists which corrects G/T mismatches back to G/C at an efficiency of about 90% (Wiebauer and Jiricny, 1989; Neddermann and Jiricny, 1993), the replication of the unrepaired deamination product of 5-methylcytosine led to the CpG depletion of vertebrate DNA during evolution. That this is the cause of the CpG deficiency is reflected by the fact that the dimers TpG and CpA, which would be expected to arise after replication through a G/T mismatch, are overrepresented in higher eukaryotic DNA (Sved and Bird, 1990).

However, about 1% of vertebrate DNA escapes this CpG depletion and is referred to as CpG islands (Bird, 1986). CpG islands are about 0.5 to 3 kilobases long, lack CpG suppression and have a high G+C content of 55 to 70% (Antequera and Bird, 1993). They are further characterized by a lack of DNA methylation. All human CpG islands identified so far are associated with genes (Larsen *et al.*, 1992)

and, all housekeeping genes have been found to have a CpG island starting 5´ of the transcription unit and covering one or more exons. Genes known for tissue-specific expression are not usually associated with CpG islands. Some CpG islands, like those in the human apoA-I and apoA-IV genes (Shemer *et al.*, 1991a) or the human glucose-6-phosphate dehydrogenase gene (Wolf *et al.*, 1984), are located in the last exon or at the 3´end of the genes.

As mentioned earlier, CpG islands are unmethylated at all times in all tissues with one exception (second exception: section 1.7): on the inactive X chromosome. One of the two X chromosomes in eutherian females becomes inactivated at early developmental stages and is cytologically known as the Barr body. It is distinguished by its heterochromatinization, hypermethylation and lack of hyperacetylated histones (Tribioli *et al.*, 1992; Jeppesen and Turner, 1993). So far, all CpG islands on the inactive X chromosome show a strict correlation between transcriptional inactivity and DNA methylation (Tribioli *et al.*, 1992).

CpG island methylation can have dramatical medical implications. For example the fragile-X syndrome is a heritable form of mental retardation in males, which is associated with a specific breakage on the X chromosome (Bell *et al.*, 1991). A CpG island close to the breakage point was found to be hypermethylated and may play a role in the triggering of the chromosomal break (Pieretti *et al.*, 1991).

De novo methylation of CpG islands has also been observed in cell lines (Antequera *et al.*, 1990), and it was concluded that genes not essential for the growth of the cells, are inactivated by CpG island methylation. This epimutation (Holliday, 1987) leads to the inactivation of genes and can, in many cases, be reversed by treatment with 5-azacytidine, leading to demethylation of the CpG island (Gounari *et al.*, 1987). However, as the same islands are never methylated in the normal cells of the organism, the analysis of CpG island methylation and demethylation by 5-azacytidine appears not to be a reliable model for the understanding of methylation mediated gene regulation.

So, how do CpG islands remain methylation free? CpG islands are substrates for the DNA methyltransferase, as the methylation of the inactive X chromosome and CpG-rich satellite DNA (Macleod and Bird, 1984) proves. CpG islands which had been introduced into transgenic mice were found to stay methylation free, regardless of their expression (Kølsto *et al.*, 1986; Shemer *et al.*, 1990). At present, an active demethylation in embryonic cells is thought to be responsible for keeping islands methylation free. This hypothesis is based on the findings by Frank *et al.* (1991) who demonstrated that the 5´ CpG island of the *in vitro*-methylated adenine phosphoribosyltransferase gene became demethylated, whereas the 3´ end showed additional *de novo* methylation in transgenic mice. The demethylation was rapid and replication independent, implying an active, enzymatic activity. Jost (1993) demonstrated such an activity in the extracts of differentiating chicken embryos, but it remains open to what extent DNA demethylation contributes to keep CpG islands "methylation-free zones".

1.4 DNA methylation and transcriptional activity

It is now a well established fact that DNA methylation inhibits the transcription of many tissue-specific and housekeeping genes (reviewed by Doerfler, 1983; Razin and Cedar, 1991). However, to date, no clear answer can be given to the how and why of this inactivation process. There seem to be at least two ways by which DNA methylation can suppress transcription.

1.4.1 DNA methylation prevents binding of transcription factors

An obvious mechanism of action of DNA methylation is that the physical presence of a methyl group on a binding site for a transcription factor prevents factor

association and thereby inhibits transcription. Several transcription factors have been found which do not bind to their methylated target sites (recently reviewed by Tate and Bird, 1993), among them the cAMP responsive element binding protein (Iguchi-Ariga and Schaffner, 1989), the activating protein 2 (Comb and Goodman, 1990), c-Myc/Myn (Prendergast *et al.*,1991) or NF-κB (Bednarik *et al.*, 1991). For most of these cases the authors could establish a direct correlation between DNA methylation, lack of factor binding and transcriptional repression. However, most of the binding studies rely on *in vitro* systems. In the case of the rat tyrosine aminotransferase (TAT) gene Becker *et al.* (1987) demonstrated that the TAT promoter shows binding of ubiquitous factors *in vivo* and is unmethylated in expressing cells, whereas in non-expressing cells lacking *in vivo* footprints methylation was observed in the promoter region. The conclusion that methylation of the promoter is responsible for the lack of factor binding was not confirmed in a subsequent study by Weih *et al.* (1991), as 5-azacytidine-induced demethylation in non-expressing cells did not result in factor binding. Other transcription factors like Sp1 (Harrington *et al.*, 1988; Höller *et al.*, 1988; Bryans *et al.*, 1992) or CTF (Ben Hattar *et al.*, 1989) are insensitive to the presence of methyl CpG in their target sites, but transcription from their promoters can be inhibited by DNA methylation (Bryans *et al.*, 1992; Ben Hattar *et al.*, 1989). Certainly, failure of transcription factor binding due to DNA methylation in their target site is not the only mechanism of methylation-mediated gene repression.

1.4.2 Specific binding of proteins to methylated DNA

The alternative scenario of methylation-induced gene repression is the specific binding of factors to methylated DNA which, in turn, prevents binding of transcription factors, thus acting as repressors. Several such factors have been identified (Huang *et al.*, 1984; Meehan *et al.*, 1989; Lewis *et al.*, 1992; Jost and Hofsteenge, 1992) and are referred to as methylated DNA binding proteins (MDBPs) or methylated cytosine

binding proteins (MeCPs). MDBP-2 requires a length of 30 nucleotides with a single methylated CpG for binding and effectively represses transcription *in vitro* (Pawlak *et al.*, 1991). In addition, it shares sequence homologies with histone H1 (Jost and Hofsteenge, 1992). MeCP1 is known to repress expression of methylated genes *in vitro* and *in vivo*. and binds to a minimum of 12 methylated, closely linked CpGs (Boyes and Bird, 1991; Boyes and Bird, 1992). MeCP2 requires only one methylated cytosine in the dinucleotide CpG for binding and is associated with heterochromatin (Lewis *et al.*, 1992). However, MeCP2 does not preferentially inhibit transcription from methylated genes *in vitro* (Meehan *et al.*, 1992). Bird and coworkers suggested a model for the possible roles of MeCP1 and MeCP2, whereby MeCP1 competes with transcription factors for binding to the methylated DNA and guides it into a heterochromatic structure, which is subsequently maintained by association of MeCP2 (Meehan *et al.*, 1992). Attractive as it is, this model can only be tested once purified MeCP1 becomes available. However, it can be assumed that more proteins exist which bind specifically to methylated DNA and some of them may play a similar role in transcriptional repression of methylated DNA.

1.5 DNA methylation and chromatin structure

Transcriptionally active (open) chromatin is known to be more sensitive to nuclease digestion than non-transcribed (closed) chromatin (reviewed by Gross and Garrard, 1988) and it is generally believed that open chromatin structure is required for transcriptional initiation (Adams and Workman, 1993). There is growing evidence that methylation of DNA is accompanied by a change in chromatin structure. Razin and Cedar (1977) demonstrated, by digestion of nuclei with micrococcal nuclease and subsequent analysis of the released DNA by mass spectrometry, that when 50% of the DNA was solubilized, over 75% of the 5-methylcytosine content of the DNA remained in the unsolubilized fraction. This indicates that 5-mC is nonrandomly distributed, but

enriched in chromatin that is relatively resistant to digestion by nucleases. In subsequent studies by Solage and Cedar (1978) and Adams *et al.* (1984) it was demonstrated that the DNA solubilized at early times of digestion had a reduced 5-methylcytosine content, indicating that linker DNA is hypomethylated compared to nucleosomal core DNA. Keshet *et al.* (1986) were among the first to provide direct evidence that DNA methylation has an effect on chromatin formation. They used methylated constructs which were integrated into the genome of L cells and observed a DNaseI-insensitive chromatin structure, characteristic for inactive (closed) chromatin. Nuclease insensitivity has been observed for a variety of methylated genes (Antequera *et al.*, 1989; Levine *et al.*, 1991; Sasaki *et al.*, 1992a) and it has been reported that this closed chromatin formation is associated with transcriptional silencing. This is confirmed by studies on the inactive X chromosome where CpG island methylation is concordant with nuclease resistance and inactivation of the respective genes (Wolf *et al.*, 1984; Hansen *et al.*, 1988).

Furthermore, Buschhausen *et al.*, (1987) used reporter gene constructs for microinjection into rat TK^- cells and observed a sharp drop in expression levels of the methylated constructs only 8 hours after injection, which coincided with the assembly of the DNA into chromatin. In addition, when constructs were assembled into chromatin *in vitro* prior to injection, gene inactivation of the methylated construct was immediate. In experiments with the γ-globin gene Busslinger *et al.* (1983) showed that *in vitro* methylation of the promoter region abolished expression of the gene after transfection into cells. The inhibition was not dependent on a specific methylated cytosine in the promoter, but the presence of three 5-mC was sufficient to repress transcription (Murray and Grosveld, 1987), pointing to an indirect inactivation mechanism.

What are the differences between "methylated" and "unmethylated" chromatin? So far, not many reports have dealt with the special nature of chromatin assembled on methylated DNA. In an early study by Felsenfeld *et al.* (1983) it was reported that a methylated CpG polymer has a twofold greater affinity for histones than a

unmethylated one, but assembly of histones into core particles on the methylated substrate was indistinguishable from assembly on the unmethylated CpG polymer. However, the choice of substrate might not have been an ideal one as methylated CpG polymer DNA does not represent a natural sequence and can easily undergo transition from B-DNA to Z-DNA (Behe and Felsenfeld, 1981). Buschhausen *et al.* (1987) used reporter gene constructs for chromatin assembly experiments and could not observe significant differences between chromatin assembled on methylated and unmethylated plasmid DNA, as tested by electron microscopy and micrococcal nuclease digestion. In a recent study by Englander *et al.* (1993) histone octamers and tetramers were assembled, *in vitro,* on differentially methylated Alu element templates. Interestingly, assembly of a histone (H3/H4)-tetramers on the methylated template resulted in new DNaseI hypersensitive sites compared to the unmethylated control, whereas naked DNA and octamer assembled DNA yielded only subtle differences for the differentially methylated templates. Furthermore, tetramer assembly preferentially inhibited the methylated template in *in vitro* transcription assays, whereas octamer assembly was equally efficient in repression of *in vitro* transcription for both, methylated and unmethylated templates. The significance of these results may be that transcription activators compete with the H2A and H2B dimers for binding to the histone H3/H4 tetramer, and that methylation of the DNA associated with tetramers favours the completion of the histone octamer, thus blocking transcription. Certainly, the recent availability of improved *in vitro* chromatin assembly systems (Workman *et al.*, 1991; Wolffe and Schild, 1991) will greatly enhance our understanding of "methylated" chromatin.

Histone H1 has long been the prime candidate to account for differences between chromatin of methylated and unmethylated DNA. In an early study by Ball *et al.* (1983) it was demonstrated that at least 80% of 5-methylcytosine is localised in nucleosomes which contain histone H1, which is a characteristic of inactive chromatin (Weintraub, 1985). In addition, in CpG island chromatin (containing DNA which is unmethylated), histone H1 was much less abundant (about 10%) than in other

chromatin (Tazi and Bird, 1990). In an interesting study by Higurashi and Cole (1991) it was found that when DNA was associated with histone H1 *in vitro*, digestion with *Msp*I, but not with other restriction enzymes was specifically inhibited on the methylated DNA substrate. In addition, they could show that histone H1 has no higher affinity for methylated than for unmethylated DNA and concluded that binding of H1 to methylated DNA changes DNA conformation, thus rendering the DNA resistant to digestion by *Msp*I. In contrast, Levine *et al.* (1993) did observe preferential binding of histone H1 to methylated DNA, thus supporting the data from Jost and Hofsteenge (1992), who reported that a methylated-DNA binding protein, MDBP-2, shares sequence homologies with histone H1. Furthermore they demonstrated, for the first time, that histone H1 is not only inhibiting transcription from unmethylated DNA (Croston *et al.*, 1991), but is an even more efficient repressor for methylated templates. The conflicting data about the role of histone H1 as a methylated-DNA binding protein reflects the different methods used by the authors to purify H1, to assemble it on DNA and to assay its binding capabilities (Clark and Thomas, 1986, 1988). Certainly, *in vitro* systems to study structural and functional aspects of histone-methylated DNA interactions are not ideal. However, as DNA methylation studies cannot take advantage of elegant yeast systems (Svaren and Hörz, 1993), they appear to be the most appropriate way to unravel the nature of the special features of chromatin assembled on methylated DNA.

1.6 The inactive X chromosome

X chromosome inactivation is a phenomenon unique to mammals and ensures that females are equivalent to XY males with respect to X chromosome dosage. Soon after fertilization both X chromosomes in the female mammalian embryo are active but in early embryogenesis (about 6.5 days *post coitum* in mice, McMahon and Monk, (1983)) one of the X chromosomes undergoes inactivation by heterochromatinization.

The inactive X chromosome (X^i) is cytologically recognizable in metaphase spreads of female chromosomes as the densely staining Barr body. The inactivation process is thought to occur in three stages: initiation of inactivation, the spreading of inactivation along the length of the chromosome and the maintenance of the inactive state of the chromosome. A role for DNA methylation is possible for all three of these processes. However, most data so far give evidence for involvement of DNA methylation in the maintenance of X chromosome inactivation.

Apart from forming facultative heterochromatin, the X^i replicates late during S phase and its CpG islands are hypermethylated. There is evidence that DNA methylation may play a role in the replication timing of the X^i. In studies where demethylation of the X^i was induced by the methylase inhibitor 5-azacytidine, it was shown that changes in DNA methylation correlate with changes in replication timing (Gregory *et al.*, 1985; Jablonka *et al.*, 1985). This phenomenon is not only confined to the X^i but was also observed for the mouse major satellite DNA, where demethylation causes a dramatic shift of the satellite replication time from very late to the middle of the S phase (Selig *et al.*, 1988). The temporal separation of replication of hypermethylated, heterochromatinized DNA could mean the exposure to a different cellular environment and thus could be involved in the maintenance of the differential methylation and chromatin structure.

Several studies linked methylation of the CpG islands on the X^i with transcriptional repression and nuclease insensitivity. In the case of the human glucose-6-phosphate dehydrogenase gene (*g6pd*) it was shown that transcription from the active X chromosome (X^a) is correlated with nuclease hypersensitivity of the unmethylated CpG islands 3′ of the gene. Furthermore, reactivation of the *g6pd* gene on the X^i by treatment with the demethylating agent 5-azacytidine revealed the appearance of nuclease sensitivity in the CpG island chromatin (Wolf *et al.*, 1984; Wolf and Migeon, 1985; Toniolo *et al.*, 1988). Similar observations were made for the hypoxanthine-guanine phosphoribosyltransferase (*hprt*) genes (Lock *et al.*, 1986; Sasaki *et al.*, 1992a). In more detailed studies by Pfeifer and colleagues on the human

X linked phosphoglycerate kinase (*pgk*) gene it was demonstrated that the 5´ CpG island on the X^a was completely unmethylated, free of nucleosomes and showed footprints for putative transcription factors, whereas the same sequence on the X^i was found to be hypermethylated, wrapped around two nucleosomes and did not reveal any transcription factor binding in genomic footprints (Pfeifer *et al.*, 1990a; Pfeifer *et al.*, 1990b; Pfeifer and Riggs, 1991; Pfeifer *et al.*, 1991; Pfeifer *et al.*, 1992).

Although an X chromosome inactivation centre has been identified and mapped at Xq13 (Brown *et al.*, 1991) the mechanisms of initiation and spreading of X chromosome inactivation remain unknown. It is known that DNA methylation can spread from sequences of viral integration into the genome (Toth *et al.*, 1989; Toth *et al.*, 1990; Orend *et al.*, 1991) and it might be conceivable that the gradual inactivation of the X chromosome is related to the spreading of DNA methylation. However, the role of DNA methylation as the initial signal for the spreading of inactivation is disputed by Lock *et al.* (1987). They studied the involvement of DNA methylation on the inactivation of the X-linked *hprt* gene and found the 5´ CpG island unmethylated prior to and several days after X inactivation. This suggests that DNA methylation may not play a role in the primary events of X chromosome inactivation, but may be required to efficiently maintain the repressed state.

1.7 Genomic imprinting

Recent studies have revealed that some autosomal loci in the developing mouse embryo are expressed in a parental-specific way, such that one locus is expressed and the other is silent, although both parental loci are genetically identical (Monk and Surani, 1990). It seems that, these loci somehow "remember" their parental origin and are regulated according to that memory. This phenomenon is known as genomic imprinting and describes the differential transcription of maternal and paternal chromosomes imposed by an epigenetic process.

Genomic imprinting has also been observed in humans and is of considerable medical importance. For example, the Prader-Willi syndrom, a form of mental retardation, always results from a mutation of the paternally inherited chromosome, whereas the Angelman syndrome is caused by a deletion on the maternal chromosome (Magenis *et al.*, 1990).

Although little is known about the molecular basis of the imprint, four characteristics have been suggested to be essential for the mechanism. First, the primary imprint must be established during gametogenesis as this is the only stage during which male and female genomes are separated and can be differentially imprinted. Second, the imprint must be clonally inherited from one cell generation to the other, thus, the imprint requires to be reimposed following DNA replication. Third, the mechanism must be reversible as reprogramming should be possible after passage through the germ line of the opposite sex. Finally, the imprint must affect the expression of the gene (in one way or the other) to account for parental-specific expression of the locus. DNA methylation fulfils all the requirements mentioned above to serve as the epigenetic signal in the process of genomic imprinting, and a link between methylation and genomic imprinting has been suggested for some time (Monk *et al.*, 1987; Surani *et al.*, 1990).

First evidence for an involvement of DNA methylation came from the differential methylation observed in X chromosome inactivation. Takagi and Sasaki (1975) reported the preferential inactivation of the paternal X chromosome in extraembryonic tissues of developing female (of course!) mice and, interestingly, the paternally derived X chromosome in female marsupials is the preferred substrate for X chromosome inactivation (VandeBerg *et al.*, 1987).

The use of transgenic mice allowed detailed analyses of autosomal genomic imprinting and revealed the proposed switch of the methylation pattern in successive generations, depending on the gamete of origin (Sapienza *et al.*, 1987; Reik *et al.*, 1987). These differentially imprinted transgenes can be expressed in a mosaic fashion, such that some cells express the transgene whereas others do not (McGowan *et al.*,

1989; Allen *et al.*, 1990). The imprinting effect can be lost after successive generations, depending on the genetic background of the mouse strain (Reik *et al.*, 1990). These effects are mediated by modifier genes which act on the DNA of the gamete after fertilization (Sapienza *et al.*, 1989; Engler *et al.*, 1991).

With the isolation of endogenous mouse genes which show genomic imprinting it has become possible to directly tackle the molecular mechanisms responsible for the imprinting process. The insulin-like growth factor II (*Igf2*) gene was found to be expressed only from the paternally inherited chromosome (DeChiara *et al.*, 1991). However, the CpG island associated with the promoter is unmethylated and shows DNaseI hypersensitive sites in both parental alleles (Sasaki *et al.*, 1992b). The *H19* gene is another imprinted gene which resides on chromosome 7, being only 90 kb away from the *Igf2* gene on the same chromosome (Bartolomei *et al.*, 1991). Interestingly, it is reciprocally imprinted, being active on the maternal and inactive on the paternal chromosome (Ferguson-Smith *et al.*, 1991). The methylation status of the *H19* gene was analysed and it was found that its 5´ CpG island was only methylated on the paternal allele, which was paralled by the formation of nuclease insensitive chromatin (Ferguson-Smith *et al.*, 1993). Methylation, however, appeared not be the primary imprinting signal as the gene was unmethylated in sperm and became *de novo* methylated after fertilization. The speculation that *H19* imprinting and *Igf2* imprinting are functionally or mechanistically correlated is not "contradicted" by the fact that the function of *H19* is yet unknown.

The third gene known to undergo genomic imprinting is the mouse insulin-like growth factor II receptor (*Igf2r*) gene, which is exclusively expressed from the maternally inherited chromosome (Barlow *et al.*, 1991). Two CpG islands have been identified and their methylation status analysed (Stöger *et al.*, 1993). Region 1 covers the promoter and is only methylated on the paternal, silent chromosome. Region 2 is located within the first intron and, surprisingly, is methylated on the active, maternal chromosome, but not on the inactive paternal. Methylation of region 2 is inherited

from the female gamete, and thus, might act as the imprinting signal, possibly by acting as a silencer and binding repressor proteins when unmethylated.

In a detailed study by Brandeis *et al.* (1993), on all three imprinted genes mentioned above, it was demonstrated that parental specific methylation is established in a progressive manner during embryogenesis. Only a few modifications at distinct sites are carried through from the gamete and may be candidates for maintaining the parental identity during development. Li *et al.* (1993) analysed the methylation status and expression of the three imprinted genes in mice deficient in DNA methyltransferase activity (Li *et al.*, 1992). They observed activation of the normally inactive paternal allele of the *H19* gene, whereas both the normally active paternal allele of the *Igf2* gene and the normally active maternal allele of the *Igf2r* gene were repressed. The significance of these novel findings, as they stand now, is difficult to assess, although the results reported by Li *et al.* (1993) provide evidence for an involvement of DNA methylation in the monoallelic expression of imprinted genes. However, the role of DNA methylation in the implementation of a genomic imprint will hopefully become clearer with the identification of more imprinted genes.

1.8 DNA methylation during differentiation and development

Distinct changes in the degree of DNA methylation have been observed during early mammalian development and it was proposed that a basic methylation profile is set up at early stages of embryogenesis (Razin and Riggs, 1980).

Specific genes in sperm DNA of mouse have been shown to be fully methylated, although the overall level of methylation of sperm DNA is relatively low (Yisraeli and Szyf, 1984; Groudine and Conkin, 1985). This can be attributed to the fact that methylation of the major mouse satellite DNA is relatively low compared to its somatic levels (Sanford *et al.*, 1984; Ponzetto-Zimmermann and Wolgemuth, 1984). In contrast, genes in the oocyte seem to be undermethylated in comparison to

sperm DNA (Monk *et al.*, 1987; Driscoll and Migeon, 1990). Kafri *et al.* (1992) confirmed the initial findings of differential methylation in germ cells by showing that some loci are unmethylated in the oocyte despite their full methylation in sperm.

In the early embryo, the pattern of methylation inherited from the parental germ cells is rapidly lost resulting in undermethylation of both extraembryonic and embryonic tissues at the preimplantation stage of the embryo (Howlett and Reik, 1991; Shemer *et al.*, 1991b; Kafri *et al.*, 1992). Interestingly, this is paralleled by a decrease in methylase activity (Monk *et al.*, 1991). Although the significance of this demethylation is unknown, it might be a necessary process prior to the establishment of the somatic methylation patterns.

Jähner and Jaenisch (1984) demonstrated the ability of implantation embryos to *de novo* methylate integrated retrovirus sequences, but *de novo* methylation was restricted to a narrow time window not extending into postimplantation stages. It is now clear that this *de novo* methylation is the first step in the establishment of gene specific methylation patterns (Sanford *et al.*, 1987; Kafri *et al.*, 1992), whereby the hypomethylated DNA undergoes methylation just prior to gastrulation. However, this *de novo* methylation is selective; CpG island DNA remains unmodified, whereas non-island DNA becomes hypermethylated. Whether CpG island DNA is protected from methylation or becomes demethylated immediately after *de novo* methylation is not clear, but recent evidence suggests the presence of an active demethylation mechanism (Frank *et al.*, 1991; Shemer *et al.*, 1991a; Jost, 1993). The second step in establishing the final methylation pattern is the gene specific and tissue specific demethylation during late embryonic development. Yisraeli *et al.* (1986) demonstrated that an *in vitro* methylated α actin construct undergoes demethylation and activation in myoblasts, but not in fibroblasts, thus indicating that the muscle-like cells have the ability to recognize and activate the tissue specific gene by demethylation. The demethylation appeared to be dependent on cis-acting sequences and was independent of replication (Paroush *et al.*, 1990).

The establishment of tissue-specific methylation patterns seems to develop by waves of demethylation and methylation at early embryonic stages, followed by the specific demethylation of gene sequences at later stages. This complicated process is not fully understood, but may have a function in implementing the activity status of specific genes in a defined developmental stage and thereby making this specific activity pattern stable and heritable for many cell generations.

1.9 Aims of the project

As described above, DNA methylation plays a role in many fundamental biological processes, most of which centre around the vital question of how DNA methylation exerts its effects on gene expression. Evidence has been gathered for three different mechanisms of transcriptional inactivation by DNA methylation. (a) The presence of methyl groups in the promoter region can inhibit transcription factor binding and thus reduces transcriptional activation. (b) Proteins which bind specifically to methylated DNA can act as repressors, restricting access of the transcriptional machinery to promoter regions. (c) the presence of methyl groups in a gene sequence results in the formation of an inactive chromatin structure, rendering the gene inaccessible for transcription.

Not all promoters are inhibited by DNA methylation and the SV40 early promoter presents the best investigated example of a methylation-insensitive promoter. The SV40 promoter is regarded as a model for a housekeeping promoter and is not normally methylated *in vivo*. Figure 1 shows the organisation of the promoter and its position in plasmid pVHCk. The promoter consists of two perfect 21 bp repeats and a degenerate 22 bp repeat. Each of these repeats contain two hexanucleotide elements known as GC boxes (Fig. 1), which are crucial for both late and early transcription (Benoist and Chambon, 1981; Gidoni *et al.*, 1984). Studies on these sequences revealed that they interact with a transcription factor known as Sp1 (Kadonaga and

Tjian, 1986). Sp1, a zinc finger protein (Kadonaga *et al.*, 1987), is an ubiquitous factor and is involved in the regulation of a number of cellular or viral genes (Dynan *et al.*, 1986; Jones and Tjian, 1985; Lee *et al.*, 1987). The SV40 enhancer sequence (Fig. 1) has been extensively studied and several proteins have been identified which bind to the enhancer elements, the best characterized being the activator proteins (AP) 1, 2, 3, and 4 (Mermod *et al.*, 1988; Mercurio and Karin, 1989). Tjian *et al.* (1987) proposed that the interplay between these factors and those that interact with the GC boxes results in the high levels of early and late transcription in SV40.

With regard to DNA methylation, the promoter contains 9 CpGs, one of them in each of the 6 GC boxes and the remaining three positioned between the TATA box and the transcription start sites. The 72 bp repeat enhancer sequence is devoid of CpG sites (Fig. 1). SV40 DNA is CpG-deficient, containing 27 methylatable sites in 5243 bp of DNA. Early transcription from SV40 is not inhibited by methylation of the single *Hpa*II site (Fradin *et al.*, 1982) or by complete methylation of all 27 CpG pairs (Grässmann *et al.*, 1983). In addition, several groups could show that binding of transcription factor Sp1 is not affected by DNA methylation (Harrington *et al.*, 1988; Höller *et al.*, 1988; Bryans *et al* , 1992). Thus, transcription from the SV40 early promoter in its natural, CpG-deficient environment is not affected by DNA methylation.

Before defining the aims of this project it is necessary to summarize the findings of Bryans (1989), who initially described in detail the effects of DNA methylation on transcription from the SV40 early promoter in transient transfection assays. A plasmid containing the SV40 early promoter linked to the chloramphenicol acetyl transferase (CAT) gene was methylated *in vitro* with a murine methylase preparation. 20-30% of all CpGs were methylated, but in transient expression assays CAT activity of these methylated plasmids was only 20-30% of the unmethylated control. At this level of methylation only 1-2 of the 9 CpGs in the promoter region will be methylated. Thus, it seems unlikely that inhibition of transcription was mediated by promoter methylation. Further support for this hypothesis came from cotransfection

experiments, where oligonucleotides, containing GC box motifs inhibited transcription regardless of their methylation status. These findings indicated that the SV40 promoter is methylation sensitive in a methylcytosine rich environment and it was speculated that methylation of the CpG rich vector sequences can drive the whole plasmid into an inactive chromatin structure, thus inhibiting transcription from an almost unmethylated promoter.

From these findings the objectives for this study were defined as follows:

- to establish the effect of complete methylation of the reporter gene construct on transient expression.

- to develop a method for regional methylation of plasmid molecules and to test the effect of regional methylation of the reporter gene construct on transient expression.

- to reveal possible differences in chromatin structure of methylated and unmethylated reporter gene constructs after transfection into cells.

- to test the effect of DNA methylation on the transcription of the reporter gene construct *in vitro*.

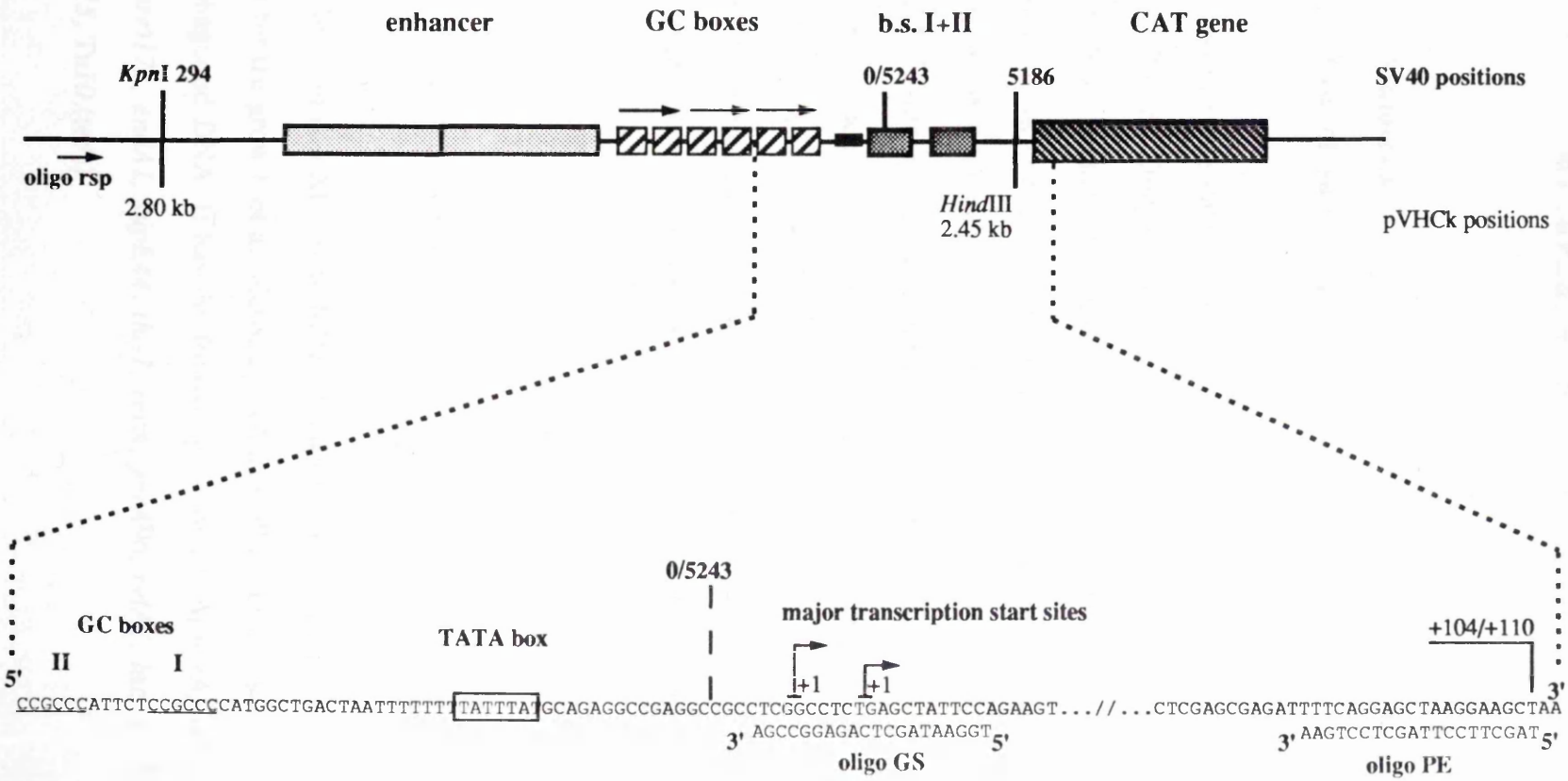


Figure 1 Map of the SV40 early promoter

The SV40 early promoter and its functional elements are shown including its position in plasmid pVHCk. b.s. I+II: binding sites for T antigen; oligo rsp: annealing site for the reverse sequencing primer; oligo GS: primer used for PCR amplification of the SV40 promoter (section 6.2); oligo PE: primer used for primer extension assay (section 7.2).

CHAPTER TWO

Materials and methods

2.1 Materials

2.1.1 List of suppliers

Unless otherwise stated all the chemicals were Analar grade, supplied by BDH Chemicals, Poole, Dorset, or Fisons Scientific, Loughborough, Leics. Radiochemicals were purchased from Amersham International plc, Aylesbury, Bucks. Growth media for bacteria were supplied by Difco Laboratories, Detroit, USA, and for the cultivation of mammalian cells by GIBCO/BRL Ltd., Paisley, Scotland. Prokaryotic DNA methylases were purchased from New England Biolabs, Beverly, MA, USA. Restriction enzymes and DNA modifying enzymes were obtained from Promega Ltd., Southampton, Pharmacia Ltd., Milton Keynes and Boehringer Mannheim Ltd., Lewes, East Sussex, if not otherwise specified. Where special chemicals, reagents or equipment were obtained from other sources, this is indicated in the text.

2.1.2 Bacterial strain

Escherichia coli XL1-Blue MRF' (Stratagene Ltd., Cambridge) was the host strain used for the growth of all plasmid DNA as well as for the isolation of single-stranded phagemid DNA. It has the following genotype: *Δ(mcrA)183, Δ(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA, gyrA96, relA1, lac,* λ-, (F', *proAB, lacI*^{q}*ZΔM15,* Tn*10,*(tet^{r})).

2.1.3 Mammalian cell lines

L929 cells (Sanford *et al.*, 1948) are mouse fibroblast cells and were used in transient expression assays.

HeLa S3 cells (Gey *et al.*, 1952), a human, cervical carcinoma cell line, were used for the preparation of nuclear extracts for *in vitro* transcription assays.

COS-1 cells are simian CV-1 cells which were transformed with an origin-defective SV40 genome (Gluzman, 1981). These cells constitutively express wild-type SV40 T antigen and drive the replication of SV40 origin-containing plasmids. They were used in transient transfection assays.

2.1.4 Bacterial growth media

LB medium	10 g bactotryptone
	10 g NaCl
	5 g yeast extract
	made up to 1 litre with dH_2O.
2x TY medium	16 g bactotryptone
	5 g NaCl
	10 g yeast extract
	made up to 1 litre with dH_2O.

The media were supplemented with 15 g bactoagar/litre for growth on plates. Media were sterilised by autoclaving for 20 minutes at 15 psi. Antibiotics were added as indicated in Methods.

2.1.5 Cell culture media

EC10 medium for L929 cells was prepared by mixing 450 ml dH_2O , 50 ml 10x Glasgow modified Eagle's minimal essential medium (GMEM), 50 ml newborn calf serum, 20 ml of 7.5% sodium bicarbonate and 5 ml of 200 mM L-glutamine.

HeLa S3 cells were grown in Eagle's minimum essential medium (EMEM) which contained 450 ml dH_2O, 50 ml 10x EMEM, 50 ml newborn calf serum, 30 ml of 7.5% sodium bicarbonate, 5 ml of 200 mM L-glutamine and 5 ml non essential amino acids.

COS-1 cells were grown in Dulbecco's minimum essential medium (DMEM), containing 450 ml dH_2O, 50 ml 10x DMEM, 50 ml foetal calf serum, 30 ml of 7.5% sodium bicarbonate, 5 ml of 200 mM L-glutamine, 5 ml non essential amino acids and 5 ml of penicillin/streptomycin (10,000 units/ml and 10,000 µg/ml, respectively).

All components were obtained sterile and freshly made media were checked for contamination 2-3 days before use.

A stock solution of versene (diaminoethanetetraacetic acid) 0.2 g/l was made up in PBS A containing 1% phenyl red and autoclaved before use.

2.1.6 DNA vectors

Plasmid pVHC1 is a pUC 8 derivative which contains the SV40 early promoter/enhancer region linked to the CAT reporter gene and the SV40 terminator region. Its construction is described in Bryans *et al.* (1992).

All other constructs are based on pBluescript II KS- (Stratagene, Cambridge), which contains an f1 origin of replication and hence allows the isolation of single-stranded DNA.

2.1.7 Synthetic oligonucleotides

Synthetic oligonucleotides for PCR or primer extension assays were made in the Department of Biochemistry by Dr. V. Math, using an Applied Biosystems 381A DNA synthesiser for the phosphite-triester method.

2.1.8 Commonly used buffers and solutions

Phosphate buffered saline (PBS)

Buffer A (pH 7.2)

NaCl	0.17 M
KCl	3.35 mM
Na_2HPO_4	10 mM
KH_2PO_4	1.84 mM

Buffer B

$CaCl_2 \cdot 6H_2O$	6.8 mM

Buffer C

$MgCl_2 \cdot 6H_2O$	4.9 mM

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

TE buffer

Tris-HCl (pH 7.9)	10 mM
EDTA	1 mM

TAE buffer, 50x

Tris	242 g
glacial acetic acid	57.1 ml
EDTA (0.5 M, pH 8.0)	100 ml

Amounts per litre of 50x TAE.

1x: 40 mM Tris-acetate (pH 7.9), 1 mM EDTA.

TBE buffer (pH 8.3)

Tris	89 mM
Boric acid	89 mM
EDTA	2 mM

SSC buffer, 20x

NaCl	3 M
Na-citrate	0.3 M

pH 7.5 with HCl

TEN buffer

Tris-HCl (pH 7.8)	20 mM
EDTA	10 mM
NaCl	12 mM

HBS buffer, 2x

HEPES-KOH (pH 7.12)	50 mM
NaCl	280 mM
Na_2HPO_4	1.5 mM
$NaH_2PO_4 \cdot 2H_2O$	1.5 mM

FSB buffer

K-acetate	10 mM
$MnCl_2 \cdot 4H_2O$	45 mM
$CaCl_2 \cdot 2H_2O$	10 mM
KCl	100 mM
$(Co[NH_3]_6)Cl_3$	3 mM
glycerol	10%

Buffer M+

Tris-HCl (pH 7.8)	50 mM
EDTA	1 mM
NaN_3	0.02%
DTT	1 mM
glycerol	10%
PMSF	0.06%

50x Denhardt's reagent

Ficoll (Type 400, Pharmacia)	1%
BSA (fraction V, Sigma)	1%
polyvinylpyrrolidone	1%

Hybridization buffer

SSC	5x
Denhardt's	5x
SDS	0.5%

RSB buffer

Tris-HCl (pH 7.4)	10 mM
NaCl	50 mM
$MgCl_2$	3 mM

oligonucleotide elution buffer

NH_4-acetate	0.5 M
Mg-acetate	10 mM
EDTA	1 mM
SDS	0.1%

***Taq* DNA polymerase buffer**

KCl	50 mM
Tris-HCl (pH 9.0 at 25°C)	10 mM
Triton X-100	0.1%

10x T4 polynucleotide kinase buffer

Tris-HCl (pH 7.6)	0.5 M
$MgCl_2$	0.1 M
DTT	50 mM
spermidine	1 mM
EDTA	1 mM

10x S1 nuclease buffer

Na-acetate (pH 4.5)	5 M
NaCl	2 M
$ZnSO_4$	10 mM
glycerol	5%

10x T4 ligase buffer

Tris-HCl (pH 7.4)	200 mM
$MgCl_2$	50 mM
DTT	50 mM
BSA	500µg/ml

10x CIP buffer

Tris-HCl (pH 8.4)	500 mM
$MgCl_2$	10 mM
$ZnCl_2$	10 mM
spermidine	10 mM

10x annealing buffer

Tris-HCl (pH 7.5)	200 mM
$MgCl_2$	100 mM
NaCl	500 mM
DTT	10 mM

10x Klenow buffer

Tris-HCl (pH 7.6)	500 mM
$MgCl_2$	100 mM
DTT	1 mM

Solutions for plasmid preparations

Solution I:	glucose	50 mM
	Tris-HCl (pH 8.0)	25 mM
	EDTA	10 mM

Solution II:	NaOH	0.2 M
	SDS	1%
Solution III:	K-acetate	60 ml
	glacial acetic acid	11.5 ml
	dH_2O	28.5 ml

DMS buffer

sodium cacodylate (pH 7.0)	50 mM
EDTA	1 mM

DMS stop solution

sodium acetate (pH 6.5)	1.5 M
β-mercaptoethanol	1 M
yeast tRNA	250 µg/ml

Hydrazine stop solution

sodium acetate (pH 6.5)	0.3 M
EDTA	0.1 mM
yeast tRNA	100 µg/ml

Sequencing gel loading buffer

formamide (deionized)	98%
EDTA	10 mM
xylene cyanol FF	0.025%
bromphenol blue	0.025%

2.1.9 DNA size markers

The following DNA standards were used as size markers for the analysis of nucleic acids via gel electrophoresis.

Bacteriophage λ DNA, digested with *Hind*III (λ^H): 23.130, 9.416, 6.557, 4.361, 2.322, 2.027, 0.564, 0.125 (size in kb).

Bacteriophage λ DNA, digested with *Pst*I (λ^P): 14.057, 11.501, 5.077, 4.749, 4.507, 2.838, 2.556, 2.459, 2.445, 2.170, 1.986, 1.700, 1.159, 1.093, 0.805, 0.514, 0.468, 0.448, 0.339, 0.264, 0.247, 0.216, 0.211, 0.200, 0.164, 0.150 (size in kb).

Bacteriophage ϕx174, digested with *Hinf*I: 726, 713, 553, 500, 427, 417, 413, 311, 249, 200, 151, 140, 118, 100, 81, 66, 48, 42, 40, 24 (size in bp).

2.2 Methods

2.2.1 General methods used in molecular biology

The following methods are standard procedures in molecular biology and essentially follow protocols described by Sambrook *et al.* (1989), adapted to the equipment available in the lab.

2.2.1.1 Phenol/chloroform extraction

Phenol, equilibrated with TE buffer and containing 8-hydroxyquinoline at 0.1% was mixed with chloroform/isoamylalcohol (24:1) at a ratio of 1:1. Extraction of an aqueous DNA solution was performed as follows: an equal volume of phenol/chloroform was added to the DNA solution, vortexed and centrifuged for 5 min

in a microfuge; the upper aqueous phase was transferred to a fresh tube and the extraction repeated if necessary.

2.2.1.2 Ethanol precipitation

A salt solution was added to the DNA solution (either Na-acetate to 0.3 M or NaCl to 0.2 M) and the DNA precipitated with 2.5 volumes of ethanol (98%) at -70°C for 15 min. The DNA was recovered by centrifugation at 10,000 rpm for 15 min at 4°C. Subsequent washes with cold 70% ethanol removed any salt which was coprecipitated with the DNA.

2.2.1.3 Quantitation of nucleic acids

The absorption of various dilutions of DNA samples was measured using quartz 1ml cuvettes in a spectrophotometer at 260 nm. An OD of 1 corresponds to approximately 50 µg/ml for double-stranded DNA, 40 µg/ml for single-stranded DNA and 20 µg/ml for oligonucleotides.

2.2.1.4 Small scale preparation of plasmid DNA

A 3 ml overnight culture was prepared from a single colony of transformed bacteria in LB medium supplemented with ampicillin (100 µg/ml). 1.5 ml bacterial suspension was transferred to an Eppendorf tube and the bacteria pelleted at 5,000 rpm for 5 min. The pellet was suspended in 100 µl solution I (see section 2.1.8). 200 µl solution II was added, vortexed and incubated for 5 min on ice. Subsequently 150 µl of solution III was added, vortexed and incubated again for 5 min on ice. The bacterial

debris was pelleted at 12,000 rpm for 10 min at 4°C. To the supernatant was added 460 µl phenol/chloroform mix; the mixture was vortexed and the phases separated at 10,000 rpm for 10 min at 4°C. To the aqueous phase was added 1 ml ethanol and the sample was incubated for 15 min at –70°C. The DNA was pelleted at 12,000 rpm for 10 min at 4°C and the pellet resuspended in 40 µl of TE buffer. 10 µl were used for a restriction digest which included RNase A at 1 µg/µl.

2.2.1.5 Large scale plasmid preparation

Transformed cells were grown overnight in 200 ml LB medium supplemented with ampicillin (100 µg/ml). The cells were harvested by centrifugation for 15 min at 6,000 rpm in a Beckman J2-21 centrifuge using a JA 14 rotor. The pellet was resuspended in 10 ml solution I (section 2.1.8). Lysis of the cells was achieved by addition of 10 ml solution II and incubation on ice for 5 min. Addition of 7.5 ml solution III precipitated out the cell debris which was sedimented at 10,000 rpm at 4°C (Beckman J2-21 centrifuge, JA 20 rotor). The supernatant was filtered through Whatman 3MM paper and 0.6 volumes of ethanol were added to precipitate the nucleic acids for 15 min at room temperature. The nucleic acids were pelleted at 3,000 rpm for 15 min at 4°C using a Beckman CS-6R benchtop centrifuge and resuspended in 2 ml TE buffer. 2 ml 5 M LiCl/50 mM Tris-HCl (pH 8.0) was added and incubated 10 min on ice to precipitate RNAs. RNAs were sedimented at 3,000 rpm for 5 min at 4°C (Beckman benchtop centrifuge) and the DNA in the supernatant precipitated with 2 volumes of ethanol for 15 min on ice. The plasmid DNA was recovered by centrifugation for 15 min at 3,000 rpm at 4°C (Beckman benchtop centrifuge) and resuspended in 500 µl TE buffer. Remaining RNAs were removed by addition of 30 µl DNase-free RNase (10 mg/ml) and incubation for 1 hour at 37°C. The DNA was ethanol precipitated after a phenol, a phenol/chloroform and a chloroform extraction and finally dissolved in 500 µl TE buffer.

2.2.1.6 Isolation of single-stranded DNA from phagemids

Single-stranded DNA from pBluescript phagemids was isolated as described by Blondel and Thillet (1991). A colony of *E. coli* XL1-Blue, harbouring the phagemid of interest and grown on LB plates supplemented with ampicillin (100 µg/ml) for selection of the phagemid and tetracycline (12.5 µg/ml) for selection of the F' episome was suspended in 20 µl 2x TY medium (section 2.1.4). The bacterial suspension was then infected with 5 µl of helper phage (VCS M13, Stratagene, Cambridge, at least 10^{10} pfu/ml) and incubated for 15 min at room temperature. 500 µl of 2x TY medium with antibiotics (ampicillin at 100 µg/ml and tetracycline at 12.5 µg/ml) was added and incubated for 1 hour at 37°C to let the helper phage express antibiotic resistance. 120 µl of the incubated cells were then added to 3 ml 2x TY medium supplemented with ampicillin (100 µg/ml), tetracycline (12.5 µg/ml) and kanamycin (75 µg/ml). The culture was then incubated with vigorous shaking for 18 to 20 hours at 37°C. 1.5 ml of bacterial suspension was then harvested by centrifugation for 7 min at 7,000 rpm in a microcentrifuge. 1.3 ml of the supernatant was carefully transferred to a fresh tube and the phage particles precipitated by addition of 200 µl 20% PEG 6000/2.5 M NaCl and incubation at room temperature for 15 min. The virion particles were pelleted by centrifugation at 12,000 rpm for 10 min in a microcentrifuge and the pellet dissolved in 100 µl TE buffer. After two phenol extractions and one chloroform extraction the single-stranded DNA was ethanol precipitated and dissolved in 30 µl TE buffer.

Several attempts to scale up this protocol failed. Therefore, multiple minipreparations were performed in parallel, checked on agarose gels and individual preparations pooled.

2.2.1.7 Restriction enzyme digests of DNA

The DNA was incubated with the appropriate restriction enzyme in the presence of the appropriate buffer (supplied together with the restriction enzyme as 10x buffer) for at least 2 hours at 37°C, with a DNA concentration not higher than 1 µg/10 µl and an enzyme concentration of about 10 units/µg DNA. The volume of the restriction enzyme did not exceed a 1/10 of the total volume, as glycerol can affect the performance of the enzyme.

2.2.1.8 Ligations

Routinely, 200 ng of linearized vector DNA were used in a ligation in a total volume of 10 µl. The vector:insert ratio was 1:3. 1 µl of 10x ligation buffer (section 2.1.8) and 1 µl of 10 mM ATP were added to the DNA and the ligation initiated by addition of 1 Weiss unit of bacteriophage T4 DNA ligase. Incubation was for 8 to 12 hours at 16°C.

2.2.1.9 Preparation of competent cells

For the preparation of competent cells a single colony of *E. coli* XL1-Blue was transferred to 20 ml of LB medium containing tetracycline (12.5 µg/ml) and incubated overnight at 37°C. 500 µl of this suspension were used to inoculate 100 ml of LB medium containing tetracycline (12.5 µg/ml). Incubation was for 2 to 3 hours until an OD_{600} of 0.45 - 0.55 was reached. The cells were then cooled down on ice for 10 min and harvested by centrifugation at 4,000 rpm for 10 min at 4°C using a Beckman J2-21 centrifuge and JA14 rotor. The pellet was carefully resuspended in 40 ml of ice cold FSB buffer (section 2.1.8) and stored for 10 min on ice. The cells were recovered

as above and resuspended in 6 ml of FSB buffer. 200 µl of DMSO were added; cells were dispensed into aliquots of 150 µl and snap-frozen in liquid nitrogen. The tubes were stored at -70°C until needed.

2.2.1.10 Transformation of bacteria

Transformation-competent cells (150 µl, section 2.2.1.9.) were thawed on ice and plasmid DNA (no more than 50 ng) was added. After a 30 min incubation on ice the tubes were transferred to a 42°C waterbath, incubated for 90 seconds and rapidly transferred to an ice bath. After 5 min 800 µl of LB medium was added and incubated for 45 min at 37°C to allow the bacteria to express the antibiotic resistance. Dilutions of the bacterial suspension were made and plated out on LB plates containing ampicillin (100 µg/ml) and tetracycline (12.5 µg/ml).

2.2.1.11 Agarose gel electrophoresis

Agarose (GIBCO/BRL) was dissolved at the desired concentration (0.8 - 2.0% [w/v]) in TAE buffer (section 2.1.8) containing 0.5 µg/ml ethidium bromide by heating in a microwave oven until boiling. A well-forming comb was inserted and the gel allowed to set. One tenth volume of loading buffer (15% Ficoll, 0.1% bromphenol blue, 0.1% xylene cyanol FF) was added to the DNA samples before being applied to the gel. Electrophoresis was carried out at 50 mA in TAE buffer containing 0.5 µg ethidium bromide.

2.2.1.12 Isolation of DNA fragments from agarose gels

It was necessary for the generation of patch-methylated constructs to isolate sufficient quantities of restriction fragments of plasmid DNA. Depending on the size of the restriction fragment to be isolated, 50 to 100 µg of plasmid DNA were digested with the desired restriction enzyme(s). Complete digestion was checked on a minigel and the bulk of the restriction digest was separated on a preparative agarose gel, where the sample was applied in one large slot. The desired band was identified and the fragment cut out of the gel. The plunger of a 1 ml (or 2 ml) disposable syringe was removed and the syringe plugged with sterile siliconised glass wool. The agarose slice was placed in the syringe and frozen at -70°C for 15 min. The DNA was recovered by centrifugation through the glass wool for 20 min at 35°C in a Beckman CS-6R benchtop centrifuge. 500 µl of boiling TE buffer (section 2.1.8) was added to the remaining agarose and the procedure of freezing and centrifugation repeated to extract any remaining DNA. The DNA solution was chloroform extracted and the DNA recovered by ethanol precipitation.

2.2.1.13 Dephosphorylation of plasmid DNA

The 5´-phosphate groups of a linear plasmid were removed by calf intestinal alkaline phosphatase (CIP) to avoid self-ligation of the vector. 1 µg of linearized vector DNA was incubated in CIP buffer (section 2.1.8) with 1 unit of calf intestinal alkaline phosphatase (Boehringer Mannheim) in a total volume of 50 µl at 37°C for 30 min. SDS and EDTA were added to final concentrations of 0.5% and 5 mM, respectively. Proteinase K (Boehringer Mannheim) was added to a final concentration of 100 µg/ml and incubated for 30 min at 55°C. The reaction was subjected to a phenol and phenol/chloroform extraction and the DNA recovered by an ethanol precipitation.

2.2.1.14 Nuclease S1 treatment of plasmid DNA

Nuclease S1 was used for the removal of protuding, single-stranded DNA ends of plasmid DNA. 5 µg of *Hind*III/*Kpn*I digested vector DNA was incubated with 4 units nuclease S1 (Boehringer Mannheim) in nuclease S1 buffer (section 2.1.8) in a total volume of 20 µl for 30 min at 30°C. SDS and EDTA were added to final concentrations of 0.5% and 5 mM, respectively. Proteinase K (Boehringer Mannheim) was added to a final concentration of 100 µg/ml and incubated for 30 min at 55°C. The reaction was subjected to a phenol and phenol/chloroform extraction and the DNA recovered by an ethanol precipitation.

2.2.1.15 Purification of oligonucleotides

60 µg (approx. 2 OD_{260}) of the oligonucleotide were lyophilized to dryness in a rotary vacuum lyophilizer to remove the NH_4OH. The pellet was then dissolved in 20 µl H_2O and 20 µl formamide was added. Before loading on a gel the DNA was heat denatured for 5 min at 55°C. Separation was on 1 mm thick, 19% denaturing polyacrylamide (acrylamide : N,N'-methylenebisacrylamide 19:1) gels in 1x TBE buffer (section 2.1.8). The gel was pre-run for 30 min at 30 mA before the oligonucleotide was loaded in 8 slots. To monitor the electrophoresis 5 µl of a 50% formamide solution of 0.05% xylene cyanol FF and 0.05% bromphenol blue was run alongside the oligonucleotide samples. After completion of the electrophoresis the apparatus was dismantled and the gel transferred to a piece of Saran Wrap. The gel was then transferred onto a flourescent thin-layer chromatography plate (Merck, Silica gel F_{254}). The gel was taken to the dark room and illuminated with a hand-held, long-wavelenght UV lamp. The oligonucleotide absorbed the UV-light and appeared as dark bands on the flourescing background of the chromatography plate. The top bands of the oligonucleotide (= full size oligonucleotide) were marked, cut out of the gel,

transferred to Eppendorf tubes and crushed using a yellow pipette tip. 300 µl of oligonucleotide elution buffer (section 2.1.8) per band were added and incubated for 16 hours at 37°C. The gel pieces were pelleted by centifugation at 12,000 rpm for 10 min in a microcentrifuge. The supernatants were pooled, ethanol precipitated and dissolved in 50 µl TE buffer. Finally, to remove any remaining gel particles the oligonucleotides were purified using a spin column as desribed in section 2.2.1.19.

2.2.1.16 Polymerase chain reaction

Because no suitable restriction sites were available to isolate the SV40 early promoter as a blunt ended fragment, a polymerase chain reaction (PCR) approach was used, as suitable oligonucletides were already available.

The 350 bp fragment was amplified in a standard PCR reaction as follows. 30 ng of plasmid pVHCk were incubated in *Taq* DNA polymerase buffer (section 2.1.8) containing the 17mer reverse sequencing primer and oligo GS (100 pmol each, see Fig.1), $MgCl_2$ at 5 mM, the four dNTPs at 100 µM and 2.5 units of *Taq* DNA polymerase (Promega). The reaction was covered with paraffin oil and carried out in a Perkin Elmer Cetus DNA Thermal Cycler 480 using the following cycle: 1x 3 min at 94°C; 25x [1.5 min at 94°C; 1 min at 55°C; 1 min at 72°C]; 2min at 72 min. The amplified fragment was gel-purified and used for constructing plasmids pPPk and pPRk (figure 6.1.).

2.2.1.17 End labelling of oligonucleotides

Synthetic oligonucleotides were endlabelled by transfer of the γ-^{32}P from [γ-^{32}P]ATP using bacteriophage T4 polynucleotide kinase (PNK). 10 pmol of oligonucleotide were incubated in T4 PNK buffer (section 2.1.8) with 5 µl of [γ-

^{32}P]ATP (5,000 Ci/mmole, 10 mCi/ml) and 10 units of T4 PNK in a total volume of 20 µl. Unincorporated radiolabel was removed using Chroma spin columns (section 2.2.1.19).

2.2.1.18 Radio labelling of DNA fragments

The Megaprime DNA labelling system from Amersham (RPN 1606) was used for the radioactive labelling of probes for hybridizations. 5 µl of primer solution (containing random nonamer primers) and water to a final reaction volume of 50 µl were added to 50 ng of purified fragment DNA. The DNA was denatured for 2 min in a boiling waterbath. Subsequently 10 µl of Megaprime reaction buffer (containing dATP, dGTP, dTTP, $MgCl_2$, 2-mercaptoethanol and Tris-HCl buffer [pH 7.5]), 5 µl [α-^{32}P]dCTP (3,000 Ci/mmole, 10 mCi/ml) and 2 µl Klenow enzyme (1 unit/µl) were added and the reaction incubated for 30 min at 37°C.

2.2.1.19 Removal of unincorporated nucleotides from radiolabelled DNA

Chroma Spin-10 columns (Clontech Laboratories, Palo Alto, CA) were used to remove free radio label from radioactively labelled DNA. The column was drained and packed by centrifugation at 2,200 rpm for 5 min at 4°C in a Beckman CS-6R benchtop centrifuge. The sample was carefully applied onto the centre of the column and centrifuged as above. The radioactively labelled sample was collected in an Eppendorf tube at the bottom of the column whereas the unincorporated nucleotides remained in the column. The specific activity of the radiolabelled sample was calculated by comparison of the radioactivity before and after removal of the free nucleotides.

2.2.1.20 Southern blot and hybridization

A combined vacuum/alkaline blot method was used to transfer DNA from agarose gels to nylon membranes. A vacuum blotting apparatus from Hybaid (Teddington, Middx.) was used to perform the alkaline Southern blot. A piece of Whatman 3MM filter paper and a piece of nylon membrane (Hybond N+, Amersham) were cut slightly bigger than the gel to be blotted and pre-wetted in 0.4 M NaOH. The paper was placed on top of the porous plate of the blotting device followed by the nylon membrane and followed by the rubber gasket, which was cut with an aperture slightly smaller than the gel to be transferred. The agarose gel was briefly soaked in 0.4 M NaOH and then placed on the nylon membrane, avoiding trapping any air bubbles between gel and membrane. The lid was then placed onto the unit and tightened by turning the four levers into their locking positions. The vacuum was then applied and the transfer buffer (0.4 M NaOH) poured in the chamber so that the gel was completely immersed. The vacuum was reduced by a valve to 80 cm of water and the transfer time was 30 min. After the transfer was completed the nylon membrane was neutralized by washing in distilled water and 2x SSC buffer.

Southern blots were prehybridized in hybridization buffer (section 2.1.8) containing 100 µg/ml heat denatured sonicated salmon sperm DNA for 1 -2 hours at 65°C. The heat denatured probe (1 - 5 pg/ml hybridization buffer, 10^8-10^9 cpm/µg) was added and incubated for 12 - 16 hours at 65°C in a shaking water bath. The filter was washed twice in 2x SSC, 0.1% SDS for 15 min at room temperature and subsequently twice in 0.1x SSC, 0.1% SDS at 65°C for 15 min. The membrane was then autoradiographed for the desired time.

If the blot was subjected to a hybridization with a second probe the membrane was never allowed to dry during or after hybridization and washing. The blot was stripped of the first probe by pouring a boiling solution of 0.5% SDS on the membrane and allowing it to cool to 50°C.

2.2.2 Methylation of plasmid DNA *in vitro*

2.2.2.1 Methylation using prokaryotic methylases

Prokaryotic methylases M.*Sss*I, M.*Hpa*II and M.*Hha*I and their assay buffers were purchased from New England Biolabs (Beverly, MA, USA). Routinely, 20 µg plasmid DNA were incubated with 20 units of methylase (60 units in the case of M.*Hpa*II) in the appropriate assay buffer for 16 hours at 37°C in a total volume of 100 µl. The concentration of the methyl-group donor S-adenosyl methionine (SAM) was 80 µM for M.*Hpa*II and M.*Hha*I and 160 µM for M.*Sss*I, respectively. For inactivation of the methylases SDS and EDTA were added to final concentrations of 0.5% and 5 mM, respectively. Proteinase K (Boehringer Mannheim) was added to a final concentration of 100 µg/ml and incubated for 30 min at 55°C. The reaction mixture was subjected to a phenol and phenol/chloroform extraction and the DNA recovered by an ethanol precipitation. For every methylation assay, a mock-methylated control was treated in the same way as described above, but omitting the methylase in the methylation assay.

2.2.2.2 Methylation using mouse methylase

DNA methylase was prepared from mouse Krebs II ascites tumour cells as described by Adams *et al.* (1986) and was kindly provided by Dr. Roger Adams. 5 µg plasmid DNA was methylated in a 70 µl reaction in buffer M+ (section 2.1.8) containing 30 µM S-adenosyl methionine (in some cases this was tritiated at 0.5 Ci/mmol to monitor incorporation of methyl groups), 0.1 mg/ml BSA and 110 units of murine DNA methylase (one unit is defined as the amount of enzyme which will incorporate one pmol of methyl groups into denatured DNA from *Micrococcus luteus* in 1 hour). After the appropriate incubation time at 37°C the enzyme was inactivated by

adding SDS and EDTA to final concentrations of 0.5% and 5 mM, respectively. Proteinase K (Boehringer Mannheim) was added to a final concentration of 100 µg/ml and incubated for 30 min at 55°C. The reaction mixture was subjected to a phenol and phenol/chloroform extraction and the DNA recovered by an ethanol precipitation. For every methylation assay, a mock-methylated control was treated in the same way as described above, but omitting the methylase in the methylation assay.

2.2.2.3 Monitoring DNA methylation using S-adenosyl-L-[methyl-^{3}H] methionine (^{3}H-SAM) as methyl group donor

When tritiated SAM was used as methyl group donor the degree of methylation was quantified using a liquid scintillation counter. The tritiated SAM was removed by washing with 5% TCA as follows. Methylated DNA (sections 2.2.2.1 and 2.2.2.2) was spotted onto filter paper discs (Whatman 3MM) and washed 5 times for 5 min with 5% TCA, allowing 10 ml TCA per 2.5 cm^2 disc. The filters were then washed twice with ethanol and once with ether in a similar fashion, dried and transferred to scintillation vials. 500 µl of 0.5 M perchloric acid were added and incubated for 60 min at 70°C. Finally, 5 ml of scintillation liquid were added and counted in a LKB 1209 Rackbeta liquid scintillation counter.

2.2.2.4 Assay of methylase activity from mammalian cells

Exponentially growing mouse L929 fibroblast cells were harvested by trypsinization and seeded at about 5x10^5 cells per 6-cm-diameter tissue culture dish in 5 ml of EC10 medium (section 2.1.5) containing 5-aza-2´-deoxycytidine at 1 µM and were incubated at 37°C in a humidified incubator in an atmosphere of 5% CO_2. The medium was renewed every 24 hours and cells harvested at the indicated time (section

4.4.2). Cells were washed twice with PBS buffer and scraped into 500 µl ice-cold buffer M+ (section 2.1.8). Collection of the cells was at 2,000 rpm for 5 min at 4°C followed by resuspension in 100 µl buffer M+ containing 1 % TritonX-100 to lyse the cells. After incubation for 5 min at 0°C nuclei were pelleted at 3,000 rpm for 5 min at 4°C and washed once in buffer M+. To prepare the nuclear extract the nuclei were resuspended in 32 µl buffer M+ containing 0.2 M NaCl and incubated at 0°C for 15 min. Nuclei were pelleted at 3,000 rpm for 5 min at 4°C and the supernatant removed. To assay methylase activity 30 µl of nuclear extract was incubated with 6 µl S-adenosyl-L-(methyl -^{3}H) methionine (4.64 mCi/µM, 23.3 µM/l) and 10 µl poly[d(I-C)·d(I-C)], (0.1 µg/µl, Sigma). The reaction was incubated for 90 min at 37°C and stopped with an equal volume of Stop-mix (2% SDS, 10 mM EDTA, 1 µg/µl Proteinase K) followed by incubation at 42°C for 30 min. After a phenol extraction and ethanol precipitation the DNA was dissolved in 50 µl NaOH (0.3 M) and incubated for 30 min at 37°C. Methylase activity was measured as acid insoluble DNA as described in section 2.2.2.3.

2.2.3 Maxam-Gilbert sequencing

The SV40 early promoter contains 9 methylatable CpG sites but no site for a methylation-sensitive restriction enzyme. To assess the degree of methylation in the promoter region it is therefore necessary to use a Maxam-Gilbert sequencing approach (Maxam and Gilbert, 1980), as methylation of a cytosine at position 5 leaves a gap in the sequencing ladder. Again, a protocol from Sambrook *et al.* (1989) was adapted to the requirements of the experiment.

2.2.3.1 Asymmetric endlabelling of a promoter restriction fragment

Plasmid pVHC1 was methylated with M.*Sss*I and murine methylase as described in section 2.2.2 and a 350 bp *Kpn*I/*Hind*III restriction fragment isolated as described in section 2.2.1.12. Because only the *Hind*III site has a 5´ overhang it was possible to endlabel the fragment specifically at this site using Klenow enzyme. 2 µg of fragment DNA were endlabelled in Klenow buffer (section 2.1.8) containing dATP and dGTP at 2.5 mM each, 50 µCi [α-^{32}P]dCTP (3,000 Ci/mmole, 10 mCi/ml) and 4 units Klenow enzyme in a total volume of 30 µl for 30 min at 37°C. After removal of unincorporated radio label and ethanol precipitation the DNA was dissolved at 10,000 cpm/µl in dH_2O.

2.2.3.2 Maxam-Gilbert sequencing reactions

The four base-specific cleavage reactions were carried out as follows:

G reaction:

5 µl radiolabeled DNA were mixed with 4 µl sonicated salmon sperm DNA (sssDNA, 1 µg/µl) and 190 µl DMS buffer (section 2.1.8). The mixture was chilled to 0°C and 5 µl of 10% DMS (Dimethyl sulphate, Aldrich) were added, mixed and incubated for 5 min at 20°C. To stop the reaction 50 µl of DMS stop solution (section 2.1.8) were added, followed by 750 µl ethanol and incubation on dry ice.

A + G reaction:

10 µl radiolabelled DNA were mixed with 4 µl sssDNA (1µg/µl) and 10 µl dH_2O and chilled to 0°C. 25 µl formic acid (98%) were added and incubated for 4 min at 20°C. The reaction was stopped by addition of 200 µl hydrazine stop solution (section 2.1.8), followed by 750 µl ethanol and incubation on dry ice.

C + T reaction:

10 µl radiolabelled DNA were mixed with 4 µl sssDNA (1µg/µl) and 10 µl dH_2O and chilled to 0°C. 30 µl hydrazine (99%, Eastman Kodak, Hemel Hempstead, Herts.) were added and incubated 7 min at 20°C. The reaction was stopped by addition of 200 µl hydrazine stop solution, followed by 750 µl ethanol and incubation on dry ice.

C reaction:

5 µl radiolabelled DNA were mixed with 4 µl sssDNA (1µg/µl) and 15 µl NaCl (5 M) and chilled to 0°C. 30 µl hydrazine were added and incubated 5 min at 20°C. The reaction was stopped by addition of 200 µl hydrazine stop solution, followed by 750 µl ethanol and incubation on dry ice.

All four reactions were then processed in the same way.

The DNA was pelleted by centrifugation at 12,000 rpm for 10 min at 4°C, the supernatant was removed to the appropriate waste bottle and the DNA dissolved in 300 µl 0.3 M sodium acetate (pH 5.4). The solution was ethanol precipitated and the pellet subsequently washed with 98% ethanol and 70% ethanol. After drying the DNA in a rotary vacuum lyophilizer (Jouan RC10.10.) it was dissolved in 100 µl piperidine (1M). Cleavage of the sugar-phosphate chain of the DNA was carried out for 30 min at 90°C. The samples were then lyophilized to dryness, dissolved in 20 µl H_2O, transferred to a fresh tube and once more lyophilized in a rotary vacuum lyophilizer. This step was repeated until all piperidine was removed (smell test!!). The DNA was then dissolved in 10 µl sequencing gel-loading buffer (section 2.1.8) of which 5 µl were used for separation on a 6% sequencing gel.

2.2.3.3 Sequencing gel

Denaturing polyacrylamide gels (0.2 mm thick) were used for the separation of the Maxam-Gilbert sequencing reaction. 50 ml of a 6% gel contained 7.5 ml acrylamide solution (40%, acrylamide:N,N'-methylenebisacrylamide 19:1), 5 ml 10x

TBE buffer and 23 g urea. The solution was filtered through a nitrocellulose filter to remove any undissolved particles and polymerization was initiated by the addition of 200 µl 10% ammonium persulfate and 50 µl TEMED. After complete polymerization (1 hour) the gel was assembled onto the electrophoresis apparatus and pre-run at 30 mA for 30 min in TBE buffer. The heat denatured samples were loaded and electrophoresis was at 40 mA for the desired time. Upon completion of the run (as monitored by the migration of the two dyes in the loading buffer) the gel plates were separated and the gel fixed for 20 min in 10% methanol/10% acetic acid, transferred to a piece of Whatman 3MM paper, covered with Saran Wrap and dried on a gel dryer for 40 - 60 min under vacuum. Exposure to X-ray film was for 16 - 24 hours.

2.2.4 Transfection of mammalian cells with plasmid DNA and analysis of CAT expression

2.2.4.1 Transfection of mammalian cells using calcium phosphate

Exponentially growing cells (L929 or COS-1 cells, section 2.1.3) were harvested by trypsinization and seeded at about $5x10^5$ cells per 6-cm-diameter tissue culture dish in 5 ml of the appropriate medium (section 2.1.5) and were incubated for 12 - 16 hours at 37°C in a humidified incubator in an atmosphere of 5% CO_2. The calcium phosphate-DNA coprecipitate for each dish was prepared as follows: 5 µg of plasmid DNA and 15 µg of sonicated salmon sperm DNA were mixed, ethanol precipitated and dissolved in 437.5 µl of sterile H_2O. After addition of 62.5 µl $CaCl_2$ (2 M) the DNA-Ca solution was added dropwise to 500 µl 2x HBS (section 2.1.8) with gentle shaking. To allow precipitate formation the mixture was incubated at room temperature for 30 min and then added to the dish. Incubation was for 12 - 16 hours at 37°C, 5% CO_2. The medium was removed, the cells carefully washed with

prewarmed PBS buffer and 5 ml of fresh medium added. Incubation was continued for a further 24 hours unless otherwise specified.

2.2.4.2 Transfection of mammalian cells using Lipofectin

The need for a highly efficient transfection procedure arose when patch-methylated constructs (Chapter 4) were used in transient transfection assays. As the construction of regionally methylated plasmids is very labour intensive and expensive, lipofectin (GIBCO/BRL, Paisley, Scotland) was used to efficiently deliver the DNA into the cells. Lipofectin is a 1:1 (w/w) mixture of N-[1-2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) and dioleoyl phosphotidylethanolamine (DOPE) in membrane filtered water. The reagent interacts with the DNA to form DNA-lipid complexes which fuse with the tissue culture cell to deliver the DNA (Felgner *et al.*, 1987).

Cells were seeded as in section 2.2.3.1, 5 µg of plasmid DNA were made up to 100 µl with OPTI-MEM (a reduced serum medium, GIBCO/BRL). In a separate tube, 40 µl lipofectin was diluted with 60 µl OPTI-MEM, the mixture was added to the DNA solution and incubated for 15 min at room temperature. The cells were washed once with 5 ml OPTI-MEM and covered with 2 ml OPTI-MEM. The lipofectin-DNA solution was then added to the cells and incubated at 37°C, 5% CO_2. The DNA solution was replaced after 5 hours with the normal growth medium of the cell line and cells harvested 48 hours after transfection.

Using a higher concentration of lipofectin only slightly increased the transfection efficiency (as measured by CAT activity of transfected plasmid). Longer exposure of the cells to the lipofectin-DNA solution (up to 12 hours) did increase transfection efficiency, but cells tended to detach from the dish, which made it difficult to obtain comparable values for duplicate dishes. Under the conditions described

transfection efficiencies were 2 - 3 times higher than for transfections with calcium phosphate-DNA coprecipitates.

2.2.4.3 Chloramphenicol acyl transferase assay

Chloramphenicol acyl transferase (CAT) activity of transiently transfected cells was assayed according to the method of Seed and Sheen (1988). The cells were harvested as follows. The medium was removed and the cell monolayer washed twice with PBS buffer. 1 ml of TEN buffer (section 2.1.8) was added and the cells incubated for 5 min at room temperature before being scraped off the dish, using a rubber policeman. The cells were pelleted by centrifugation at 2,000 rpm at 4°C for 5 min and resuspended in 90 µl 0.25 M Tris-HCl (pH 7.8). The cells were lysed by three cycles of freeze/thawing by incubation on dry ice for 5 min followed by incubation at 37°C for 2 min. The cell debris was pelleted by centrifugation at 6,000 rpm for 4 min at 4°C in a microcentrifuge and the supernatant removed to a fresh tube. 40 µl of this cell extract were used in a CAT assay containing 10 µl 1 M Tris-HCl (pH 7.8), 10 µl 2.5 mM butyryl CoA and 8 µl [^{14}C]-chloramphenicol (Amersham, 55 mCi/mmol) in a total volume of 100 µl. After incubation at 37°C for 1 hour, the reaction was terminated by the addition of 210 µl of a 2:1 mixture of tetramethylpentadecane:xylene and mixed vigorously by vortexing for 30 seconds. After centrifugation for 2 min at 12,000 rpm at room temperature in a microcentrifuge 180 µl of the upper, organic phase was removed to a fresh tube and extracted with 100 µl of 100 mM Tris-HCl, (pH 7.8). The solutions were mixed as before and the organic layer removed to a scintillation vial and counted.

2.2.5 Analysis of transfected plasmid in isolated nuclei

2.2.5.1 Isolation of nuclei from mammalian cells

For the analysis of conformational changes and chromatin structure of plasmid DNA in mammalian cells it was necessary to isolate the nuclei of cells which had been transfected as described in section 2.2.4. If not otherwise indicated, cells were harvested 2 days after transfection. Cells were washed three times with 1 ml of prewarmed (37°C) PBS buffer and then scraped off the dish in 1 ml PBS buffer (0°C) using a rubber policeman. The dish was rinsed with a further 500 µl PBS buffer to collect any remaining cells and cells were pelleted at 1,500 rpm at 4°C for 2 min in a microcentrifuge. Lysis of cells was carried out by resuspending in 1 ml of RSB (section 2.1.8) containing 0.5% Nonidet P-40 for 5 min on ice. Nuclei were collected by centrifugation at 2,000 rpm for 5 min at 4°C, washed three times with 500 µl RSB (without Nonidet P-40) and finally resuspended in the appropriate buffer.

2.2.5.2 Isolation of nuclear DNA from mammalian cells

Nuclei from transfected cells were isolated as in section 2.2.5.1 and resuspended in 500 µl SDS buffer (25 mM Tris-HCl [pH 7.5], 0.15 M NaCl, 25 mM EDTA, 0.5% SDS, 500 µg/ml Proteinase K) and incubated for 30 min at 55°C. Nuclear DNA was purified and isolated by two phenol-, two phenol/chloroform- and two chloroform-extractions followed by ethanol precipitation, using no extra salt.

2.2.5.3 Digestion of mammalian nuclei with Micrococcal nuclease

Nuclei from transfected mammalian cells were isolated as described in section 2.2.5.1 and resuspended in 400 µl RSB (section 2.1.8). The nuclei suspension was split into 4 tubes of 100 µl each, 5 µl of $CaCl_2$ (50 mM) was added and the digest initiated by addition of 5 µl of *Staphylococcus aureus* nuclease (Boehringer Mannheim, 10 units/µl). The reaction was stopped at 0, 30, 60 and 120 seconds by the addition of an equal volume of 2x SDS buffer (section 2.2.5.2) and incubated for 30 min at 55°C. Isolation of the DNA was as in section 2.2.5.2.

2.2.5.4 Digestion of mammalian nuclei with restriction enzymes

Nuclei were isolated as described in section 2.2.5.1 and resuspended in 1 ml of *Msp*I buffer (10 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM DTE) or *Alu*I buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM DTT). Samples of 150 µl were digested by addition of increasing amounts of restriction enzyme (ranging from 0.1 to 20 units for *Msp*I in 10 µl and from 1 to 15 units for *Alu*I in 10 µl) for 35 min at 37°C. Each incubation was stopped by addition of an equal volume of 2x SDS buffer and processed as in section 2.2.5.2.

2.2.6 *In vitro* transcription assays

All solutions for the preparation of nuclear extracts and for the *in vitro* transcription assay were made up with DEPC-treated water and were autoclaved when possible, all glassware was incubated for at least 16 hours at 80°C and disposable plasticware was subjected to multiple autoclaving to reduce the risk of RNase contamination.

2.2.6.1 Preparation of nuclear extracts from HeLa cells

Nuclear extracts for *in vitro* transcription assays were prepared according to Dignam *et al.* (1983). HeLa S3 cells were grown in spinner flasks at 37°C in EMEM medium (section 2.1.5) to a density of 4 to 6 x 10^5 cell/ml. A total of about 10^9 cells were used for a typical extract preparation. Cells were harvested by centrifugation for 10 min at 2,000 rpm and room temperature (Beckman J2-21 centrifuge, JA 14 rotor). Pelleted cells were suspended in 5 volumes of PBS (4°C) and collected by centrifugation as above. Subsequent steps were performed at 4°C. The cells were suspended in 5 packed cell volumes of buffer A (10 mM HEPES-KOH [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) and incubated on ice for 10 min. Cells were collected by centrifugation as before and resuspended in two packed cell volumes of buffer A and lysed by 10 strokes of a Kontes all glass Dounce homogenizer, using the B type pestle. The homogenate was centrifuged as before to pellet the nuclei. The nuclear pellet was subjected to a second centrifugation for 20 min at 15,000 rpm (Beckman J2-21 centrifuge, JA 20 rotor) at 4°C to remove any residual cytoplasmic material. These crude nuclei were resuspended in 3 ml buffer C (20 mM HEPES-KOH [pH 7.9], 25% [v/v] glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) per 10^9 cells and homogenized as above. The resulting suspension was stirred gently for 30 min at 4°C and centrifuged for 30 min at 15,000 rpm at 4°C. The resulting clear supernatant was dialyzed against 50 volumes of buffer D (20 mM HEPES-KOH [pH 7.9], 20% [v/v] glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0 5 mM DTT) for 5 hours and the dialysate centrifuged at 15,000 rpm for 20 min at 4°C. The supernatant was snap frozen as 50 µl aliquots in liquid nitrogen and stored at -70°C.

2.2.6.2 *In vitro* transcription assay

In vitro transcription assays were carried out without any radio-labelled nucleotides as transcripts were quantified using a primer extension approach. In pilot experiments an optimal $MgCl_2$ concentration of 4 mM and a DNA saturating concentration of 500 ng/25 µl assay were established and differences between supercoiled and linear plasmid DNA were not observed.

A standard *in vitro* transcription assay was carried out as follows:

HeLa nuclear extract	11 µl
$MgCl_2$ (50 mM)	2 µl
ATP-CTP-GTP-UTP-mix (pH 7.0, each at 10 mM)	1 µl
plasmid DNA (50 - 500 ng)	11 µl
	25 µl

Incubation was for 1 hour at 30°C and was stopped by addition of 200 µl stop buffer (0.3 M Tris-HCl [pH 7.4], 0.3 M sodium acetate, 0.5% SDS, 2 mM EDTA, 3 µg/ml tRNA). After a phenol/chloroform extraction and ethanol precipitation the RNA was dried and dissolved in 10 µl of dH_2O.

2.2.6.3 Primer extension reaction

To assay the level of the specific mRNA, first strand cDNA is synthesized from the transcripts using an end-labelled primer complementary to the target RNA. The amount of cDNA obtained is a measure of the level of transcription of that particular gene. An oligonucleotide (oligo PE, Fig. 1), complementary to the mRNA of the CAT gene was end-labelled (section 2.2.1.17) and diluted to 50 fmol/µl. 1 µl of primer (10^4 cpm) was mixed with 10 µl RNA and 5 µl 2x PE buffer (100 mM Tris-

HCl [pH 8.3], 100 mM KCl, 20 mM DTT, 1 mM spermidine) and incubated for 20 min at 65°C, followed by 10 min at 42°C. To this was added 2 µl $MgCl_2$ (50 mM), 1 µl dNTP-mix (each at 10 mM) and 1 µl AMV reverse transcriptase (Promega, 1.5 units/µl). Incubation was for 30 min at 42°C, the reaction was directly ethanol precipitated, dissolved in 8 µl formamide loading buffer and separated on a 10% denaturing polyacrylamide gel (section 2.2.3.3).

CHAPTER THREE

In vitro methylation of CAT reporter gene constructs and its effect on transient expression

3.1 Introduction

In the following experiments methylases from different sources were used to methylate two similar CAT reporter gene constructs. Methylation levels were assessed by digestion with methylation sensitive restriction enzymes and by Maxam-Gilbert sequencing. The effect of DNA methylation of these constructs on reporter gene expression was established in transient transfection assays. In earlier studies by Bryans (1989) a pUC 8 based plasmid, pVHC1 (Fig. 3.1), containing the SV40 early promoter linked to the chloramphenicol acetyltransferase gene and the SV40 terminator region was used in similar experiments, but *in vitro* methylation was carried out using murine DNA methylase only. For reasons which will become clear in Chapter 4, the promoter-CAT gene-terminator cassette was cloned as a *Kpn*I/*Eco*RI fragment (see Fig. 3.1) into pBluescript KSII–, generating plasmid pVHCk (Fig. 3.2). Both vectors are very similar, the main difference being the presence of the f1 origin of replication in pVHCk, which allows isolation of single-stranded DNA. The experiments in this chapter were performed either with pVHC1 or with pVHCk. For each experiment it is indicated which plasmid was used. Table 1 lists the main features of both vectors and shows that transcription of the reporter gene is equally inhibited in both plasmids by complete CpG methylation.

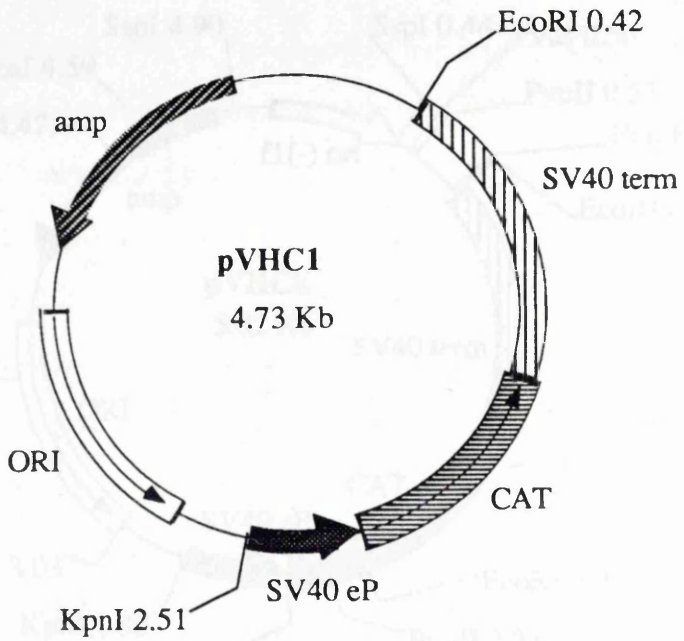


Figure 3.1 Map of plasmid pVHC1

Construction of plasmid pVHC1 was previously described by Bryans (1989) and Bryans *et al.* (1992). amp: ampicillin resistance gene; ORI: origin for replication in *E. coli*; SV40eP: SV40 early promoter/enhancer region; CAT: chloramphenicol acetyltransferase gene; SV40 term: SV40 terminator region; sites for restriction enzymes *Kpn*I and *Eco*RI are indicated. For details of the promoter region see figure 1.

	pVHC1	pVHCk
bp	4736	5025
CpGs	216	241
CAT activity (% of mock-methylated control)	5 - 10	5 - 10

Table 1 Comparison of plasmids pVHC1 and pVHCk

A standard transient transfection assay (section 2.2.4.2 and 2.2.4.3) with 5 μg plasmid gave a CAT activity of approximately 50,000 cpm for the unmethylated plasmid with a background of <1,000 cpm for untransfected cells. Transcriptional activities of methylated plasmids were calculated as per cent of mock-methylated control plasmids after subtraction of background levels. This applies to all transient transfection assays described in this thesis.

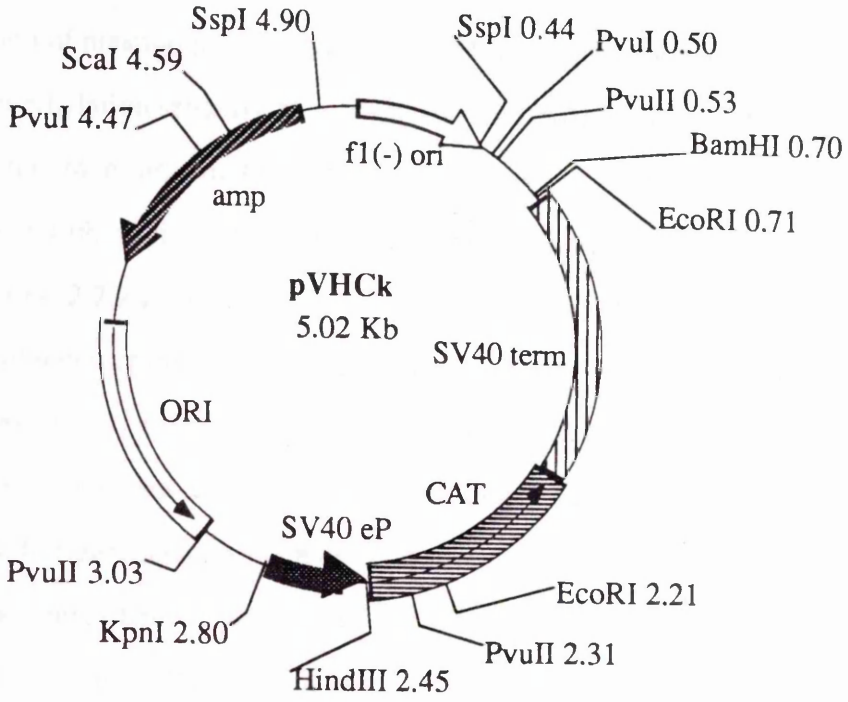


Figure 3.2 Map of plasmid pVHCk

Plasmid pVHCk was constructed by cloning the 2.09 kb *KpnI-EcoRI* fragment containing the chloramphenicol acetyltransferase gene under the control of the SV40 promoter/enhancer and the SV40 terminator region from pVHC1 (Fig. 3.1) into pBluescriptKSII–. f1(–)ori: bacteriophage f1 origin of replication in (-) orientation; other abbreviations as in figure 3.1. Locations of relevant restriction sites are indicated. For details of the promoter region see figure 1.

3.2 Methylation using M.*Sss*I or murine DNA methylase

Previously, *in vitro* methylation of DNA at the dinucleotide CpG was achieved by means of mammalian methylase preparations (Adams *et al.*, 1986; Pfeifer *et al.*, 1985). This had two major disadvantages: preparation of the enzyme was time consuming and high methylation levels were difficult to obtain. Both problems were overcome by use of the prokaryotic methylase from *Spiroplasma sp.* (Renbaum *et al.*, 1990) which methylates DNA at the dinucleotide CpG and hence mimics eukaryotic DNA methylation. The enzyme is commercially available (New England Biolabs) and highly active in *in vitro* methylation assays. Figure 3.3 shows a time-course of

methylation of plasmid pVHC1. The degree of methylation was assessed by digestion with the methylation sensitive restriction enzyme *Hpa*II and clearly shows, that in this assay, after 24 hours, methylation of the vector was complete. In comparison, methylation with murine DNA methylase isolated from mouse Krebs II ascites tumour cells (section 2.2.2.2) methylation levels of only 30 - 60 % were obtained (Bryans, 1989). Prolonged or multiple incubations of DNA with the mouse enzyme preparation did not result in higher methylation levels, which may be due to the formation of unproductive, covalent complexes between the enzyme and substrate DNA (Adams *et al.*, 1993). In figure 3.4 plasmid pVHC1 was methylated with murine DNA methylase for 5 h as described in section 2.2.2.2. *Hpa*II digestion of the population of methylated plasmids showed some molecules that were largely resistant to digestion, whilst others were completely digested by *Hpa*II.

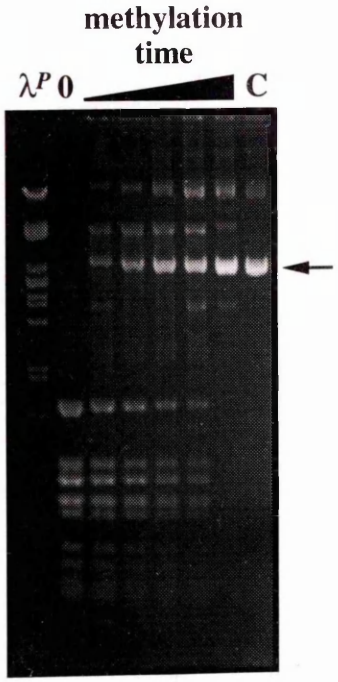


Figure 3.3 Complete methylation of plasmid pVHC1 with M.*Sss*I

Plasmid pVHC1 was methylated for 1, 3, 6, 9 and 24 hours using M.*Sss*I as described in section 2.2.2.1. Aliquots of plasmid DNA (500 ng) were digested with restriction enzyme *Hpa*II and were separated on a 1.5% agarose gel to reveal the degree of DNA methylation. λ*P*: λ-DNA, digested with *Pst*I; 0: no methylation; C: no digestion with *Hpa*II; arrow indicates supercoiled plasmid DNA.

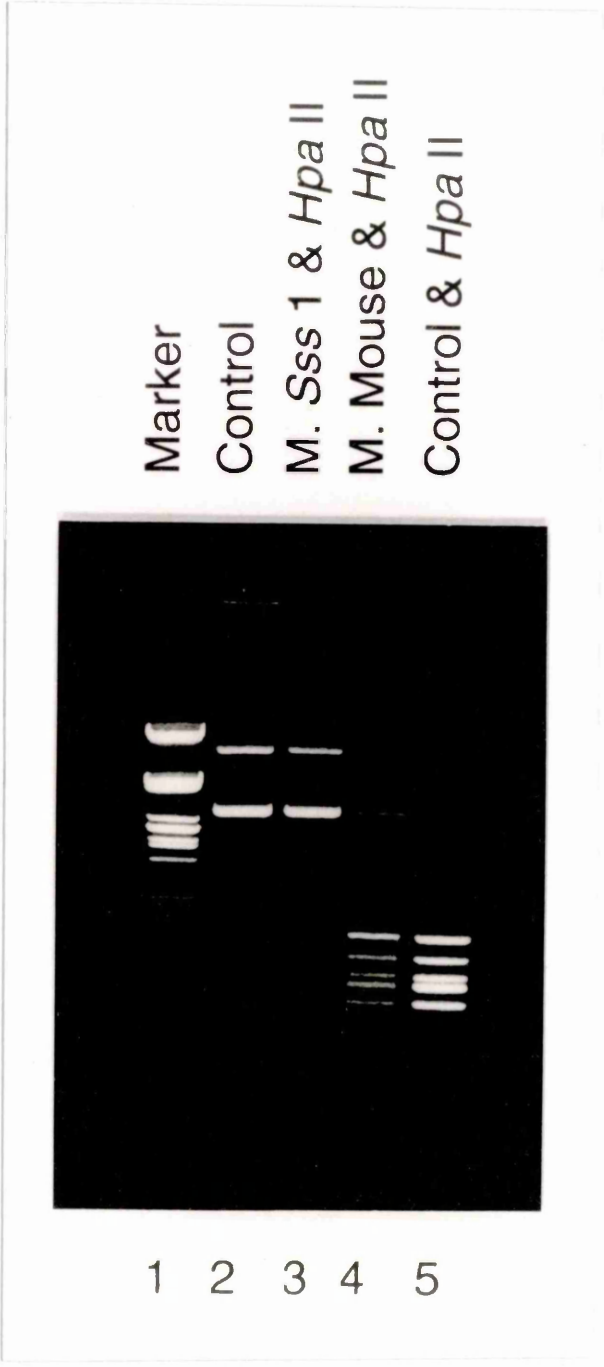


Figure 3.4 Assessment of the extent of methylation by M. *Sss*I and murine methylase

Plasmid pVHC1 was methylated using M.*Sss*I or murine methylase for 5 hours as described in section 2.2.2.1 and 2.2.2.2, respectively and aliquots were digested with *Hpa*II and resolved on a 1.5% agarose gel to establish the extent of DNA methylation. Lane 1: marker DNA λ/*Pst*I-digested; lane 2: undigested plasmid; lane 3: plasmid methylated with M.*Sss*I and *Hpa*II-digested; lane 4: plasmid methylated with murine methylase and *Hpa*II-digested; lane 5: control plasmid digested with *Hpa*II.

It is not possible to use methylation sensitive restriction enzymes to assess promoter methylation of the CAT vectors as there are no suitable sites in the promoter region. Therefore, a Maxam-Gilbert sequencing approach (Maxam and Gilbert, 1980) was used to determine the degree of *in vitro* methylation of the SV40 promoter. In the chemical cleavage reaction, hydrazine does not react with 5-methylcytosine in the presence of NaCl (section 2.2.3). Hence, methylation of a cytosine leaves a gap in the C-ladder of a Maxam-Gilbert sequencing gel. A Maxam-Gilbert sequencing reaction was carried out on plasmid pVHC1, methylated with M.*Sss*I, murine methylase and unmethylated plasmid. From figure 3.5 it can be seen that all 6 CpGs in the GC boxes of the promoter (see Fig. 1) were methylated by M.*Sss*I, whereas there is little evidence for methylation by murine methylase. It is not clear from the result with the mouse methylase whether a mixture of fully methylated and unmethylated molecules is present or whether plasmid molecules with a range of methylation have been produced, although the latter is probably the case.

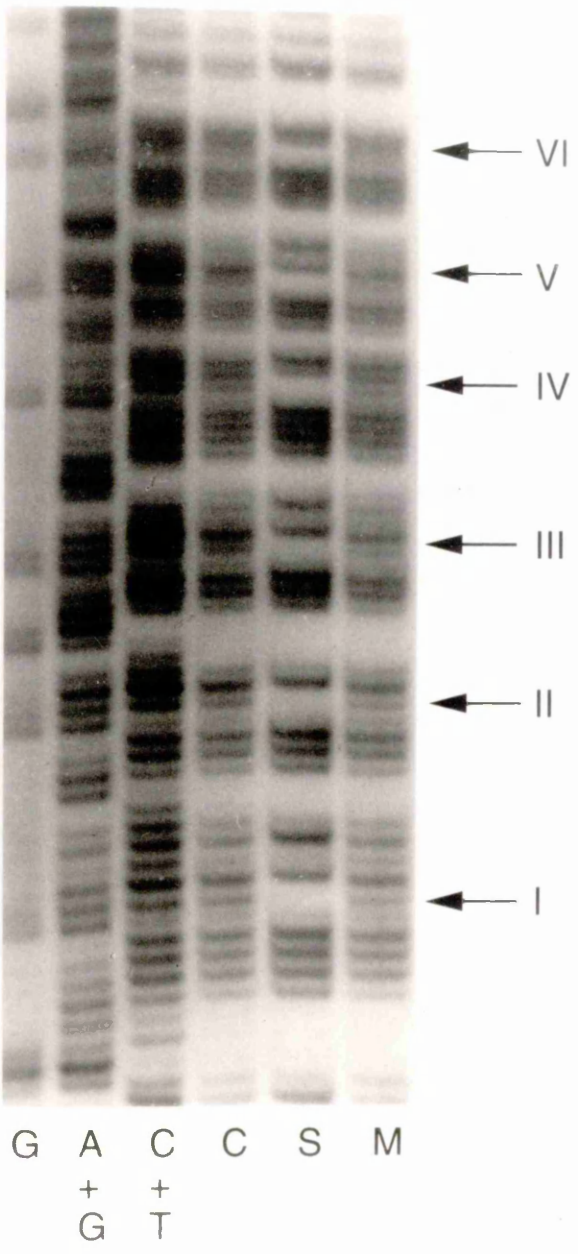


Figure 3.5 Assessment of the extent of promoter methylation using Maxam-Gilbert sequencing

The lanes represent the G-reaction; the A+G-reaction; the C+T-reaction and the C-reaction on the 350 bp *Kpn*I-*Hind*III fragment containing the SV40 early promoter (Fig. 1) from the unmethylated plasmid and the C-reaction carried out on the fragment from the plasmid methylated with M.*Sss*I (S) or the murine methylase (M). Plasmid pVHC1 was methylated as in figure 3.4 and the *Kpn*I-*Hind*III fragment isolated and asymmetrically endlabelled as described in section 2.2.1.12 and 2.2.3.1. Maxam-Gilbert sequencing reactions were performed as described in section 2.2.3. Arrows indicate the position of the cytosines in CG dinucleotides in the six GC boxes of the SV40 promoter (see also Fig. 1).

3.3 Reporter gene expression of plasmid pVHC1 methylated with murine DNA methylase or M.*Sss*I

Plasmid pVHC1, methylated in a time course with mouse methylase and methylase *Sss*I was used in transient transfection assays to establish the effect of DNA methylation on transcription from this plasmid. The DNA was introduced into mouse L929 cells using the calcium-phosphate coprecipitation method (section 2.2.4.1) and CAT activity was assayed two days after transfection. As figure 3.6 shows, transcription from these plasmids was progressively inhibited. Complete methylation by M.*Sss*I led to about 90% inhibition, whereas at a methylation level of about 30% using mouse methylase (Fig. 3.4), around 80% of transcriptional inhibition was observed.

The next experiment was designed to establish at what time after transfection the inhibitory effect of DNA methylation is apparent. Completely methylated plasmid and unmethylated control plasmid pVHC1 were transfected into L929 mouse fibroblasts and CAT activity assayed at 6, 24 and 48 hours after transfection as described in section 2.2.4.3. The result is shown in figure 3.7 and reveals that the degree of inhibition by DNA methylation is dependent on the time after transfection. At 6 hours, CAT activity of both, methylated and unmethylated plasmid was very low and no differences could be observed between methylated and unmethylated constructs. Less than 50% inhibition was found at 24 hours whilst over 90% inhibition was observed at 48 hours after transfection.

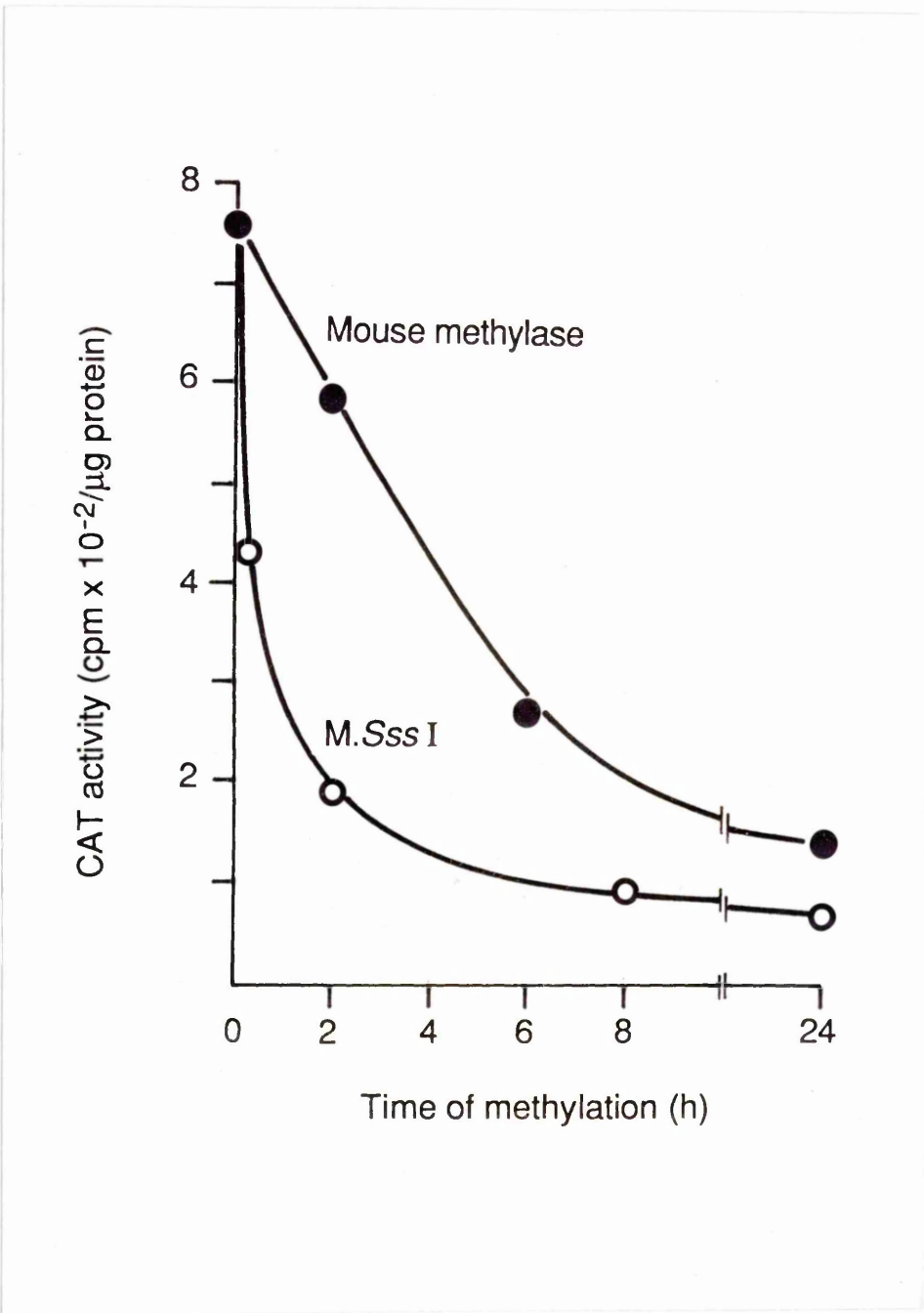


Figure 3.6 Time course of methylation of pVHC1 and the effect on transcription

5 µg samples of plasmid pVHC1 were methylated as described in section 2.2.2. After the indicated time the DNA was reisolated and used to transfect mouse L929 cells (section 2.2.4.1). CAT activity was assayed 48 hours after transfection of the cells as described in section 2.2.4.3 and is expressed as cpm/µg of protein per dish.

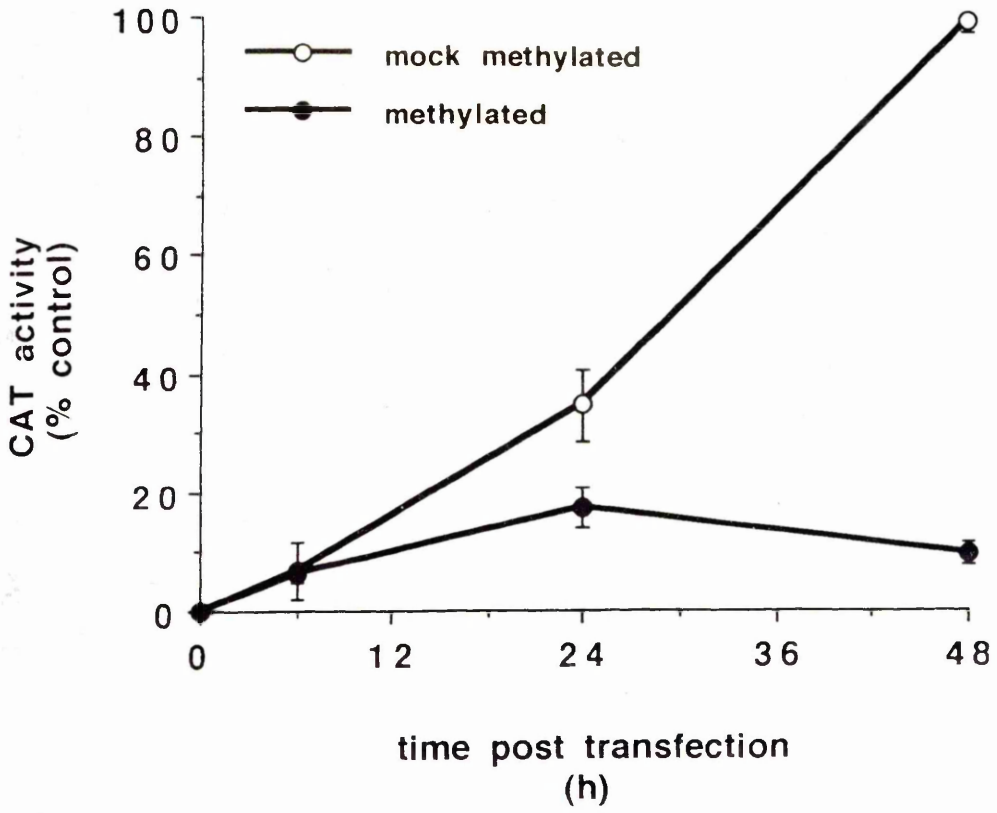


Figure 3.7 Time course of expression of CAT activity

Mouse L929 fibroblasts were transfected with plasmid pVHC1, mock-methylated or methylated to completion with M.*Sss*I and CAT activity determined at 6, 24 and 48 hours after transfection. Inhibition of CAT activity by DNA methylation was calculated by comparison to the values obtained for the control at 48 hours after transfection (=100%).

3.4 Reporter gene expression of plasmid pVHCk methylated with M.*Hpa*II and/or M.*Hha*I

In addition to the two CpG methylases, two different prokaryotic methylases were employed to investigate the effect of methylation on transcription in transient transfection assays. M.*HpaII* methylates the inner cytosine in the recognition site 5´-CCGG-3´, M.*HhaI* acts at the inner cytosine of the sequence 5´-GCGC-3´. Plasmid pVHCk has 16 *Hpa*II sites and 14 *Hha*I sites, but none of them lies in the promoter region. Figure 3.8 shows complete methylation of plasmid pVHCk with these

methylases (as established by digestion with the appropriate restriction enzyme), although complete methylation by M.*Hpa*II was only possible after incubation with a large excess of enzyme.

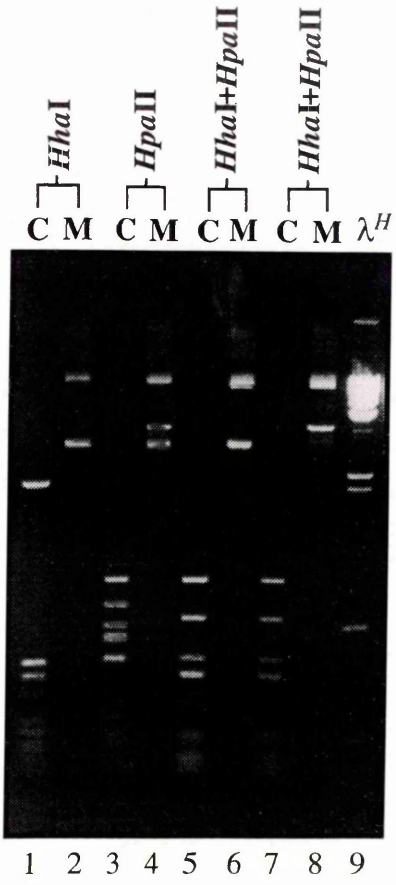


Figure 3.8 Methylation of pVHCk using M.*Hha*I and/or M.*Hpa*II

Plasmid pVHCk was methylated with M.*Hha*I, M.*Hpa*II and M.*Sss*I as described in section 2.2.2.1. Aliquots (500 ng) were subjected to digestion with the appropriate restriction enzyme(s) and resolved on a 1.5% agarose gel to exhibit the degree of methylation. C: mock-methylated control plasmid; M: methylated plasmid; lane 2: methylation with M.*Hha*I; lane 4: methylation with M.*Hpa*II; lane 6: methylation with M.*Hha*I and M.*Hpa*II; lane 8: methylation with M.*Sss*I; lane 9: λ/*Hind*III DNA marker; digestion with restriction enzymes is indicated on top of the lanes.

These methylated plasmids were then used in transient transfection assays and showed reduced CAT activity (48 hours after transfection) compared to the unmethylated control DNA (Fig. 3.9). Methylation by M.*Hpa*II inhibited transcription down to 76% of the control and methylation with M.*Hha*I down to 70%, whereas a combination of both methylases reduced CAT activity down to 67% of the unmethylated control.

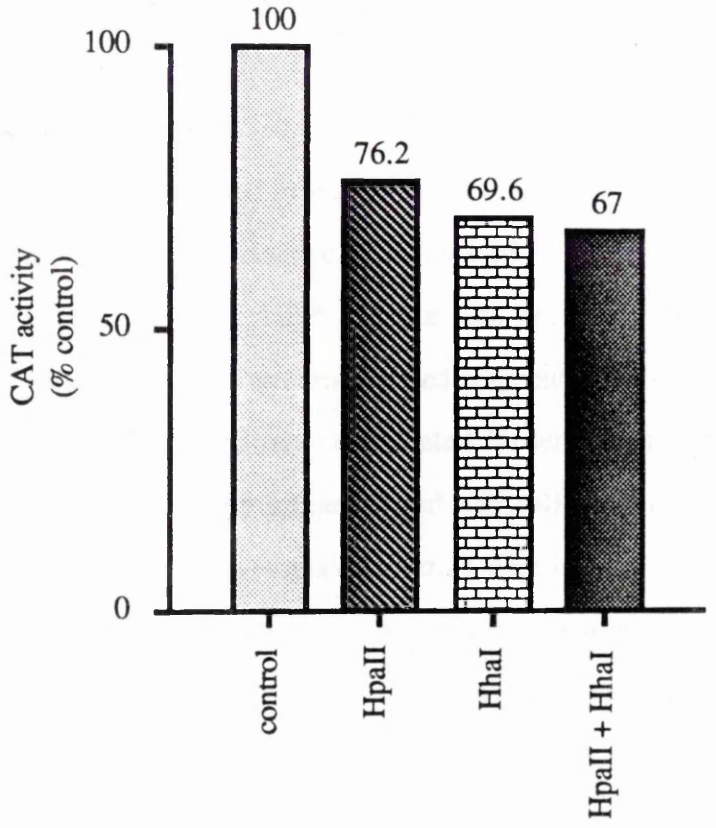


Figure 3.9 The effect of methylation with M.*Hha*I and/or M.*Hpa*II on transcription from pVHCk

Plasmid pVHCk was methylated using M.*Hha*I and/or M.*Hpa*II and 5 µg were transfected into L929 cells as described in section 2.2.2.1 and 2.2.4.2. CAT activity was determined 48 hours after transfection as described in section 2.2.4.3. Inhibition of CAT activity by DNA methylation was calculated as percent CAT activity relative to the mock-methylated control and is shown as a figure above the appropriate column.

3.5 Cotransfection of pVHCk with methylated or unmethylated competitor DNA

It was previously reported that transcriptional inhibition by methylation was overcome by cotransfection with methylated, untranscribable competitor DNA (Levine *et al.*, 1991). To test whether this applies to the system described above, calcium-phosphate coprecipitation transfections were carried out, using methylated and unmethylated pBluescript as carrier DNA. Plasmids pVHCk and pBluescript KS– were completely methylated with M.*Sss*I and mixed to a total of 20 µg at a ratio of 1:3 or 1:6 (recombinant DNA:competitor DNA). The DNA mixture was then transfected into mouse L929 cells as described in section 2.2.4.1. CAT activity of unmethylated pVHCk was not affected by the presence of methylated competitor (data not shown). Transcriptional activity of methylated pVHCk was not reactivated at the lower concentration of either methylated or unmethylated competitor DNA (Fig. 3.10) but, at a ratio of 1:6 (methylated recombinant : methylated carrier DNA), CAT activity was significantly higher (26% of the unmethylated control) than with unmethylated competitor DNA (7.5% of the unmethylated control). This indicates that the inhibitory effect of DNA methylation can be overcome by cotransfection with methylated DNA. However, it was not established whether the methylated CAT gene construct could be completely reactivated as higher concentration of methylated competitor DNA than those described were not used.

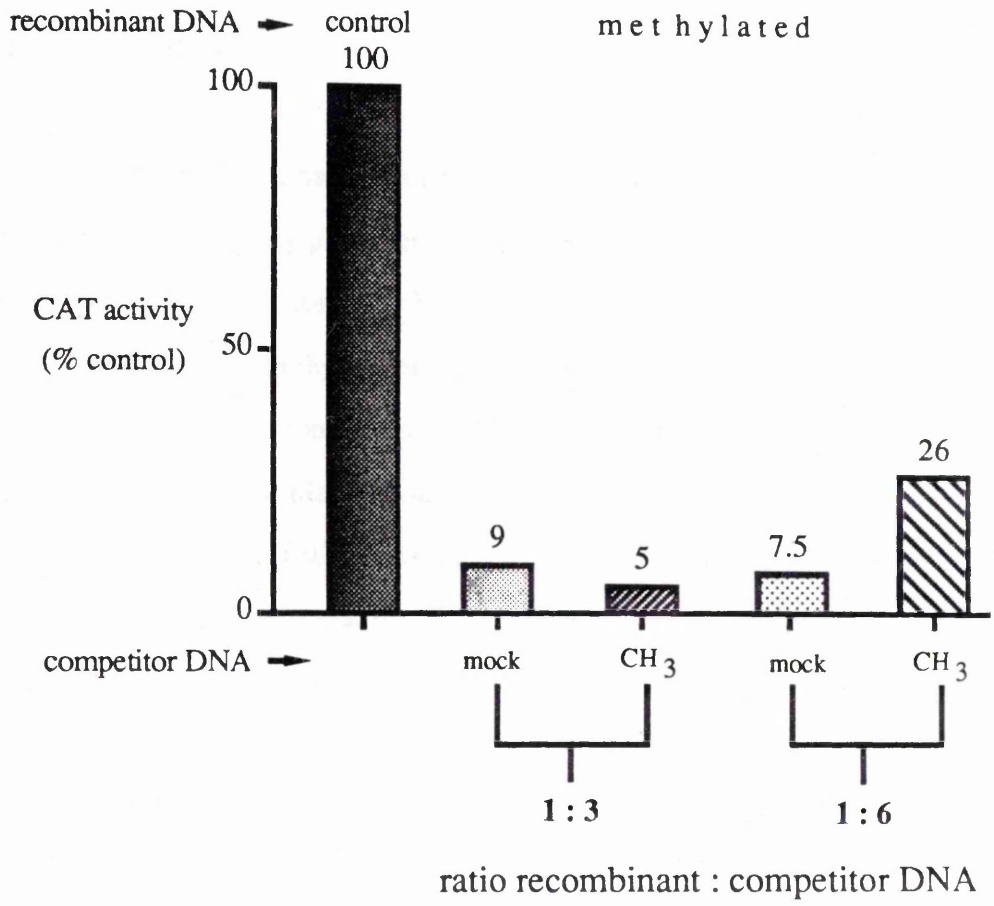


Figure 3.10 The effect of cotransfected, methylated carrier DNA on transcription from methylated pVHCk

Plasmids pVHCk and pBluescript were completely methylated with M.*Sss*I as described in section 2.2.2.1 and transfected into mouse L929 cells using the calcium phosphate coprecipitation method (section 2.2.4.1). A total of 20 µg of DNA was transfected per dish at ratios of 1:3 and 1:6 (pVHCk:pBluescript). CAT activity was assayed 48 hours after transfection. Inhibition of CAT activity by DNA methylation was calculated by comparison to the values obtained for the mock-methylated recombinant DNA control, which was cotransfected at the same ratio with the same competitor DNA.

3.6 Discussion

In this chapter it has been demonstrated using two different plasmids, both containing the SV40 early promoter linked to the CAT reporter gene, that transcription in transient transfection assays is inhibited by DNA methylation.

Using a DNA methylase prepared from mouse Krebs II ascites tumour cells relatively low methylation levels of 20 - 40% were obtained (Fig. 3.4 and 3.5). However, when such a plasmid, clearly showing less than 30% methylation, was introduced into mouse L929 cells transcription was reduced to about 20% of the unmethylated control. Plasmid pVHC1 contains a total of 216 CpG pairs, 9 of which are contained within the SV40 encoded promoter region. If methylation were random, then at this level of methylation fewer than 0.1% of plasmid molecules would have all GC boxes in the promoter methylated and 90% of molecules would still have four unmethylated GC boxes. It seems unlikely that, in the present situation, inhibition of transcription can be mediated via promoter methylation.

Methylase *Sss*I has the same specificity as mammalian methylases in that it methylates all cytosines in CpG dinucleotides (Renbaum *et al.*, 1990). But it acts in a *de novo* manner, which results in a much faster and more efficient transfer of methyl groups to the DNA. Hence, it is possible to efficiently methylate CpGs in the plasmids used for the expression assays, as is demonstrated by *Hpa*II digestion and Maxam-Gilbert sequencing (Fig. 3.4 and 3.5). Complete methylation of the plasmids with M.*Sss*I led to almost complete inhibition of transcription from the SV40 early promoter (Fig. 3.6 and 3.7). This effect was only apparent when CAT activity was assayed two days after transfection. One day after transfection inhibition by methylation was less than 50% and 6 hours after transfection no differences in transcriptional activity between methylated and control plasmid were observed (Fig. 3.7). This is in concordance with results reported by Buschhausen *et al.* (1987), where methylated DNA, microinjected into nuclei of TK^- cells, was transcriptionally inactive only after chromatin formation. Methylation with the prokaryotic methylases

*Hpa*II and *Hha*I also reduced transcriptional activity of the plasmid (Fig. 3.9), despite the fact that there are no methylation sites for these enzymes in the promoter region. Thus, it appears, that promoter methylation is not required to inhibit transcriptional activity. Previously it was shown that cotransfection of unmethylated plasmid pVHC1 with oligonucleotides containing binding sites for Sp1 (the transcription factor which binds to the GC boxes) resulted in inhibition of transcription, regardless of the methylation state of the oligonucleotides (Bryans *et al.*, 1992). This indicates similar affinities of Sp1 for methylated and unmethylated DNA. In contrast, cotransfection of methylated plasmid pVHCk with methylated, CpG-rich pBluescript DNA could partially overcome the inhibitory effect imposed by DNA methylation (Fig. 3.10). This indicates that, in this system, transcriptional inhibition by DNA methylation functions via a non-specific mechanism, possibly involving the formation of chromatin and/or the assembly of methylated-DNA binding proteins (Meehan *et al.*, 1989; Jost and Hofsteenge, 1992). This is further supported by the fact that binding of Sp1 is not impaired by the methylation status of its target sites (Bryans *et al.*, 1992, Harrington *et al.*, 1988, Höller *et al.*, 1988). As Sp1 is involved in the activation of many housekeeping genes it would be detrimental if these genes were to be inactivated by random methylation. On such grounds one would not expect promoters of housekeeping genes to be controlled by DNA methylation. It is also pertinent that the SV40 enhancer is free of CpGs and so is not susceptible to inactivation by methylation. A previous study on the effect of methylation on SV40 showed that methylation of the SV40 DNA had no effect on early gene expression when the viral DNA was introduced into cells (Grässmann *et al.*, 1983). The SV40 genome is very deficient in CpGs and they are unevenly distributed. 15 (of a total of 27) CpG pairs are located in the region spanning the promoter and origin of replication (Buchman *et al.*, 1980). However, DNA methylation has a dramatic effect on transcription if the SV40 early promoter is in the environment of a CpG-rich vector. Greater inhibition is seen the greater the extent of methylation (Fig. 3.6) and this inhibition is independent of promoter methylation. The transcriptional repression may be mediated by the

formation of inactive chromatin, a condition which is characteristic for chromatin containing methylated DNA (Keshet *et al.*, 1986). The fact that inhibition upon methylation is not immediate after transfection of the DNA into cells (Fig. 3.7), may reflect the time required to allow the formation of inactive chromatin.

In conclusion, a plasmid containing the SV40 promoter linked to the CAT reporter gene is transcriptionally inhibited by DNA methylation. This is despite the fact that the SV40 promoter in its natural environment is described as being methylation insensitive. The results presented in this chapter indicate that transcriptional silencing upon DNA methylation is not dependent on promoter methylation but might be caused by the methylation of the CpG-rich vector sequences.

CHAPTER FOUR

Patch methylation

4.1 Introduction

The data from Chapter 3, together with the results described by Bryans (1989), suggest that methylation of the CpG-rich vector sequences might be responsible for the transcriptional silencing of the SV40 early promoter-CAT gene construct in transient transfection assays.

To test this hypothesis an approach was sought to specifically methylate regions of the plasmid construct and to investigate the effect of this regional DNA methylation on reporter gene expression.

Three different methods were considered to obtain regionally methylated plasmid molecules.

The first approach took advantage of the observation that binding to and methylation of double-stranded DNA by murine DNA methylase is strongly inhibited in the presence of 0.1 M NaCl (Adams *et al.*, 1990). Hence, murine DNA methylase can be directed to specifically methylate a single-stranded region in a gapped duplex molecule. The single-stranded gap can then be filled in and after transfection into cells the maintenance methylase would generate regionally methylated plasmid molecules. However, in pilot experiments it was not possible to completely inhibit the methylation of double-stranded DNA in the presence of 0.1 M NaCl as monitored by using S-adenosyl-L-[methyl-^{3}H] methionine (^{3}H-SAM) as methyl group donor (section 2.2.2.3).

The second approach involved the ligation of methylated restriction fragments into unmethylated vector DNA. Plasmid DNA was methylated to completion with M.*Sss*I, digested with the appropriate restriction enzymes and the desired fragment(s) isolated. The methylated fragments were then ligated into the appropriate, unmethylated vector fragment to re-establish the original format of the plasmid. Unfortunately, this method suffered from the fact that ligation efficiencies between parallel samples varied considerable as was monitored by transformation of bacteria.

As the two approaches mentioned above did not generate regionally methylated plasmids in sufficient quality and quantity they were discontinued and a third method employed.

4.2 Construction of regionally "patch" methylated plasmids

4.2.1 Addition of T4 gene 32 protein to methylation assays of patch duplex plasmids

Methylase *Sss*I preferentially methylates double-stranded DNA (dsDNA) and does not act on single-stranded DNA (Fig. 4.1, Renbaum *et al.*, 1990). This fact was exploited for the construction of regionally methylated DNA. As mentioned earlier, plasmid pVHCk carries the f1 origin of replication which allows the isolation of single-stranded phagemid DNA. To this single-stranded DNA (ssDNA) was then annealed a restriction fragment to generate a patched duplex molecule. The double-stranded patch can then be methylated using M.*Sss*I. Unfortunately, DNA methylation is inhibited in a mixture of dsDNA and ssDNA to around 40% of the level of dsDNA (Fig. 4.1). A possible explanation of this finding is that, although M.*Sss*I is not methylating ssDNA it binds to it and, therefore, has a reduced activity on dsDNA.

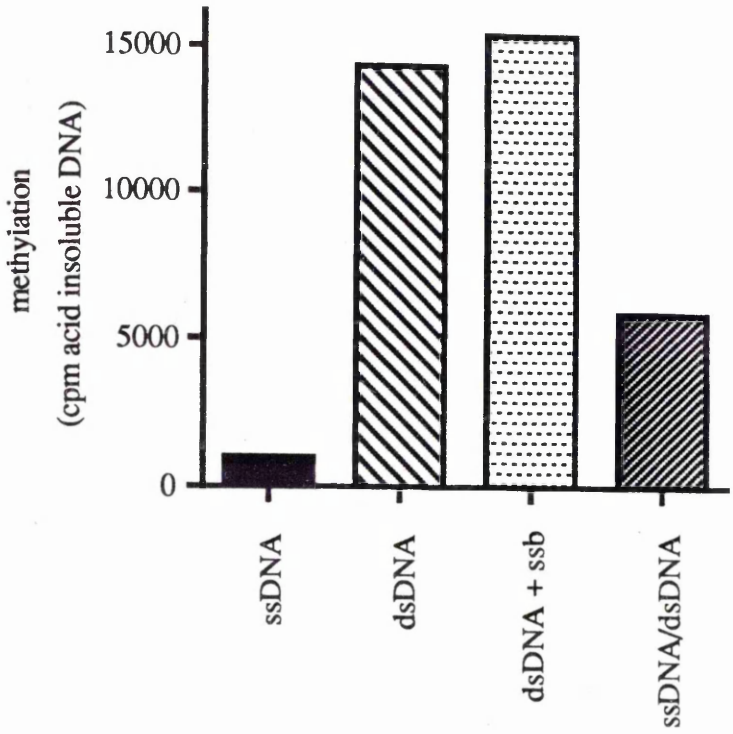


Figure 4.1 Methylation of double-stranded and single-stranded plasmid pVHCk

100 ng of DNA (single-stranded [ssDNA] or double-stranded [dsDNA] pVHCk) were methylated for 16 hours in a total volume of 20 µl with 2 units of M.*Sss*I in the presence of 5 µl S-adenosyl-L-(methyl-^{3}H) methionine (4.64 mCi/µmole, 23.3 µmole/l) under conditions described in section 2.2.2.1. The degree of methylation was quantified as acid insoluble radioactive DNA as described in section 2.2.2.3. "+ ssb" indicates the addition of 10 µg T4 gene 32 protein (Boehringer Mannheim) to the methylation assay.

However, this effect can be overcome by the addition of a protein, which binds specifically to ssDNA. This is shown in figure 4.2, where a mixture of ssDNA and dsDNA was methylated with M.*Sss*I in the presence of T4 gene 32 protein (Boehringer Mannheim). Methylation levels of the dsDNA increased about three times with increasing amounts of ssDNA binding protein. At a concentration of 25 µg/µg ssDNA a saturation level was reached as no increase in methylation was observed at higher concentrations of ssDNA binding protein. T4 gene 32 protein alone had no negative effect on methylation of dsDNA as can be seen in figure 4.1.

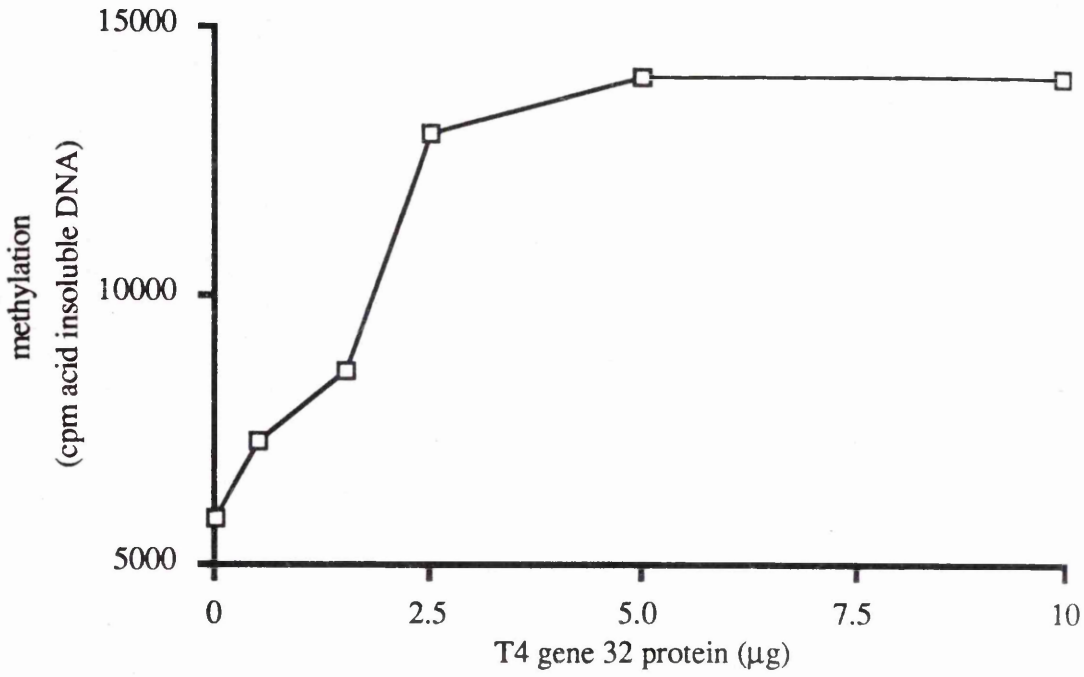


Figure 4.2 Methylation of a mixture of single-stranded and double-stranded plasmid pVHCk in the presence of T4 gene 32 protein

A mixture of single-stranded and double-stranded pVHCk (100 ng of each) was methylated as in figure 4.1 in the presence of increasing amounts of T4 gene 32 protein as indicated. The degree of methylation was quantified as acid insoluble radioactive DNA as described in section 2.2.2.3.

In turn, addition of T4 gene 32 protein reduced the low methylation levels of ssDNA by about 60% (Fig. 4.3) at concentrations of 25 µg protein per µg ssDNA.

The addition of T4 gene 32 protein in the methylation of a patched duplex molecule proved to be an essential step for the specific methylation of the double-stranded region.

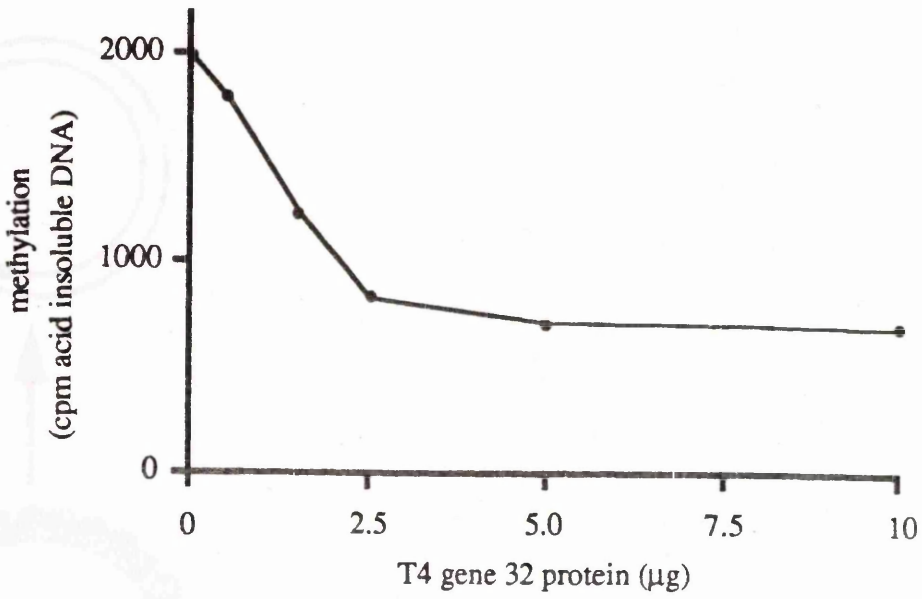


Figure 4.3 Methylation of single-stranded pVHCk in the presence of T4 gene 32 protein

100 ng of single-stranded pVHCk was methylated as in figure 4.1 in the presence of increasing amounts of T4 gene 32 protein as indicated. The degree of methylation was quantified as acid insoluble radioactive DNA as described in section 2.2.2.3.

4.2.2 Protocol for the generation of patch methylated plasmids

A schematic representation of the construction of patch methylated plasmid DNA is outlined in figure 4.4.

In detail, 5 μg (3.1 pmol) of single-stranded pVHCk were used for the generation of regionally methylated DNA. A restriction fragment was annealed at a molar ratio of 3:1 (fragment DNA:ssDNA) as follows. The ssDNA and fragment DNA were mixed, ethanol precipitated and dissolved in 50 μl annealing buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT). The DNA was heated for 2 min at 95°C and allowed to cool down from 70°C to 30°C in about one hour. After recovering the DNA by ethanol precipitation the methylation was carried out under the following conditions. The final reaction volume was 150 μl in a buffer containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT. After

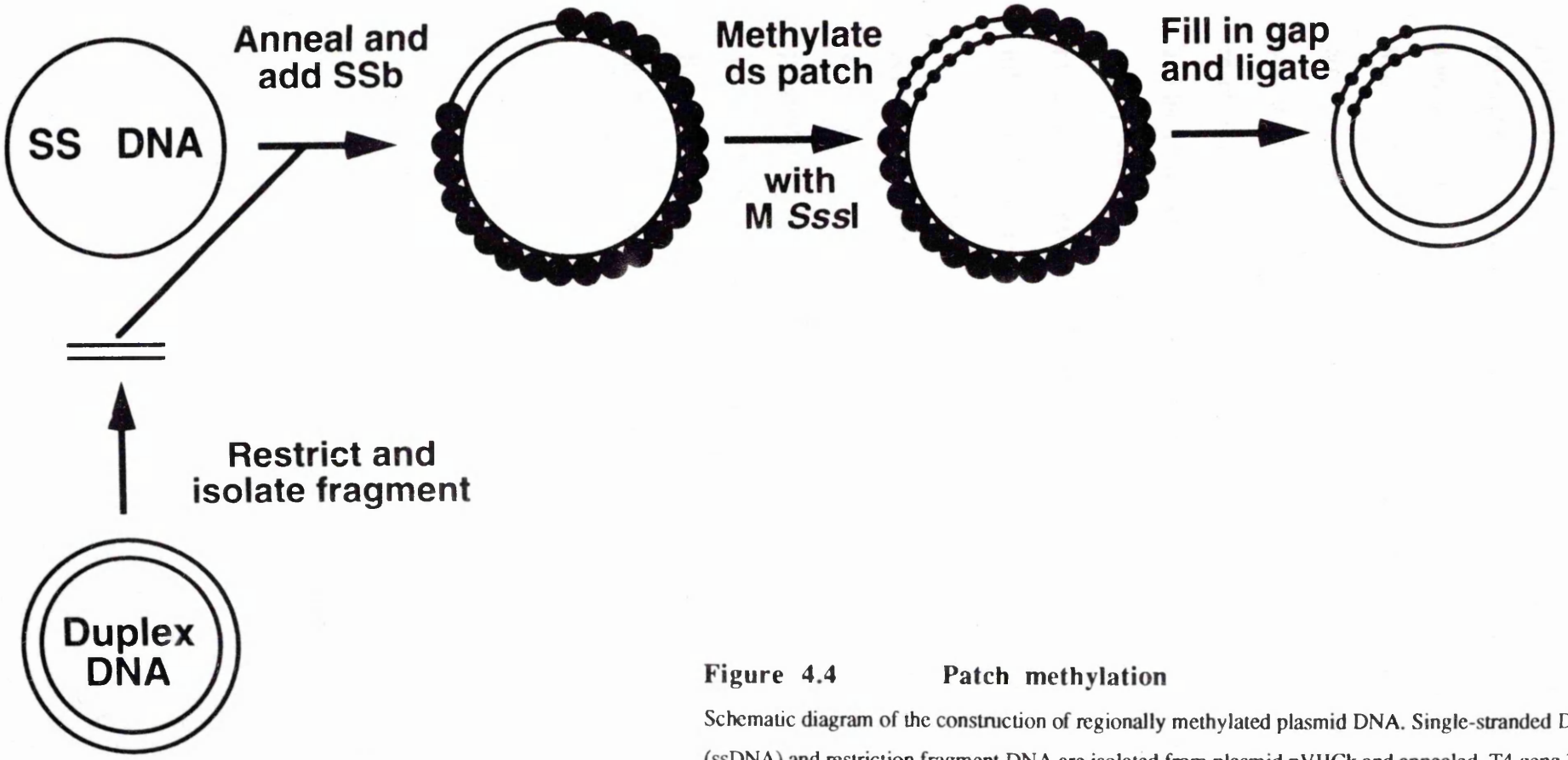


Figure 4.4 Patch methylation

Schematic diagram of the construction of regionally methylated plasmid DNA. Single-stranded DNA (ssDNA) and restriction fragment DNA are isolated from plasmid pVHCk and annealed. T4 gene 32 protein (ssb: single-stranded DNA binding protein) is added and the double-stranded (ds) patch methylated with M.*Sss*I. The plasmid is then filled in and religated.

preincubation with T4 gene 32 protein (10 μg/μg of ssDNA) at 37°C for 15 min methylase *Sss*I was added (20 units) and the reaction initiated by addition of S-adenosyl-L-methionine to a final concentration of 160 μM. Incubation was for 16 hours at 37°C. The enzyme was inactivated by adding SDS and EDTA to final concentrations of 0.5% and 5 mM, respectively. Proteinase K (Boehringer Mannheim) was added to a final concentration of 250 μg/ml and incubated for 30 min at 55°C. The reaction mixture was subjected to two phenol and two phenol/chloroform extractions and the DNA was recovered by an ethanol precipitation. The remaining single-stranded gap of the constructs was filled in under the following conditions. The reaction mixture contained 100 μM each of dATP, dCTP, dGTP and dTTP, 1 mM DTT, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 15 units of Klenow enzyme in a total volume of 100 μl. After incubation for 1 hour at 37°C, 10 units of T4 DNA ligase and ATP (1 mM) were added and the mixture incubated for 12 hours at 16°C. The DNA was purified by phenol/chloroform extraction and concentrated by ethanol precipitation. The amount of patch-methylated DNA generated in this manner was sufficient for the transfection of duplicate dishes of mouse L929 cells using the lipofection method (section 2.2.4.2). For every methylated construct, a mock-methylated control was generated in the same way as described above, but omitting the methylase in the methylation assay.

4.2.3 Southern blot analysis of patch methylated constructs

To test whether methylation was complete in the patched region the DNA was digested immediately or two days after transfection with the appropriate restriction enzymes to release the methylated region and then subjected to digestion with *Hpa*II. The patches referred to are described in Table 2 and will be considered in more detail in the next section. Figure 4.5 shows an agarose gel of the restriction digest of a patch-methylated construct. It can be seen clearly that the methylated region remains undigested by restriction enzyme *Hpa*II (Fig. 4.5, lane 6). To reveal methylation patterns of the patch-methylated plasmid two days after transfection the gel from figure 4.5 was subjected to Southern blot analysis. Figure 4.6a and figure 4.7a (using a different patch-methylated construct) show that upon *Hpa*II digestion, the methylated DNA remains undigested (lane 6), whereas control plasmid (lane 2) and mock-methylated controls (lane 4) reveal the expected digestion pattern. Furthermore, after transfecting the DNA into cells (lanes 7-10), the same digestion patterns were observed, indicating that methylation was maintained for at least two days after transfection. Before transfection, small amounts of *Hpa*II digestion products of methylated DNA are seen (lane 6). These are probably due to insufficient methylation of the large excess of fragment DNA (Fig. 4.5) used to generate double-stranded patches. This DNA becomes degraded during the transfection process and is therefore not detectable in *Hpa*II digests of methylated DNA after transfection into cells (Fig. 4.6a and 4.7a). The same blots were reprobed with DNA fragments corresponding to the unmethylated regions of the constructs (Fig. 4.6b and 4.7b). Identical digestion patterns to those seen before transfection were observed for methylated and unmethylated constructs , indicating that the regions of methylation remained unchanged even two days after transfection into cells.

* However, a small proportion of the patches did remain unmethylated (Fig. 4.6a, lane 10 and 4.7a, lane 10). This DNA could have contributed to the residual CAT activity of the patch-methylated constructs presented in Table 2.

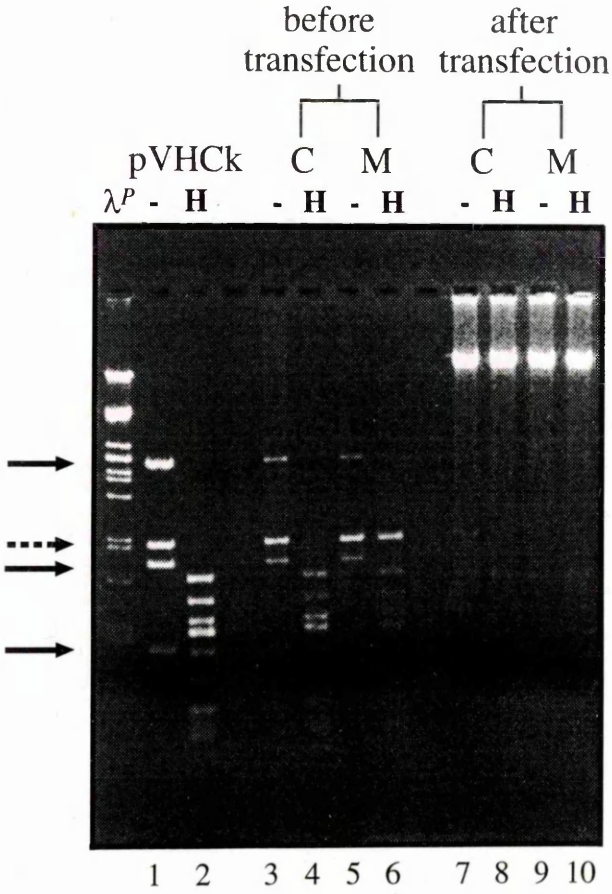


Figure 4.5 Agarose gel analysis of a patch-methylated construct

A 1.14 kb *Sca*I-*Eco*RI fragment (e in table 2) was used to generate regionally methylated plasmid DNA as described above. The DNA was restricted with the appropriate restriction enzymes as indicated and resolved on a 1.2% agarose gel. Lanes 1 and 2: control plasmid pVHCk; lanes 3 to 6: mock-methylated (C) and methylated (M) constructs before transfection into cells; lanes 7 to 10: total DNA isolated from L929 cells which had been transfected with mock-methylated (C) and methylated (M) constructs. Isolation of the DNA was 2 days after transfection into the cells and is described in section 2.2.5.2. All samples were digested with *Sca*I and *Eco*RI; arrows indicate *Sca*I-*Eco*RI digestion products; dashed arrow indicates the 1.14 kb *Sca*I-*Eco*RI fragment. H indicates digestion with *Hpa*II. λ^P indicates λ DNA digested with *Pst*I.

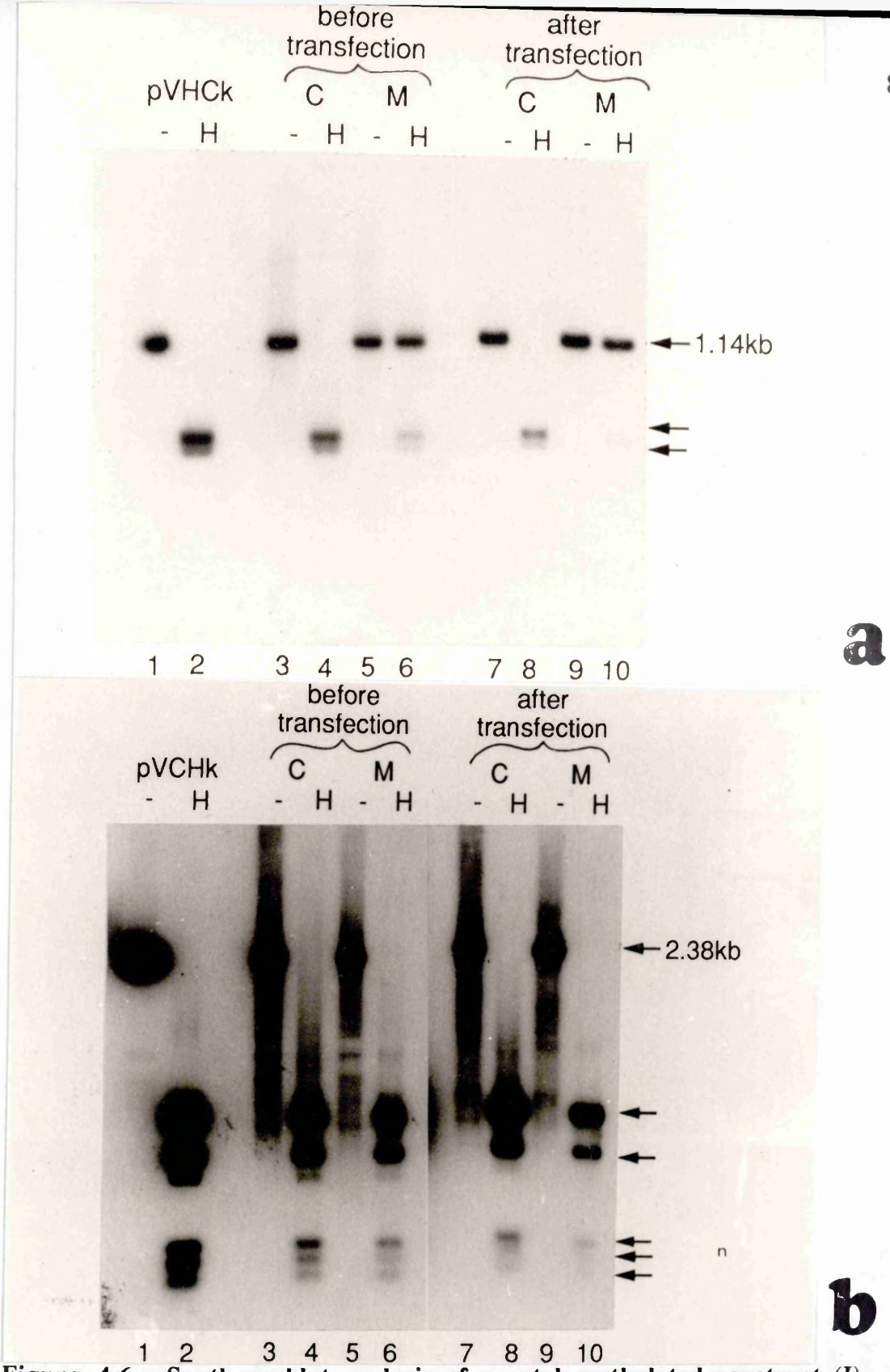


Figure 4.6 Southern blot analysis of a patch-methylated construct (I)
The agarose gel from figure 4.5 was subjected to Southern blot analysis (section 2.2.1.20). (a) The blot was hybridized with fragment e (table 2). (b) The blot from panel (a) was treated with boiling SDS solution (section 2.2.1.20) to remove the probe and reprobed with the 2.38 kb *Eco*RI-*Sca*I fragment (l in table 2). Lanes 1 and 2: control plasmid pVHCk; lanes 3 to 6: mock-methylated (C) and methylated (M) constructs before transfection into cells; lanes 7 to 10: total DNA isolated from L929 cells which had been transfected with mock-methylated (C) and methylated (M) constructs. Isolation of the DNA was 2 days after transfection into the cells and is described in section 2.2.5.2. All samples were digested with *Sca*I and *Eco*RI. H indicates digestion with *Hpa*II. Exposure of the X-ray film was longer for lanes 7 to 10 in order to obtain equal band intensities.

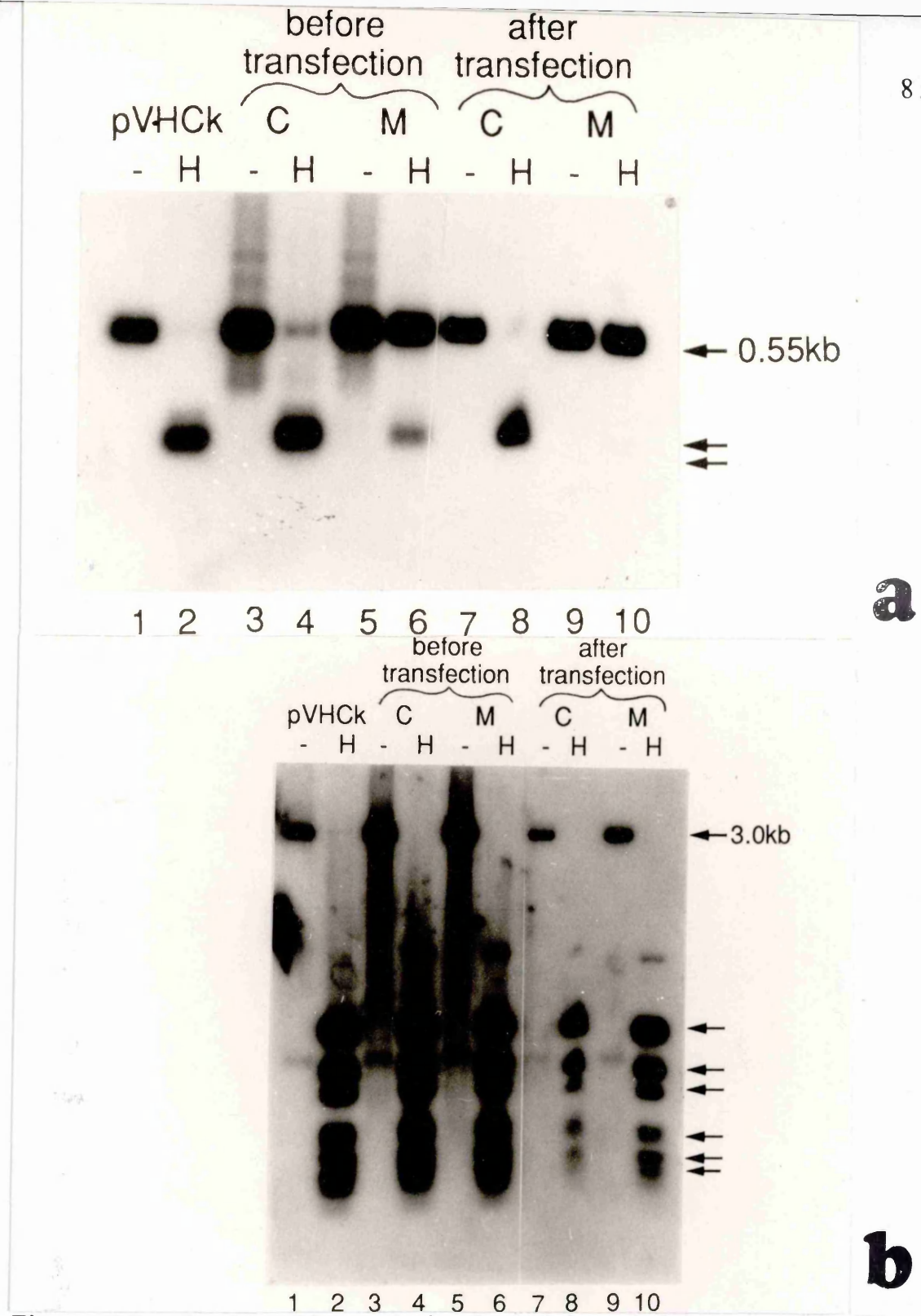
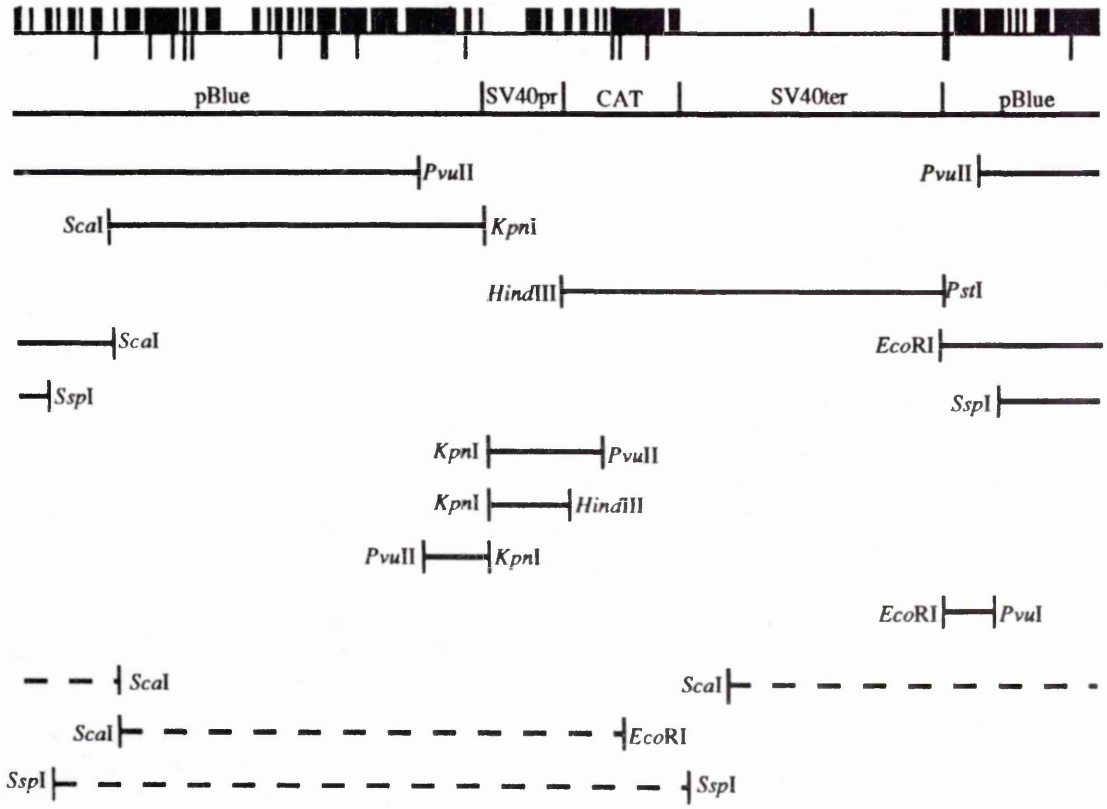


Figure 4.7 Southern blot analysis of a patch-methylated construct (II)
A regionally methylated construct was generated using a 0.55 kb *Ssp*I fragment (f in table 2). The DNA was restricted with the appropriate restriction enzymes as indicated and resolved on a 1.2% agarose gel. (**a**) The gel was subjected to Southern blot analysis and hybridized with fragment f (table 2). (**b**) The blot from panel (a) was treated with boiling SDS solution (section 2.2.1.20) to remove the probe and reprobed with a 3 kb *Ssp*I fragment (m in table 2). Lanes 1 and 2: control plasmid pVHCk; lanes 3 to 6: mock-methylated (C) and methylated (M) constructs before transfection into cells; lanes 7 to 10: total DNA isolated from L929 cells which had been transfected with mock-methylated (C) and methylated (M) constructs. Isolation of the DNA was 2 days after transfection into the cells and is described in section 2.2.5.2. All samples were digested with *Ssp*I. H indicates digestion with *Hpa*II. Exposure of the X-ray film was longer for lanes 7 to 10 in order to obtain equal band intensities.

4.3 Transient expression assays with regionally methylated plasmids

Nine different restriction fragments were used to methylate different regions of plasmid pVHCk. The patch methylated constructs were transfected into mouse L929 fibroblasts using lipofectin (section 2.2.4.2) and the effect on transcription was assayed in transient transfection assays. Table 2 gives a linear representation of the CpG-*Hpa*II map of plasmid pVHCk and the location of the restriction fragments used to generate regionally methylated plasmids. Fully methylated plasmid DNA shows around 5% CAT activity compared with the unmethylated control (Table 2, fragment a). All patch methylated constructs (fragments b-j) revealed reduced reporter gene expression in comparison with the mock-methylated constructs. Inhibition appeared not to be dependent on promoter methylation, and the location of the methylated sequences did not seem to have a significant effect. Inhibition, rather, was a function of the length or the methylcytosine content of the fragments and was independent of their location on the plasmid (Fig. 4.8).



fragment	bp	CpGs	CAT activity
a	5025	241	5 %
b	2513	164	18 %
c	1780	110	19 %
d	1770	40	20 %
e	1142	82	27 %
f	553	39	36 %
g	505	16	49 %
h	349	9	69 %
i	218	11	96 %
j	207	21	85 %
k	2140		
l	2376		
m	3008		

Table 2 Map of plasmid pVHCk and the effect of localised methylation on reporter gene expression

Above and below the top line, vertical lines show the distribution of CpG dinucleotides and *Hpa*II-*Msp*I sites, respectively. Horizontal solid lines show the restriction fragments used for the regional methylation (b to j; a is the complete plasmid); dashed lines show fragments (k to m) used as probes for hybridizations. Size, number of CpGs, and CAT activity (expressed as a percentage of the activity of the mock-methylated control) are indicated for each fragment.

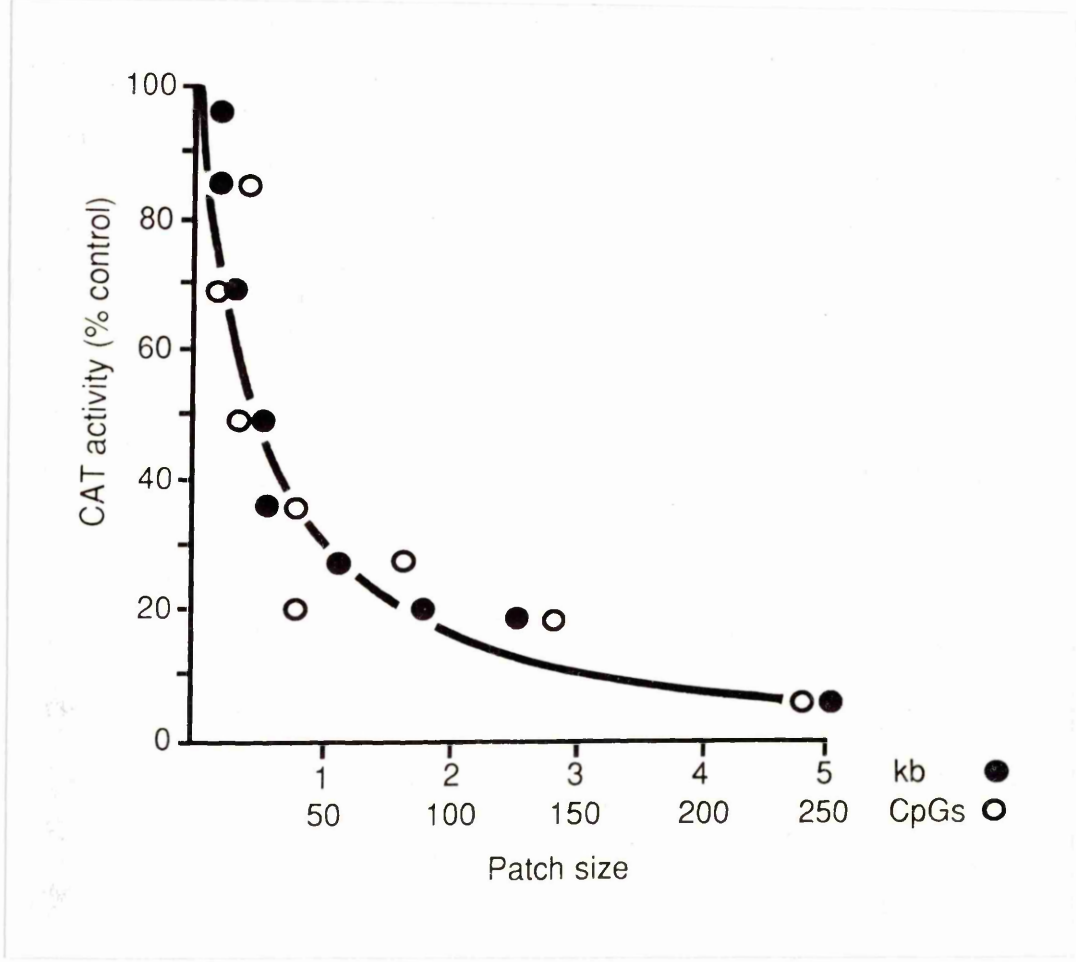


Figure 4.8 Inhibition of CAT activity is independent of the location of the methylated region

Graphical interpretation of the data from table 2 showing how CAT activity depends on the length of the methylated region (•) and its CpG content (o).

4.4 The use of patch-hemimethylated plasmids in transient expression assays

It has been reported that a hemimethylated gene construct was transcriptionally inhibited only after the formation of chromatin (Deobagkar *et al.*, 1990). Construction of regionally hemimethylated plasmid DNA can be achieved by adaptation of the protocol in section 4.2. Hence, such a construct was used to establish the effect of patch-hemimethylation by transfection into 5-aza-2'deoxycytidine treated cells.

4.4.1 Construction of patch-hemimethylated plasmid DNA

The construction of patch-hemimethylated plasmid DNA was carried out by annealing a methylated DNA fragment to unmethylated, single-stranded plasmid pVHCk as described in section 4.2. The annealed DNA was a 2.5 kb *Pvu*II fragment (b in Table 2) which was isolated from plasmid pVHCk completely methylated with M.*Sss*I. An unmethylated control construct was generated by annealing the equivalent unmethylated fragment to the ssDNA vector. The remaining gap was filled in and the constructs processed as described in section 4.2. Aliquots were digested with restriction enzymes *Pvu*II and *Hpa*II and analysed on an agarose gel. Figure 4.9 shows that the hemimethylated region (lane 4, 2.5 kb) is resistant to digestion by *Hpa*II, i.e. hemimethylated, whereas the remaining plasmid is unmethylated and therefore restricted.

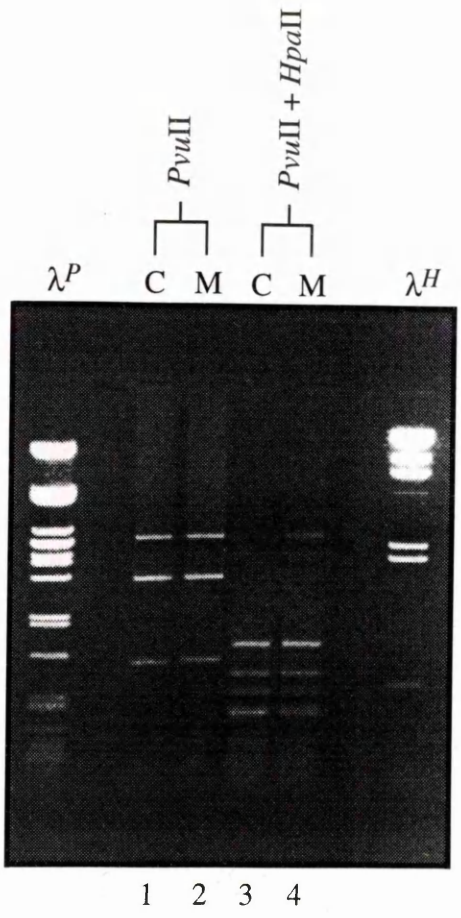


Figure 4.9 Analysis of a patch-hemimethylated construct

A 2.5 kb *Pvu*II fragment (b in table 2) was used to generate patch-hemimethylated DNA as described in section 4.4.1. Aliquots of the constructs were digested with *Pvu*II and *Hpa*II and resolved on a 1.5% agarose gel. Lanes 1 and 2: digestion with *Pvu*II; lanes 3 and 4: digestion with *Pvu*II and *Hpa*II; M indicates methylated; C indicates mock-methylated control; size markers are λ-DNA digested with *Pst*I (left) and *Hind*III (right), respectively.

4.4.2 Methylase activity of L929 cells treated with 5-aza-2'-deoxycytidine

Hemimethylated DNA is the preferred substrate for mammalian DNA methyltransferases (Adams and Burdon, 1985) and it has been reported that

hemimethylated plasmid DNA, transfected into cells can become symmetrically methylated even in the absence of replication (Sandberg *et al.*, 1991). It is therefore necessary to efficiently inhibit the maintenance methylase of the cells to maintain the methylation status of the hemimethylated plasmid DNA after transfection. The methylase inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) was used to reduce this activity when hemimethylated plasmids were used in transient transfection assays. 5-aza-dC is incorporated into replicating DNA and inhibits DNA methylase by immobilizing the enzyme, thus reducing subsequent methylation of newly synthesized or externally introduced DNA (Jones *et al.*, 1982, Taylor and Jones, 1982; section 1.1.3).

To test the effect of the methylase inhibitor under conditions used for the transfection of plasmid DNA, L929 cells were grown as described in section 2.2.4.1 in the presence of 5-aza-dC (1 μM). Cells were harvested after 24 and 48 hours exposure to 5-aza-dC and methylase activity assayed as described in section 2.2.2.4. Figure 4.10 shows that after 24 hours exposure to 5-aza-dC methylase activity of the extracts was reduced to around 20% of the control and that this inhibition increased only slightly after 48 hours exposure (17% of control). Methylase activity appears to be considerably reduced in 5-aza-2'-deoxycytidine treated cells. Increased overall levels of methylase activity at 48 hours of exposure to 5-aza-dC are due to the continuing growth of the cells.

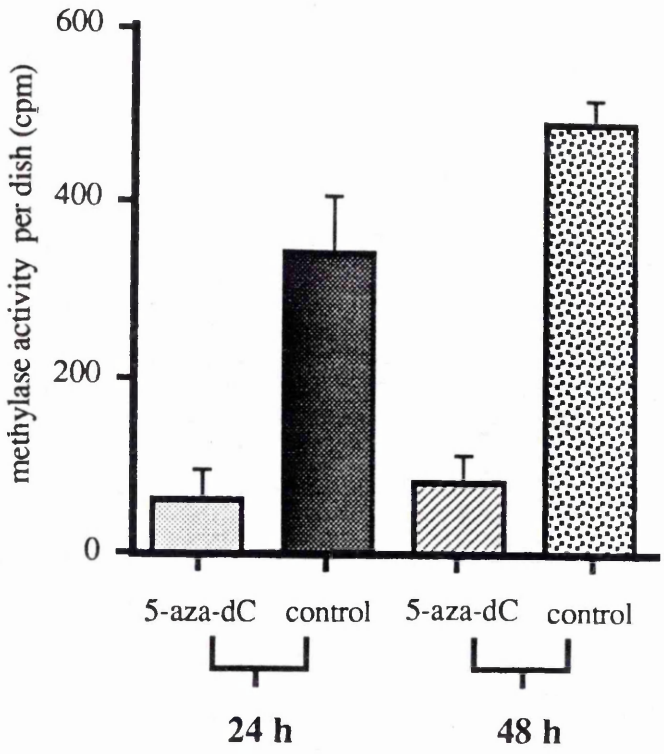


Figure 4.10 Methylase activity of cells treated with 5-aza-2'-deoxycytidine (5-aza-dC)

Mouse L929 cells were seeded as described in section 2.2.4.1 and grown in the presence of 1 µM 5-aza-dC. Cells were harvested and methylase activity assayed 24 and 48 hours after exposure to 5-aza-dC (section 2.2.2.4). Methylase activity was assayed per dish of cells and is expressed as acid insoluble radioactive DNA (cpm). Control indicates cells grown without 5-aza-dC.

4.4.3 Transient expression assays with patch-hemimethylated plasmids

Plasmid pVHCk, hemimethylated at a region of 2.5 kb length (fragment b, table 2) was introduced into mouse L929 fibroblast cells grown in the presence or absence of the DNA methylase inhibitor 5-aza-2'-deoxycytidine (section 4.4.2). Cells were harvested and CAT activity determined as described in section 2.2.4.3. Figure 4.11 shows that hemimethylation of the 2.5 kb region of plasmid pVHCk reduces transcription from the CAT gene to 26% in the presence of 5-aza-dC. Cells transfected without the methylase inhibitor showed CAT activity of 35% compared to the mock-

methylated control. However, transcriptional activity of the cells treated with 5-aza-dC was only about 80% of that of the non-treated, which is due to the toxicity of the drug at the concentrations used (Jones, 1984).

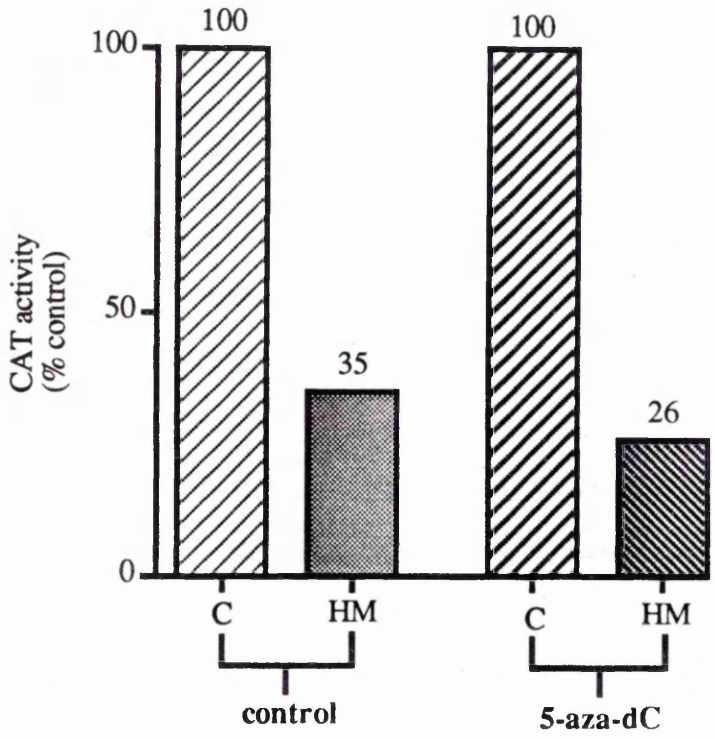


Figure 4.11 Transcriptional activity of patch-hemimethylated constructs

5 µg of patch-hemimethylated construct (section 4.4.1) was transfected into mouse L929 fibroblasts and CAT activity assayed 48 hours after transfection as described in sections 2.2.4.2 and 2.2.4.3. Inhibition of CAT activity by hemimethylation (HM) is expressed as percentage of the activity of the mock-methylated control (C). control: cells grown without 5-aza-dC; 5-aza-dC: cells grown in the presence of 5-aza-dC.

4.5 Discussion

Previously, several groups (Busslinger *et al.*, 1983; Keshet *et al.*, 1985; Murray and Grosveld, 1987) used a method to generate regionally methylated constructs whereby single-stranded M13 templates were used to synthesise, *in vitro*,

hemimethylated DNA by incorporation of 5-methyl dCTP and the three other deoxynucleotide triphosphates into the minus strand of the constructs. The unmethylated region of these constructs was defined by the location and the size of the primer (oligonucleotide or restriction fragment). The resulting molecules contained methyl residues at every cytosine (not only at CpG dinucleotides) of the newly synthesised DNA strand, while the template strand remained unmodified. After stable transfection of these constructs into cells the template strand of this hemimethylated DNA became methylated by the cellular DNA methyltransferase at most of their recognition sites, that is predominantly at CpG residues (Stein *et al.*, 1982). Furthermore, methylation at sites other than CpG was lost and, once established, the methylation pattern was stably maintained in successive cell generations.

This method required the stable integration of the hemimethylated construct into the cellular DNA and therefore was not suitable for transient transfection assays.

A new method to methylate specific regions in a plasmid molecule has been described in this chapter. Restriction fragments were annealed to ssDNA and T4 gene 32 protein, a protein which binds specifically to ssDNA was added to prevent methylase *Sss*I from binding to and methylating the single-stranded gap. The double-stranded patch was then methylated with M.*Sss*I and the remaining gap filled in and ligated (Fig. 4.4). The method yields plasmids with methylated patches (i.e. patches of DNA sequence in which all CpGs are methylated on both strands). The methylation is confined to the patched region and is stable in the preimposed pattern for at least 2 days *in vivo* (Fig. 4.6 and 4.7). Hence, regionally methylated plasmids generated in this manner are suitable for use in transient transfection assays.

The hypothesis that methylated, CpG-rich vector sequences impair the transcriptional activity of the reporter gene constructs was confirmed in transient expression assays using patch-methylated plasmids. Nine different constructs were generated to establish the effect of DNA methylation of non-promoter sequences on transcription from the SV40 early promoter. All of them showed reduced CAT activity

compared with the mock-methylated controls (Table 2). The extent of inhibition appeared to be independent of the position of the methylated patch, as exemplified by patches f and g (Table 2); these patches are of similar size, but the latter covers the promoter region whereas the former is at the opposite site of the plasmid. In addition, it seems that the contribution of CpG density is a secondary effect as the CpG-rich fragment j is less inhibitory than the 2.5-fold larger fragment g of lower CpG density. This is further supported in figure 4.8, which shows percentage inhibition as a function of both fragment length and CpG content. Although most points fall on a smooth curve, deviations are found with constructs having patches of similar CpG content but of different size.

The experiments in section 4.4 demonstrate that even hemimethylation of a region in plasmid pVHCk inhibits transcription in transient transfection assays. Patch-hemimethylation of a 2.5 kb *Pvu*II fragment results in 26% CAT activity compared to the control (Fig. 4.11), whereas full methylation of this region reduces transcriptional activity to 18% of the mock-methylated construct (Table 2). This is in concordance with results reported by Deobagkar *et al.* (1990), who showed that a hemimethylated construct containing the herpes simplex virus thymidine kinase gene was not expressed when microinjected into cells. However, inhibition was only evident after chromatin formation had occurred, either *in vitro* or *in vivo*. In contrast, Sasaki *et al.* (1992a) found a correlation between hemimethylation and *Msp*I hypersensitivity for the 5´CpG island of the hypoxanthine phosphoribosyltransferase (*hprt*) gene and concluded that the formation of inactive chromatin requires symmetrically methylated DNA. For the experiment described in figure 4.11 it was not established whether inhibition of the cellular DNA methyltransferase by 5-aza-dC was sufficient to prevent maintenance methylation of the patch-hemimethylated plasmid (although methylase activity in treated cells was down to less than 20% compared to untreated cells). The effect of hemimethylated DNA on gene expression is even less understood than that of symmetrically methylated DNA and it remains to be seen whether it contributes to transcriptional silencing of vertebrate genes.

There is conflicting evidence as to whether DNA methylation of non-promoter sequences has a negative effect on transcription. Previously it has been reported that vector methylation does not affect the expression of the human γ-globin or the hamster *aprt*-gene when these are integrated into the host chromosome, whereas methylation of the 3′ region of the herpes *tk*-gene does inhibit its expression (Busslinger *et al.*, 1983, Keshet *et al.*, 1985). In addition, DNA methylation of single *Hpa*II sites within the coding region of the HSV-*tk* gene caused inactivation after microinjection into TK$^-$ cells (Grässmann *et al.*, 1993) and Yisraeli *et al.* (1988) showed that both the 5′ and the 3′ region of the β-globin gene down regulate transcription if methylated. More recently, Levine *et al.* (1992) used M.*Hpa*II, M.*Hha*I, M.*Fnu*DII but not M. *Sss*I to methylate whole constructs having inserts within the preinitiation domain of the SV40 promoter. This resulted in transcriptional inhibition whereas the effect of methylation of inserts further upstream or downstream of the TATA box was lower. Binding of TFIIA and TFIID was not affected by DNA methylation suggesting an indirect mechanism of inhibition. In contrast, the results presented in this chapter, which also point to an indirect effect, suggest that promoter methylation in a CpG rich methylated environment is not required for transcription inhibition. Non-promoter regions methylated using M.*Hpa*II (16 sites) and M.*Hha*I (14 sites) reduce transcriptional activity by around 30% (section 3.4), those methylated by M.*Sss*I, which simulates eukaryotic methylases in methylating all CpGs, lead to gene inactivation.

In summary, evidence is presented in this chapter for the causal relationship between vector methylation and transcriptional inactivation of the SV40 promoter in transient transfection assays.

CHAPTER FIVE

Chromatin structure of methylated plasmids

5.1 Introduction

Several studies suggest that methylation of DNA is accompanied by a change in chromatin structure, and that there is a causal relationship between chromatin structure and transcriptional inhibition by methylation (section 1.5).

The SV40 early promoter has been reported to be methylation insensitive (Fradin *et al.*, 1982; Grässmann *et al.*, 1983) but, placed in a methylated, CpG-rich environment, transcription is strongly inhibited (Chapter 3 and 4). This points to indirect inactivation, which might be mediated by the formation of inactive chromatin.

In this chapter several experiments are presented, which aim to unravel the state of chromatin of transiently transfected plasmids, and to highlight any differences between methylated and unmethylated plasmid chromatin. The analysis of chromatin structure often involves the use of DNA nucleases, as active (open) chromatin is known to be nuclease hypersensitive, whereas inactive (closed) chromatin shows a lesser degree of sensitivity (Gross and Garrard, 1988). Therefore, different nucleases were used to investigate the chromatin structure of transiently transfected plasmids.

5.2 Analysis of the DNA conformation of methylated plasmids *in vivo*

The following experiment was carried out to exclude the possibility that methylated plasmid DNA forms a conformation which is different from that of

unmethylated plasmid DNA after transfection into cells. Plasmid pVHCk (completely methylated or unmethylated) was transfected into mouse L929 fibroblasts and nuclear DNA was isolated two days after transfection (section 2.2.5.2). The DNA was resolved on an agarose gel and transfected plasmid visualized by Southern blot analysis (Fig. 5.1).

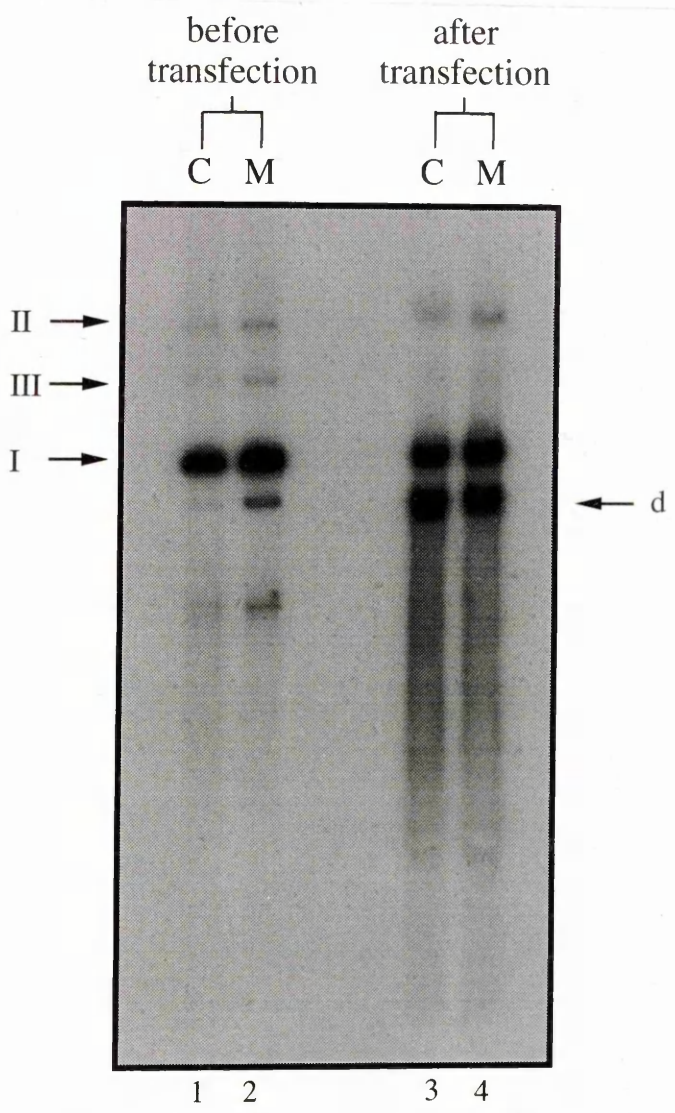


Figure 5.1 DNA conformation of transiently transfected plasmids

Plasmid pVHCk was transfected into mouse L929 fibroblasts using the calcium phosphate coprecipitation method (section 2.2.4.1). Total DNA was isolated 48 hours after transfection (section 2.2.5.2) and resolved on a 0.7% agarose gel. The gel was subjected to Southern blot analysis (section 2.2.1.20) using plasmid pVHCk as a hybridization probe. Lanes 1 and 2: plasmid before transfection; lanes 3 and 4: plasmid after transfection; C: control; M: methylated. I, II, and III refer to supercoiled, relaxed circular, and linear forms of pVHCk, respectively. d refers to (possibly) degraded DNA.

Although changes in conformation between plasmid DNA before and after transfection were observed, no differences were visible between methylated (lane 4) and unmethylated plasmid (lane 3) after transfection into cells. The nature of the plasmid DNA in band "d" (lane 3 and 4) is unknown, but may represent degraded DNA caused by nucleases present in the DNA preparation.

5.3 Micrococcal nuclease analysis of methylated plasmids

Nuclear DNA is organized in a nucleosomal structure, with repeating units of nucleosome core particles alternating with stretches of linker DNA. A nucleosomes core particle consists of 146 bp of DNA wrapped approximately twice around an octamer of core histones. To reveal the pattern of repeating nucleosomal subunits, micrococcal nuclease is widely used as it cleaves chromatin in the most accessible DNA, that is the linker DNA. Therefore, digestion of nucleosomal DNA with micrococcal nuclease under mild conditions generates bands that are separated from each other by a single nucleosome repeat length of DNA, known as the nucleosomal ladder (Wolffe, 1992).

The intention of the following experiment was to reveal whether plasmid DNA, transfected into mouse L929 fibroblasts adopts a nucleosomal chromatin structure and if so, whether differences can be observed between methylated and unmethylated plasmid DNA.

Mouse L929 cells were transfected with methylated and mock-methylated plasmid pVHCk. Cells were harvested 2 days after transfection and nuclei isolated as described in section 2.2.5.1. Digestion with micrococcal nuclease was for 0, 1 or 2 min as described in section 2.2.5.3. The purified DNA was then separated on an agarose gel (Fig. 5.2) which showed the expected nucleosomal ladder for the cellular DNA. However, when the gel was subjected to Southern blot analysis (Fig. 5.3) it was apparent that neither methylated nor unmethylated plasmid DNA followed that

pattern. However, the plasmids were not readily degraded by micrococcal nuclease but a resistant region of about 300 to 700 bp was apparent for both, methylated and unmethylated plasmid.

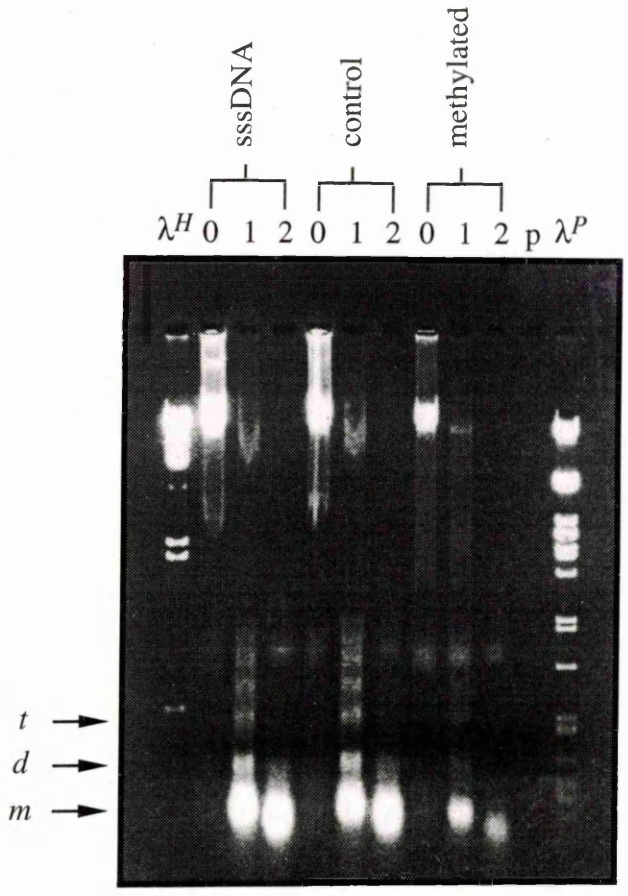


Figure 5.2 Micrococcal nuclease digestion of transfected nuclei

L929 mouse fibroblasts were transfected with completely methylated or mock-methylated (control) plasmid pVHCk (or carrier DNA-"sssDNA") and nuclei isolated 48 hours after transfection as described in section 2.2.4.1 and 2.2.5.1. Nuclei were digested with micrococcal nuclease for 0, 1, or 2 min as described in section 2.2.5.3. Total DNA was isolated from the nuclei and separated on a 1.5% agarose gel. λ^H and λ^P: λ-DNA digested with *Hind*III (left) and *Pst*I (right), respectively; p: pVHCk; sssDNA: sonicated salmon sperm DNA; *m, d, and t*: mono, di, and trinucleosomes.

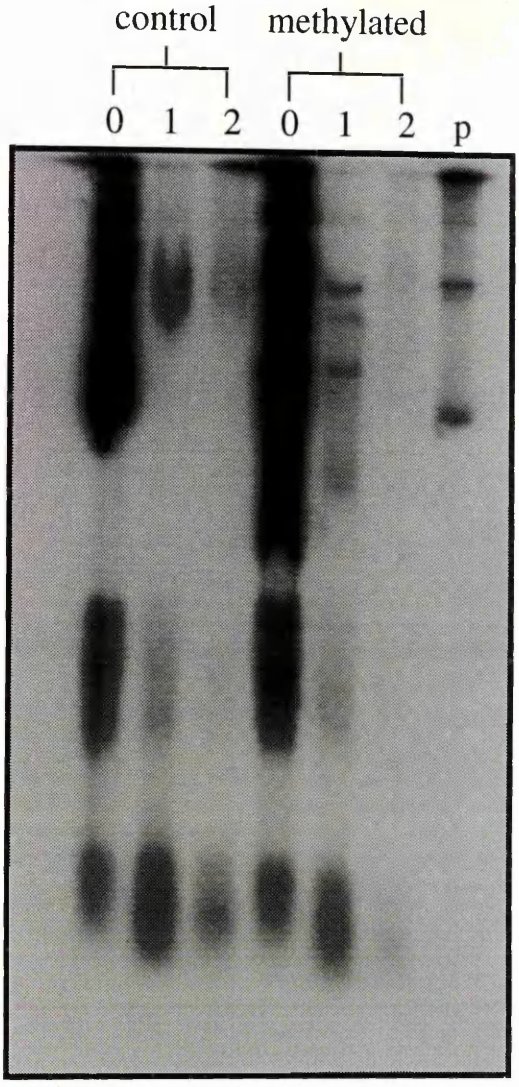


Figure 5.3 Micrococcal nuclease digest of transfected plasmid

The gel from figure 5.2 was subjected to Southern blot analysis and hybridized using plasmid pVHCk as a probe. 0, 1, 2: min digestion with micrococcal nuclease; p: pVHCk; (the left part of the autoradiograph [Fig. 5.2, sssDNA] was omitted as no signals were obtained).

5.4 *Msp*I digestion assay of methylated plasmids

Chromatin containing methylated DNA is relatively insensitive to digestion by *Msp*I and this has been used to advantage in a number of studies (Antequera *et al.*, 1989; Levine *et al.*, 1991; Sasaki *et al.*, 1992a). *Msp*I cleaves the sequence 5′-CCGG-3′ but is not affected by methylation of the inner cytosine. The use of *Msp*I therefore

allows one to distinguish between the nuclease sensitivity of methylated and unmethylated DNA at methylation sites *in vivo*.

5.4.1 *Msp*I digestion of methylated plasmids at different times after transfection

It was shown in section 3.3 that transcriptional inhibition upon DNA methylation was apparent only 24 to 48 hours after transfection, and it was argued that this time might be required to allow the formation of inactive chromatin. To address this question, cells were transfected with methylated and unmethylated plasmid pVHCk and harvested at three different times after transfection. Isolated nuclei were then subjected to digestion with *Msp*I as described in section 2.2.5. Total DNA was isolated from the nuclei and separated on agarose gels. Figure 5.4 is a photograph of a typical gel (the gel from the Southern blot in Fig. 5.5c) and shows that the CpG-deficient cellular DNA in chromatin becomes only slightly digested by *Msp*I under the conditions used. To visualize the restriction pattern of the plasmid DNA the gels were subjected to Southern blot analysis.

The sequence of events is confirmed by the results shown in figure 5.5. At 6 hours after transfection (Fig. 5.5a), the plasmids are still extremely sensitive to digestion with *Msp*I and there is no difference between methylated and mock-methylated DNA. By 24 hours (Fig. 5.5b), both the methylated and unmethylated plasmids are clearly more resistant to digestion than at 6 hours after transfection, but only minor differences can be observed between control and methylated samples. At 48 hours after transfection (Fig. 5.5c), the methylated plasmid adopts a highly resistant chromatin structure compared with the mock-methylated control, which coincides with its low transcription levels (Fig. 3.7).

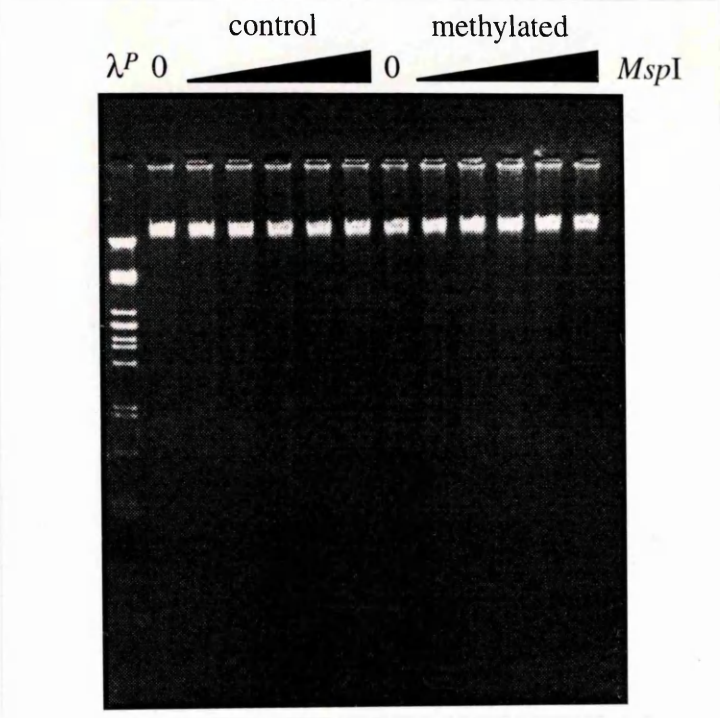


Figure 5.4 *Msp*I digestion of transfected nuclei
L929 mouse fibroblasts were transfected with completely methylated and mock-methylated (control) plasmid pVHCk and nuclei isolated 48 hours after transfection as described in section 2.2.4.2 and 2.2.5.1. Nuclei were digested with increasing amounts of *Msp*I (0.75, 1.5, 4.5, 7.5, and 15 units/150 µl) as described in section 2.2.5.3 and total DNA was isolated, and separated on a 1.5% agarose gel. λ^P: λ-DNA digested with *Pst*I.

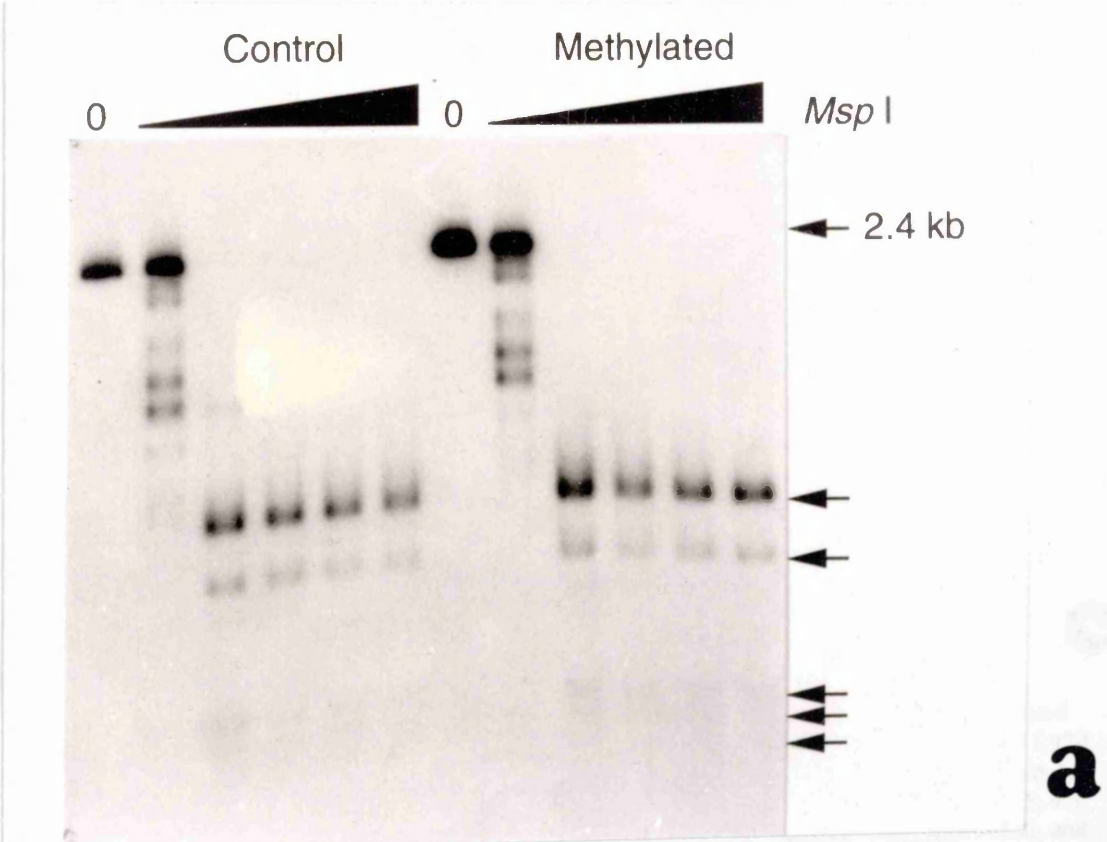


Figure 5.5a **for legend see next page**

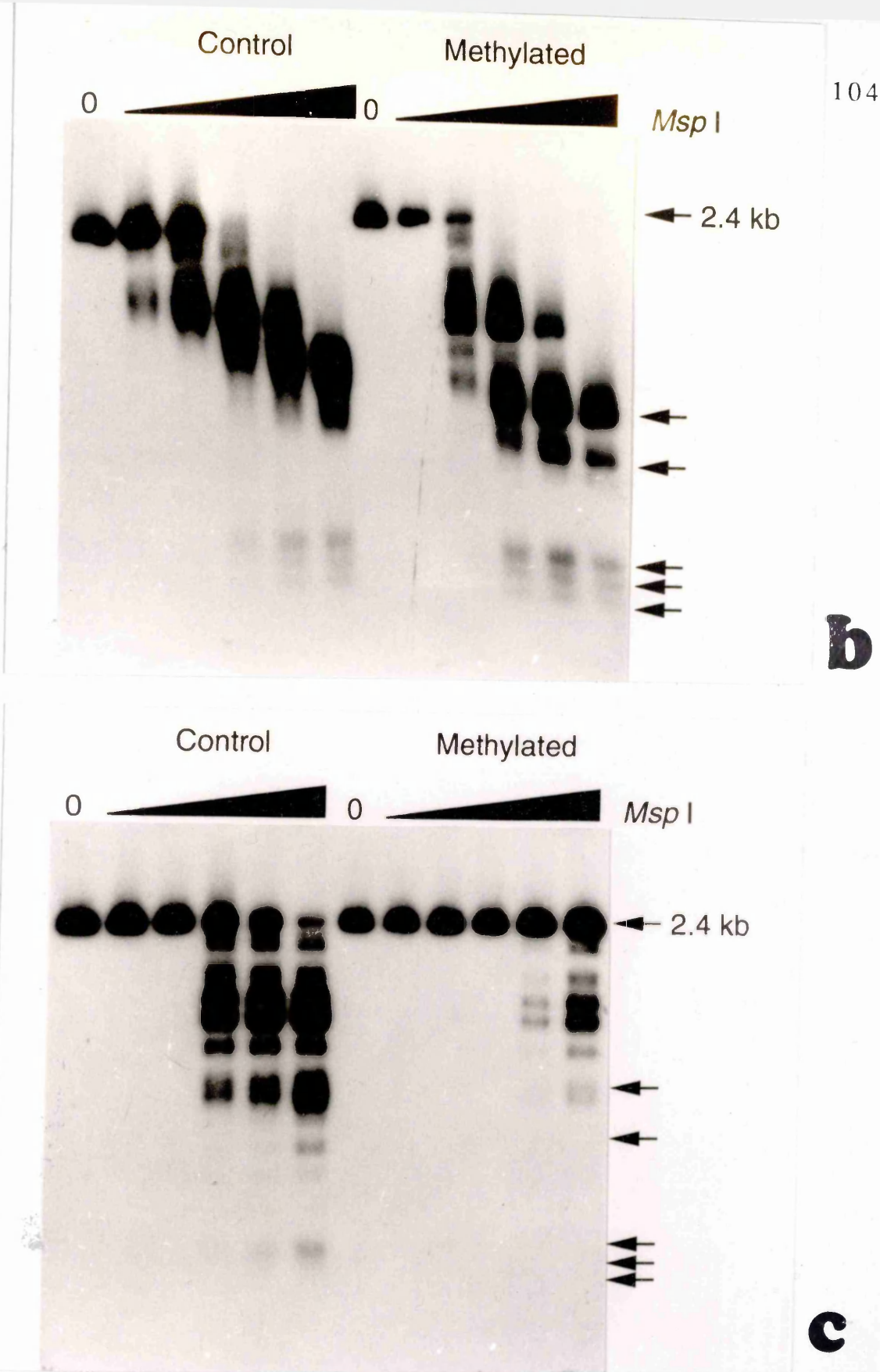
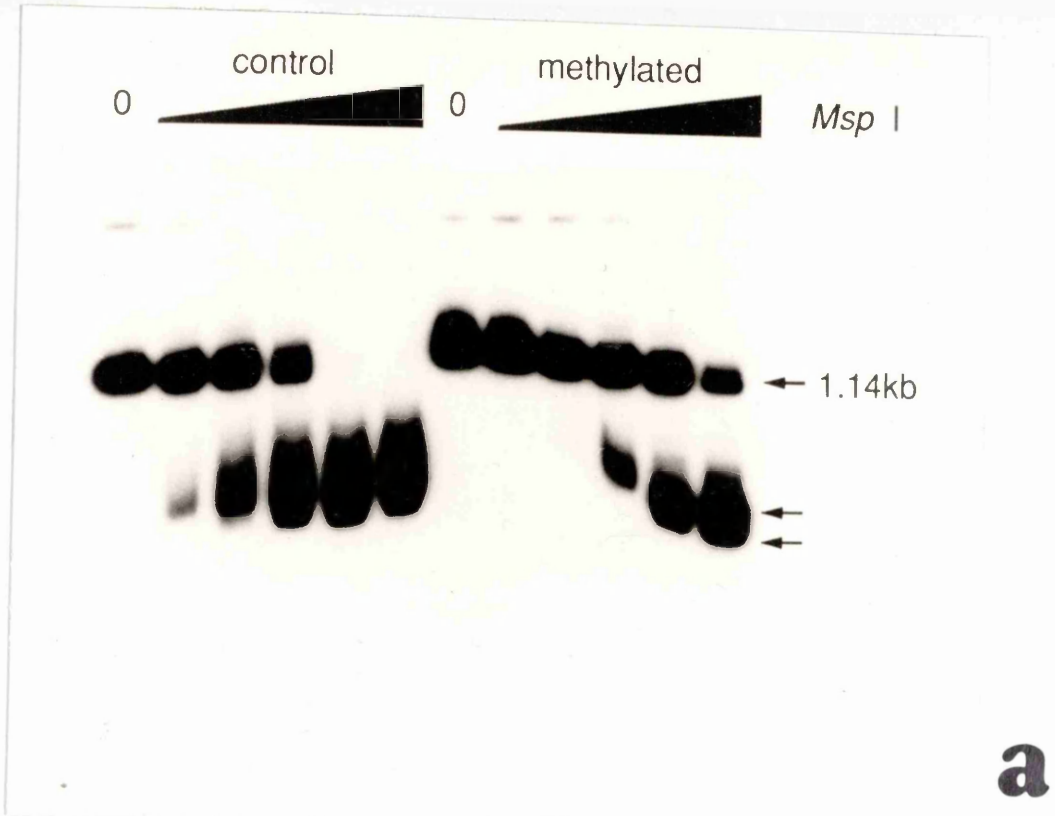


Figure 5.5 Time course chromatin formation of transfected plasmid
Completely methylated and mock-methylated (control) plasmid pVHCk was transfected into L929 cells as described in section 2.2.4.2. Cells were harvested 6 hours (**a**), 24 hours (**b**) and 48 hours (**c**) after transfection. Nuclei were isolated and digested with increasing amounts of *Msp*I (0.75, 1.5, 4.5, 7.5, and 15 units/150 µl, section 2.2.5.3), total DNA was purified and digested with *Eco*RI-*Sca*I, and the resulting Southern blot was probed with fragment 1 (Table 2).

5.4.2 *Msp*I digestion of regionally methylated plasmids

The results from section 5.4.1 strongly suggest that completely methylated plasmid DNA develops a nuclease resistant chromatin structure two days after transfection into mouse L929 cells. To test the effect of regional methylation on the chromatin structure of the gene and its promoter, patch-methylated constructs (section 4.2) were used in *Msp*I protection assays (section 2.2.5).

Figures 5.6, 5.7 and 5.8 give three examples of the results, which show clearly that the methylated region of a patch-methylated construct has adopted an *Msp*I-resistant, inactive chromatin structure compared with the mock-methylated control construct (Fig. 5.6a, 5.7a and 5.8a). The Southern blots were then stripped off and hybridized with a second probe to reveal the digestion patterns of the unmethylated regions in the patch-methylated constructs (Fig. 5.6b, 5.7b and 5.8b). Surprisingly, these regions too were less sensitive to digestion with *Msp*I than were the unmethylated controls. Therefore it appears that methylation in one region of a plasmid can affect the chromatin structure of an unmethylated region on the same plasmid.



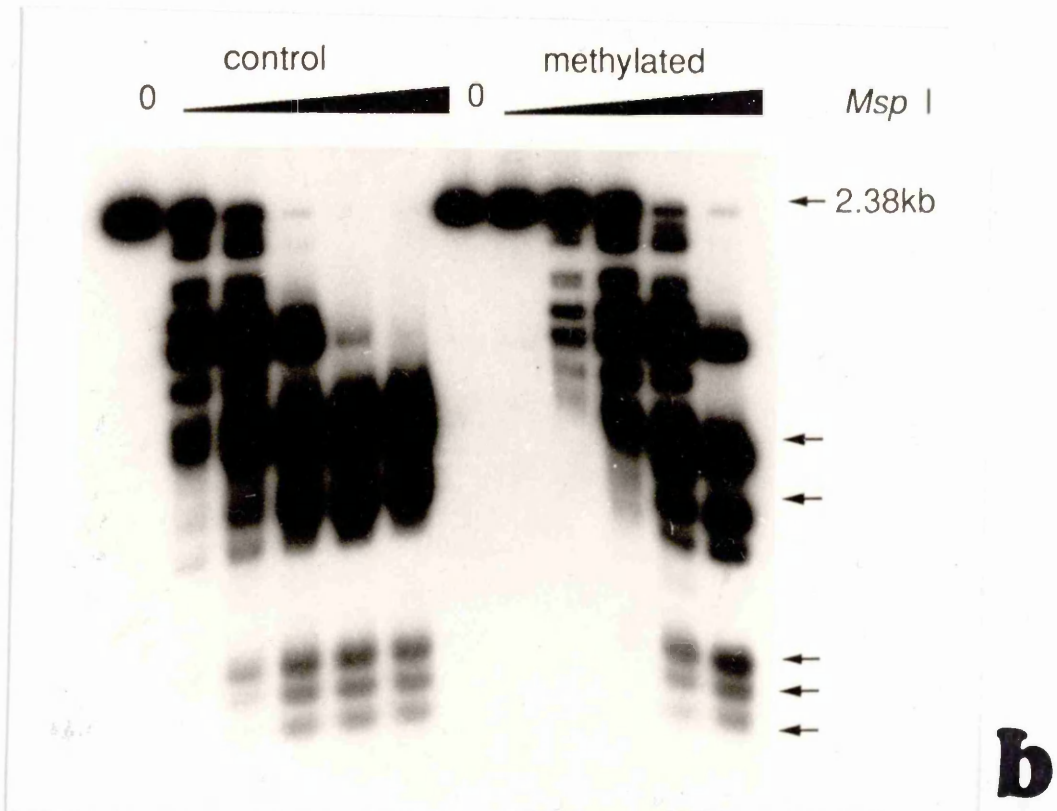


Figure 5.6 ***Msp*I digestion of nuclei transfected with patch methylated constructs (I)**

L929 cells were transfected with 5 µg of regionally methylated or mock-methylated (control) constructs (using fragment e, Table 2) as described in section 2.2.4.2. At 48 hours after transfection, nuclei were isolated and aliquots digested with increasing amounts of *Msp*I (0.75, 1.5, 4.5, 7.5, and 15 units/150 µl; section 2.2.5.3). Total DNA was purified and digested with *Eco*RI-*Sca*I, separated on a 1.5% agarose gel and subjected to Southern blot analysis. (**a**) The blot was probed with fragment e (table 2). (**b**) The blot from panel (a) was treated with boiling SDS solution (0.5%) to remove the probe and reprobed with a 2.38 kb *Eco*RI-*Sca*I fragment (I in Table 2) in order to show *Msp*I digestion patterns of unmethylated sequences of the constructs.

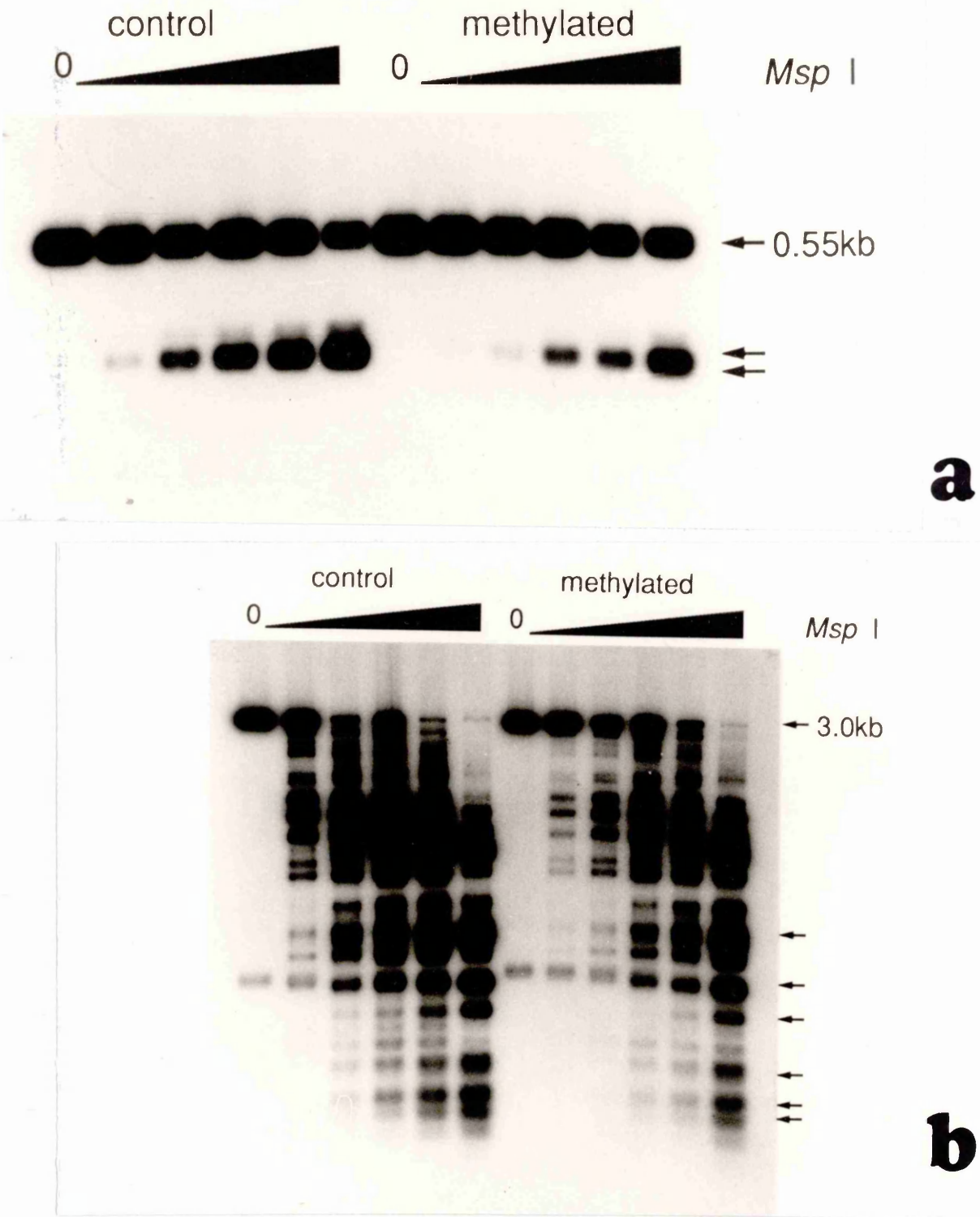


Figure 5.7 ***MspI* digestion of nuclei transfected with patch methylated constructs (II)**

As figure 5.6 except that a 0.55 kb *Ssp*I fragment (f in Table 2) was used to construct regionally methylated plasmids. L929 cells were transfected with 5 µg of regionally methylated or mock-methylated (control) constructs as described in section 2.2.4.2. At 48 hours after transfection, nuclei were isolated and aliquots digested with increasing amounts of *Msp*I (0.15, 0.75, 1.5, 7.5, and 15 units/150 µl section 2.2.5.3). Accordingly, isolated total DNA was digested with *Ssp*I, separated on a 1.5% agarose gel and subjected to Southern blot analysis. (**a**) The blot was probed with fragment f (Table 2). (**b**) The blot from panel (a) was treated with boiling SDS solution (0.5%) to remove the probe and reprobed with a 3 kb *Ssp*I fragment (m in table 2) in order to show *Msp*I digestion patterns of unmethylated sequences of the constructs.

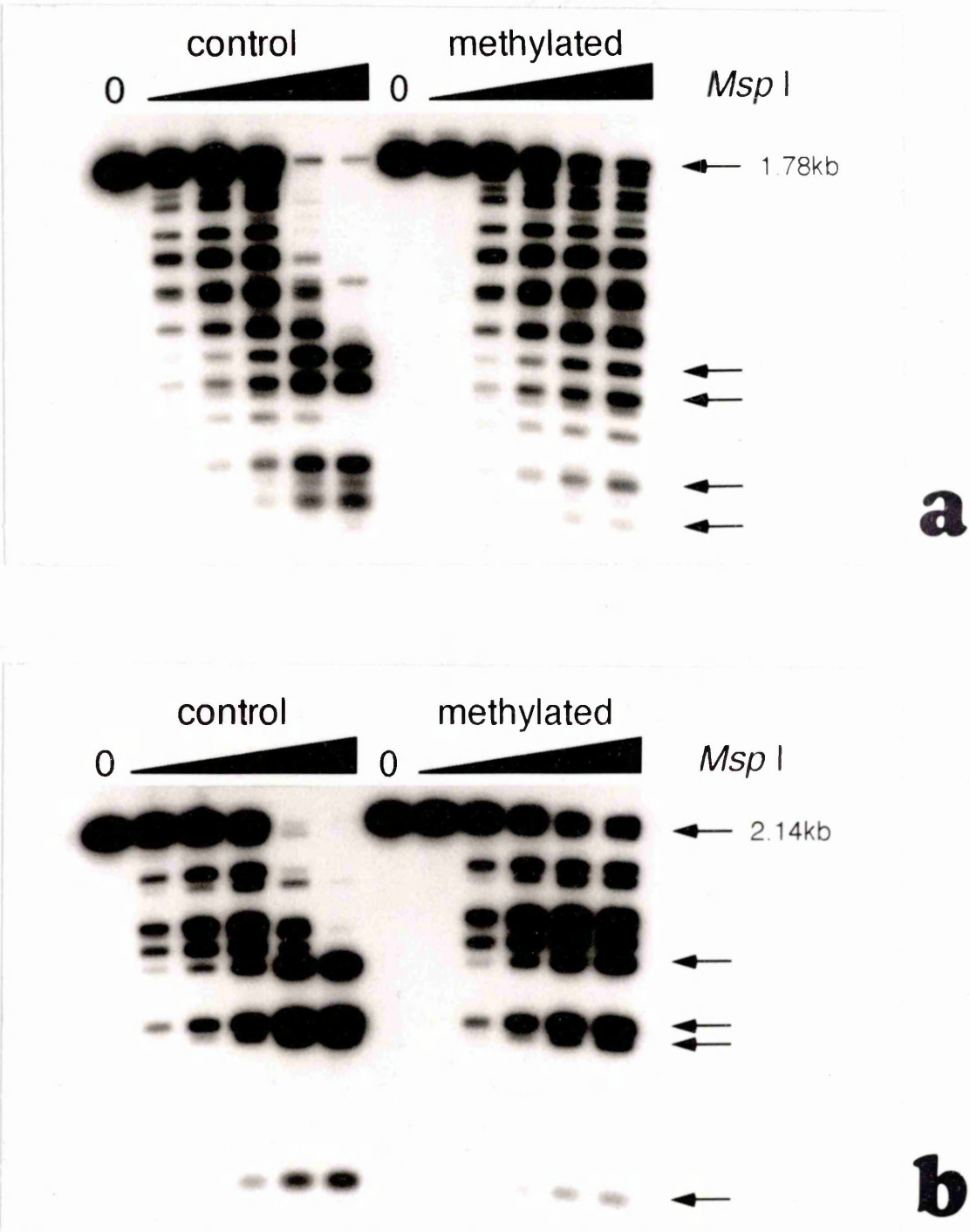


Figure 5.8 ***Msp*I digestion of nuclei transfected with patch methylated constructs (III)**

As figure 5.6 and 5.7 except that a 1.78 kb *Sca*I-*Kpn*I fragment (c in Table 2) was used to construct regionally methylated plasmids. L929 cells were transfected with 5 µg of regionally methylated or mock-methylated (control) constructs as described in section 2.2.4.2. At 48 hours after transfection, nuclei were isolated and aliquots digested with increasing amounts of *Msp*I (0.1, 0.2, 1, 5, and 20 units/150 µl section 2.2.5.3). Accordingly, isolated total DNA was digested with *Sca*I and *Kpn*I, separated on a 1.5% agarose gel and subjected to Southern blot analysis. **(a)** The blot was probed with fragment c (table 2). **(b)** The blot from panel (a) was treated with boiling SDS solution (0.5%) to remove the probe and reprobed with a 2.14 kb *Sca*I fragment (k in Table 2) in order to show *Msp*I digestion patterns of unmethylated sequences of the constructs.

5.5 *Alu*I digestion of methylated plasmids

To test whether there are differences in nuclease accessibility between methylated and unmethylated nuclear plasmid DNA not only at *Msp*I sites, but also at non-CpG sites, restriction enzyme *Alu*I was used in a nuclease protection assay as described in section 2.2.5.4. The recognition sequence for *Alu*I is 5´-AGCT-3´ and hence is not directly associated with the dinucleotide CpG. Plasmid pVHCk has 29 *Alu*I sites and was used in the following experiment.

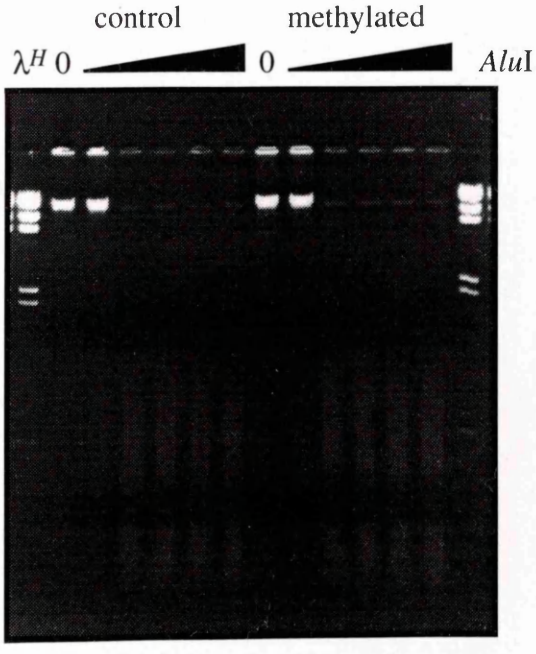


Figure 5.9 *Alu*I digestion of nuclei transfected with plasmid pVHCk

L929 mouse fibroblasts were transfected with completely methylated and mock-methylated (control) plasmid pVHCk and nuclei isolated 48 hours after transfection as described in section 2.2.4.2 and 2.2.5.1. Nuclei were digested with increasing amounts of *Alu*I (1, 3, 5, 10, and 15 units/150 µl) as described in section 2.2.5.3 and total DNA was isolated, linearized with *Pst*I and separated on a 1.5% agarose gel. λ^H: λ-DNA digested with *Hind*III.

Mouse L929 cells were harvested two days after transfection and nuclei isolated and digested with *Alu*I as described in section 2.2.5. Total DNA was isolated and fractionated on an agarose gel (Fig. 5.9). The gel revealed that the cellular DNA is more readily digested with *Alu*I than with *Msp*I (compare Fig. 5.4 with 5.9). The gel was subjected to Southern blot analysis to exhibit *Alu*I digestion patterns of the methylated and unmethylated plasmid DNA. No differences were observed in the rates of digestion with *Alu*I between methylated and mock-methylated plasmid (Fig. 5.10). Thus, it appears that differences in nuclease accessibility between methylated and unmethylated plasmid are present only at CpG sites.

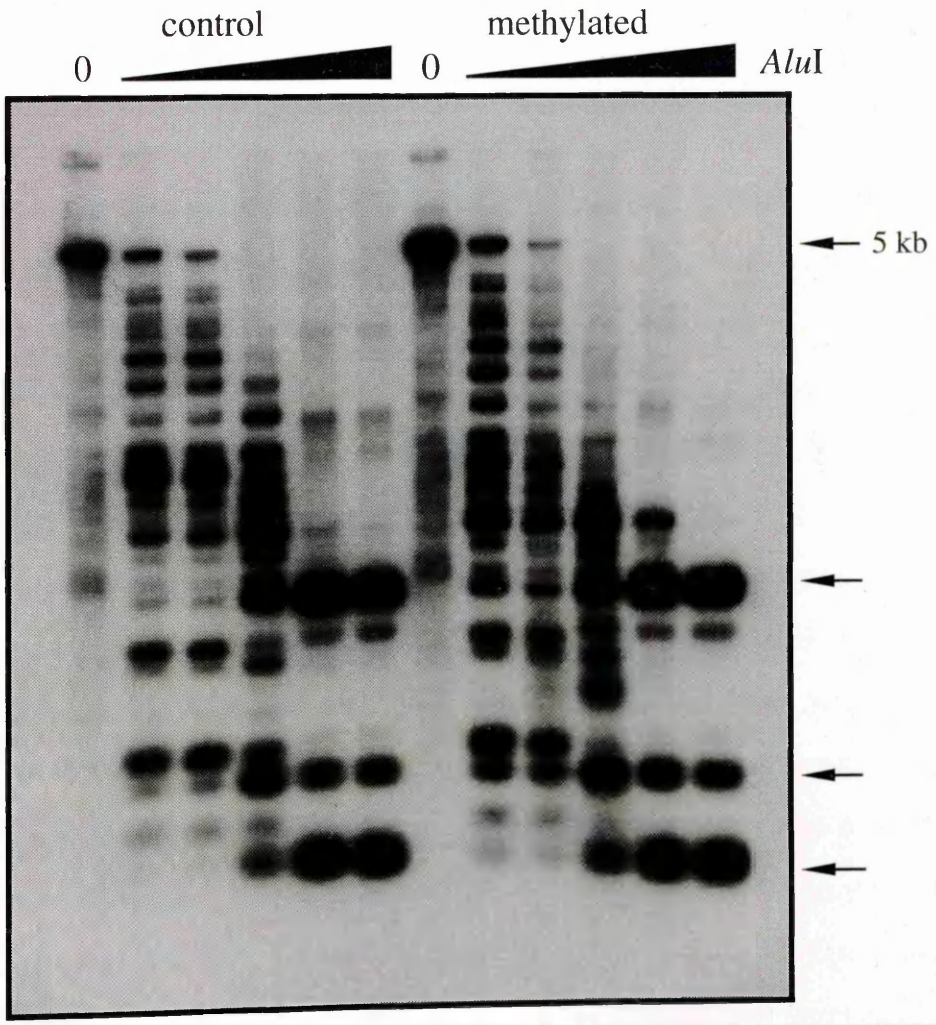


Figure 5.10 *Alu*I digestion patterns of transfected plasmid pVHCk
The gel from figure 5.9 was subjected to Southern blot analysis using plasmid pVHCk as a hybridization probe. 5 kb indicates linear plasmid DNA. Arrows indicate final digestion products.

5.6 Discussion

Transient transfection of non-replicating plasmid vectors into mammalian cells is routinely used to assess transcriptional activity of promoters connected to a reporter gene. However, not many studies have tried to establish whether these plasmids assemble into a nucleosomal chromatin structure. Reeves *et al.* (1985) reported that although plasmids form minichromosomes after transfection into cells, the efficiency of the nucleosomal assembly can be very low (10 - 30%) and is highly dependent on the transfection conditions. In a recent study by Archer *et al.* (1992) no evidence for a nucleosome repeat pattern on transiently transfected plasmid was found. From figure 5.3 it appears that probably only a minor proportion of the nuclear plasmid DNA is assembled into a regular array of nucleosomes two days after transfection and the situation was not different three days after transfection (data not shown). Interestingly, a region of about 300 to 700 bp is not readily degraded by mild digestion with micrococcal nuclease, probably indicating the assembly of nuclear proteins in an irregular manner on the plasmid DNA.

However, the nuclear plasmid DNA becomes increasingly resistant to digestion with restriction enzyme *Msp*I. At 24 hours after transfection, the plasmid becomes less accessible to *Msp*I (Fig. 5.5b) than at 6 hours post-transfection (Fig. 5.5a), but differences between methylated and unmethylated DNA are only subtle (Fig. 5.5b, lanes 2 and 8). At 48 hours after transfection (Fig. 5.5c), both methylated and unmethylated plasmids are further protected from digestion with *Msp*I. In addition, the difference in the rate of digestion between the methylated and unmethylated samples has dramatically increased compared with that at 24 hours, which is concordant with its transcriptional silencing (Fig. 3.7). Although from the results in section 5.3 it appears that the transfected DNA is not assembled into a regular array of nucleosomes, *Msp*I resistance increases post-transfection. It can be assumed that histones or other nuclear proteins with a high binding affinity to DNA are responsible for this phenomenon. The second effect, the increasing *Msp*I resistance of the methylated

plasmid, may be due to the specific binding of proteins to methylated DNA. Several of these proteins have been identified (Huang *et al.*, 1984; Meehan *et al.*, 1989; Jost and Hofsteenge, 1992; Lewis *et al.*, 1992) and at least two of them, MeCP1 and MDBP-2, are involved in the transcriptional repression upon DNA methylation (Boyes and Bird, 1991; Jost and Hofsteenge, 1992). MeCP2 does not specifically inhibit the transcription of methylated DNA *in vitro*, but has been shown to be associated with inactive heterochromatin (Lewis *et al.*, 1992; Meehan *et al.*, 1992). In addition, MDBP-2 shares sequence homologies with histone H1 (Jost and Hofsteenge, 1992) and might act as a transcriptional repressor. All these data point to a strong involvement of these methylated-DNA binding proteins in chromatin formation *and* transcriptional repression of methylated genes. This might explain both, the transcriptional silencing and the *Msp*I resistance of the patch-methylated constructs (section 4.3 and 5.4). It appears, that regional methylation of CpG-rich sequences can drive the whole plasmid into an inactive chromatin formation and thereby inhibit the transcription of the reporter gene. *Msp*I resistance of methylated DNA has been reported in many studies (Wolf and Migeon, 1985; Antequera *et al.*, 1989; Levine *et al.*, 1991) and Sasaki *et al.* (1992a) could show that reactivation of a methylation-silenced gene is correlated with increasing *Msp*I sensitivity.

Differences in nuclease sensitivity were observed only at CpG sites and not with the non-CpG restriction enzyme *Alu*I (Fig. 5.10). Similar observations were reported by Antequera *et al.* (1989), where *Alu*I did not distinguish between the active (unmethylated) and inactive (methylated) loci of the mouse *hprt* gene. Again, this makes an involvement of proteins, which bind specifically to methylated DNA very likely.

In summary, no conformational differences could be observed between methylated and unmethylated plasmid DNA after transfection into mammalian cells. Plasmid DNA appeared not to be associated with a regular array of nucleosomes, but increasing *Msp*I resistance after transfection probably reflects the specific binding of chromatin factors. The involvement of methylated DNA binding proteins seems very

likely as methylated regions in a plasmid can drive the whole molecule into an *Msp*I inaccessible chromatin formation. It appears that the inactive chromatin formation can spread from a focus of methylation.

CHAPTER SIX

Promoter involvement in the spreading of inactive chromatin

6.1 Introduction

From the data in Chapter five it appears that chromatin, inaccessible to nuclease *Msp*I is formed at a region of methylation. This chromatin formation can then spread to contiguous unmethylated sequences and thereby inhibit transcription from an unmethylated promoter. As there is only one active promoter in the plasmid pVHCk, the inactivation process spreading in one direction could be blocked by bound transcription factors, yet the gene could still be inactivated by spreading of an inactive chromatin conformation from the other direction. To establish whether bound transcription factors could protect the gene and the promoter from the inactivation brought about by the spreading of inactive chromatin from the focus of methylation, plasmids were constructed which contained a second SV40 promoter/enhancer region immediately following the terminator region. As no suitable restriction sites for cloning were present but primers were already available, a PCR approach was used to generate the two-promoter constructs (Fig. 6.1). Briefly, the SV40 early promoter/enhancer region was amplified from plasmid pVHCk in a standard PCR reaction (section 2.2.1.16) using the 17mer reverse sequencing primer and oligo GS (see Fig. 1). The gel-purified 350 bp promoter fragment was phosphorylated using T4 polynucleotide kinase (section 2.2.1.17) and subsequently ligated (section 2.2.1.8) to phosphorylated *Bam*HI linker (5´-CGGATCCG-3´). After digestion with *Bam*HI the linkered promoter fragment was ligated into *Bam*HI restricted, dephosphorylated (section 2.2.1.13) plasmid pVHCk. The two resulting vectors were termed pPPk (promoters in

same orientation) and pPRk (second promoter in reverse orientation), respectively and can be distinguished from each other by digestion with restriction enzyme *Kpn*I (Fig. 6.1).

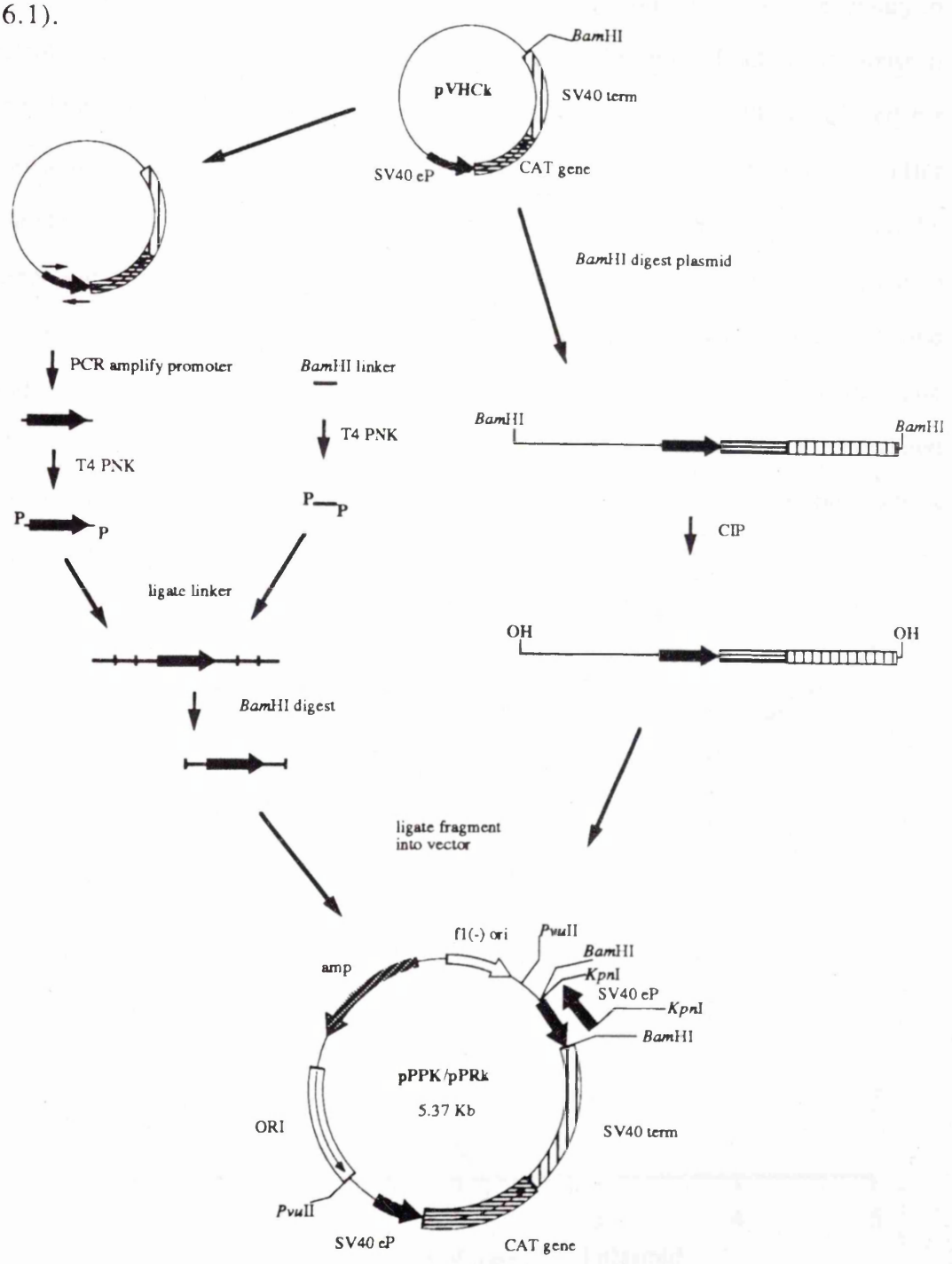


Figure 6.1 Construction of plasmids pPPk and pPRk

The construction of the two vectors is described in more detail in section 6.1. PCR: polymerase chain reaction; T4 PNK: T4 polynucleotide kinase; P: phosphorylated 5´-ends; OH: dephosphorylated 5´-ends; CIP: calf intestinal alkaline phosphatase; amp: ampicillin resistance gene; f1(–)ori: bacteriophage f1 origin of replication in (–) orientation; ORI: origin for replication in *E. coli*; SV40eP: SV40 early promoter/enhancer region; CAT gene: chloramphenicol acetyltransferase gene; SV40 term: SV40 terminator region; sites for relevant restriction enzymes are indicated.

6.2 Two-promoter constructs in transient transfection assays

As plasmids pPPk and pPRk both contain two promoters it was necessary to establish the plasmid DNA concentrations which give a linear CAT activity response in transient transfection assays. Increasing amounts of plasmid pPPk were used for transient transfection of L929 cells and CAT activity assayed two days after transfection as described in sections 2.2.4.2 and 2.2.4.3. From figure 6.2 it can be seen that the amount of plasmid DNA is linear to the CAT activity at least to concentrations of 5 µg of plasmid per assay. This is correct for both the unmethylated and completely methylated form of plasmid pPPk. In addition, complete methylation of the plasmid reduces transcriptional activity to around 13% of the mock-methylated control, indicating similar inactivation mechanisms for the two-promoter constructs as for pVHCk.

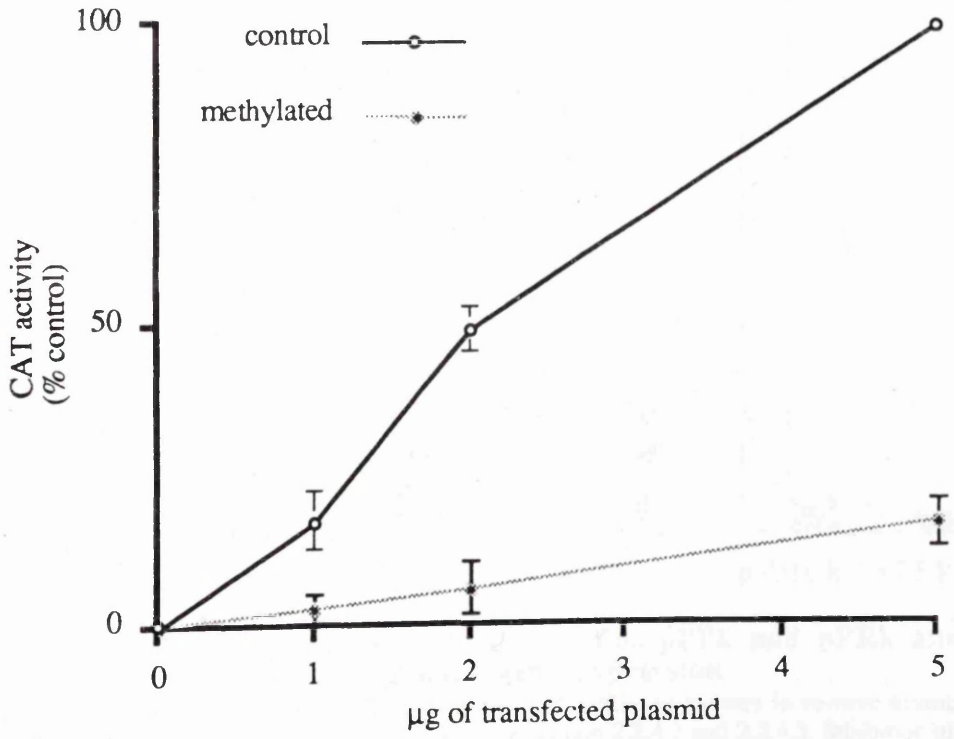


Figure 6.2 DNA response curve of transient expression of plasmid pPPk

Increasing amounts of completely methylated and mock-methylated (control) plasmid pPPk were transfected into mouse L929 fibroblasts and CAT activity assayed two days after transfection as described in section 2.2.4.2 and 2.2.4.3. CAT activity is expressed as percentage of the activity of 5 µg mock-methylated plasmid.

Under transfection conditions in which CAT activity is directly proportional to the DNA concentration it can be assumed that both promoters in plasmids pPPk and pPRk are equally likely to bind the normal complement of transcription factors. When fully methylated, transcription from these plasmids was inhibited to 13% (pPPk, promoters in same orientation) and 20% (pPRk, promoters in reverse orientation), respectively, compared with the unmethylated control (Fig. 6.3). With patch-methylation (using a 2.5 kb *Pvu*II fragment; table 2, fragment b), transcription was considerably reduced (to 28 and 33% relative to mock-methylated control). Thus the presence of a second promoter in either orientation only slightly protects the gene from inactivation (Fig. 6.3).

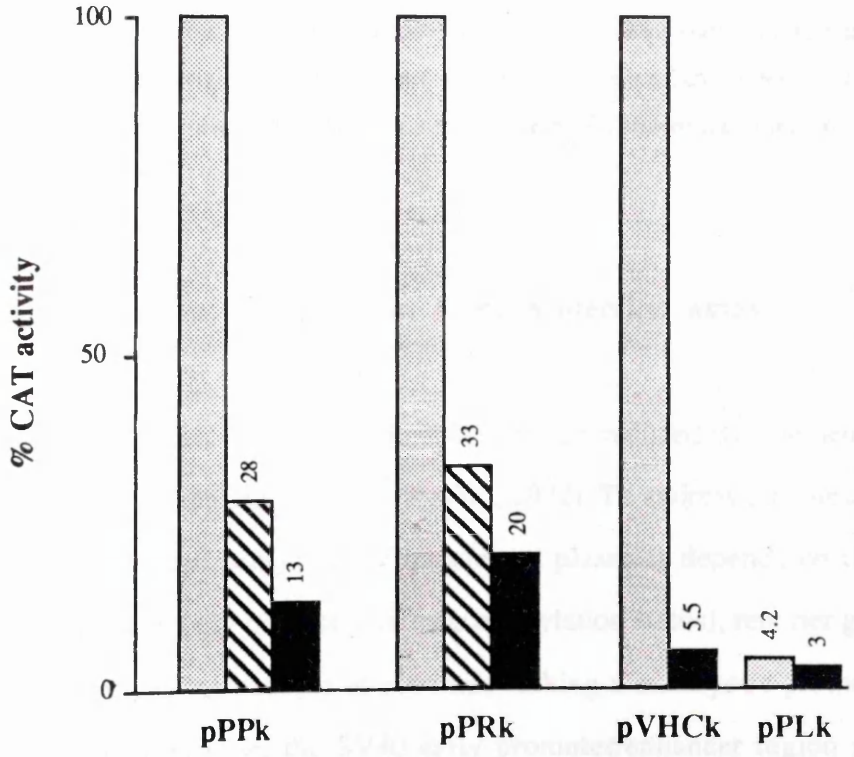


Figure 6.3 Regional methylation of plasmids pPPk and pPRk and the effect on reporter gene expression

Plasmids pPPk (both promoters in same orientation), and pPRk (promoters in reverse orientation) were used in transient expression assays as described in section 2.2.4.2 and 2.2.4.3. Inhibition of CAT activity by DNA methylation was calculated by comparison with the values obtained for the mock-methylated constructs (= 100%). CAT activity of pPLk (promoter less construct, Fig. 6.4) was compared to that of mock-methylated pVHCk. Stippled columns: mock-methylated control; hatched columns: patch-methylated constructs (a 2.5 kb *Pvu*II fragment [b in table 2] was used to generate regionally methylated plasmids pPPk and pPRk as described in section 4.2); closed columns: fully methylated plasmids.

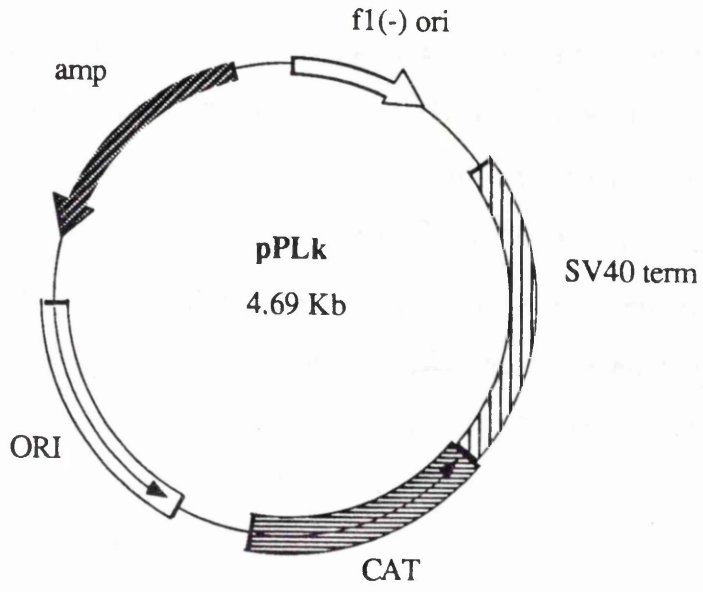


Figure 6.4 Map of plasmid pPLk

Construction of plasmid pPLk (promoter less) is detailed in section 6.3. amp: ampicillin resistance gene; f1(–)ori: bacteriophage f1 origin of replication in (–) orientation; ORI: origin for replication in *E. coli*; CAT gene: chloramphenicol acetyltransferase gene; SV40 term: SV40 terminator region.

6.3 Promoter-less constructs in *Msp*I protection assays

Transcriptionally active chromatin is known to have a reduced complement of nucleosomes (Clark and Felsenfeld, 1992; Felsenfeld, 1992). To address the question whether the differential *Msp*I sensitivity of transfected plasmids depends on their transcriptional activity (and only indirectly on their methylation status), reporter gene expression and *Msp*I digestion patterns of plasmids lacking a eukaryotic promoter were examined. For this purpose, the SV40 early promoter/enhancer region was deleted from plasmid pVHCk (Fig. 3.2) by digestion with *Kpn*I and *Hind*III. The remaining vector was then treated with S1 nuclease to generate blunt ends (section 2.2.1.14) and religated. The promoter-less version of plasmid pVHCk was named pPLk and is shown in figure 6.4. In transient expression assays pPLk produced only insignificant levels of CAT activity, regardless of its methylation status (Fig. 6.3),

excluding the possibility of transcription from cryptic sites within the plasmid. However, when such a plasmid was fully methylated it formed nuclease-insensitive chromatin, as established by *Msp*I digestion of transfected nuclei (Fig. 6.5a). Use of the prokaryotic, CpG-dense vector pBluescript in *Msp*I protection assays revealed an even more profound difference in *Msp*I sensitivity between methylated and unmethylated plasmid (Fig. 6.5b). Hence, it appears that the presence of an inactivated promoter is not necessary for the formation of nuclease resistant chromatin.

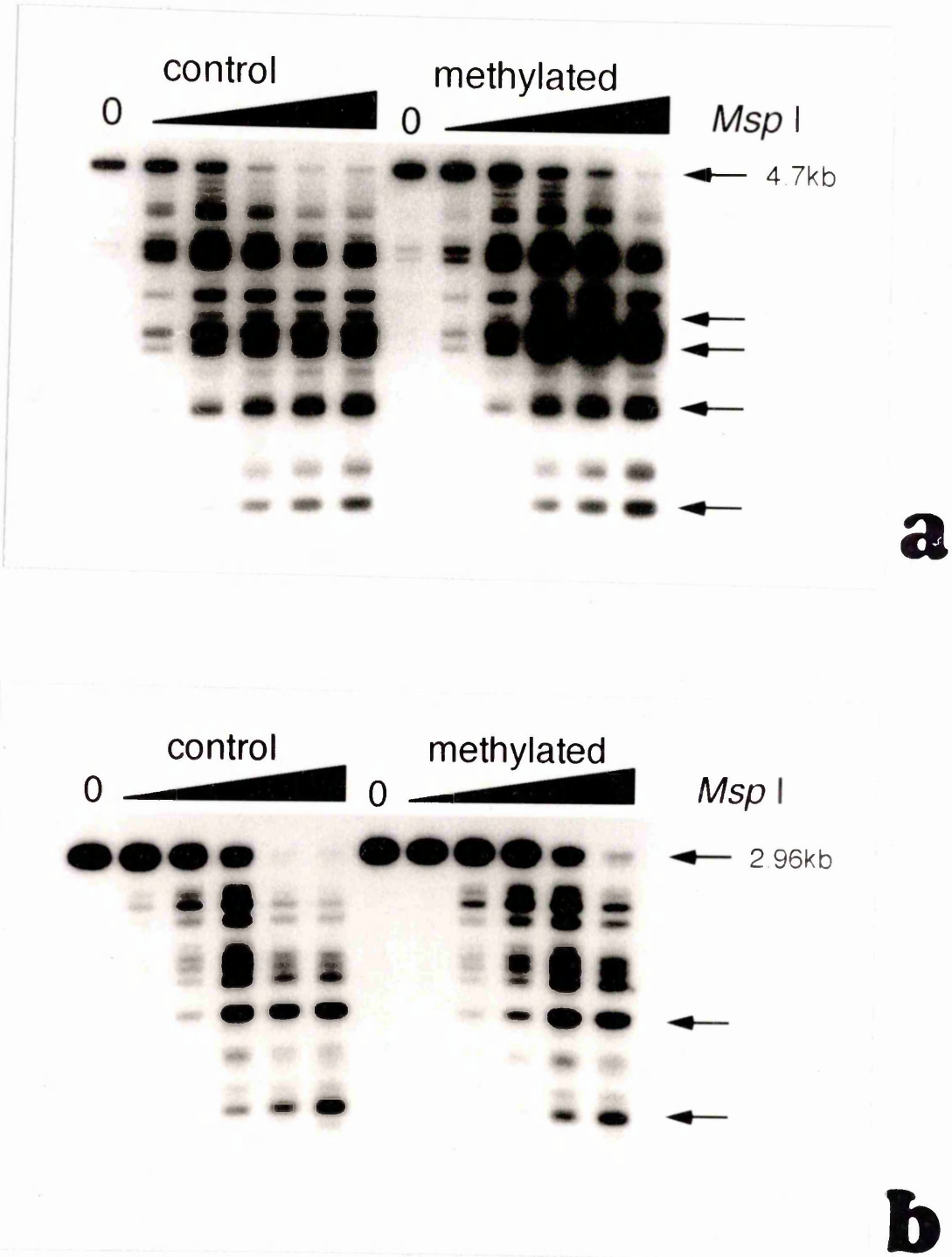


Figure 6.5 ***Msp*I digestion patterns of transfected plasmids pPLk and pBluescript**

Completely methylated and mock-methylated (control) plasmids were transfected into L929 cells as described in section 2.2.4.2. Nuclei were isolated 48 hours after transfection and treated with increasing amounts of *Msp*I (0.1, 0.2, 1, 5, and 20 units/150 µl) as described in section 2.2.5.3. Total DNA was isolated and digested with *Pst*I to linearize the plasmids. The DNA was separated on a 1.5% agarose gel and subjected to Southern blot analysis. Hybridization was with fragment b (table 2). (**a**) Plasmid pPLk; (**b**) plasmid pBluescript KSII–.

6.4 Discussion

The results from Chapter 5 strongly suggest that specific DNA methylation in a region of plasmid pVHCk can influence the chromatin structure of a neighbouring, unmethylated sequence in *cis*. Boyes and Bird (1992) reported that the presence of a strong promoter or enhancer could override the inhibitory effect of DNA methylation of a sparsely methylated gene, but not of a densely methylated gene. They concluded that the intensity of repression is proportional to the density of methylation. To investigate whether the presence of a potentially active promoter is able to overcome this inactivation mechanism, constructs were generated with a second promoter immediately 3´ of the terminator region. However, insertion of this promoter could only partially overcome the transcriptional inactivation (Fig. 6.3). The relative inhibition of transcription of patch-methylated and fully methylated constructs was comparable in all three constructs, i.e., that with a single promoter (pVHCk; table 2) and those with two promoters (pPPk and pPRk; Fig. 6.3). It appears that, at least in this system, active promoters cannot block the spreading of inactive chromatin from methylated to unmethylated sequences. The methylated patch from figure 6.3 is CpG-rich, as it consists entirely of pBluescript DNA (Table 2, fragment b) and upon complete methylation of this region transcription is inhibited. It was not tested whether sparse methylation of this region has an effect on transcription from the two-promoter constructs but it may well be that the inhibitory effect is not apparent at low-density methylation.

To establish whether DNA methylation is the direct cause of the formation of inaccessible chromatin or whether it is dependent only on the transcriptional status of the plasmid DNA, transcriptionally incompetent vectors (Fig. 6.3) were used in *Msp*I protection assays (Fig. 6.5). Both, the promoterless plasmid pPLk and pBluescript revealed increased sensitivity to *Msp*I in the unmethylated form, indicating that DNA methylation (and not transcriptional silencing) is the sole reason for the formation of nuclease inaccessible chromatin. This in agreement with Keshet *et al.*, (1986), who

reported that methylated M13 sequences, integrated into the genome, revealed a decreased DNase I sensitivity compared to the unmethylated controls.

The results in this chapter give further evidence for the formation of inactive chromatin upon DNA methylation. It appears that the spreading of this chromatin from methylated to unmethylated sequences is not impaired by the presence of potentially active promoters. In addition, nuclease inaccessible chromatin structure is caused directly by DNA methylation and is not a consequence of transcriptional inactivity.

CHAPTER SEVEN

Methylated plasmid pVHCk in different systems: COS cells and *in vitro* transcription

7.1 The use of COS-1 cells in transfection assays

COS-1 cells are simian cells permissive for lytic growth of SV40. They are derived from CV-1 cells by transformation with an origin-defective mutant of SV40 and express T antigen (Gluzman, 1981). Recombinant plasmids, carrying the SV40 viral origin region, are assembled into nucleosomal chromatin and replicate as minichromosomes in the nucleus of COS-1 cells (Innis and Scott, 1983; Cereghini and Yaniv, 1984). Thus, it was considered that transfection of plasmid pVHCk, which contains the SV40 origin of replication region (Fig. 1) into COS-1 cells might provide new information about the effect of chromatin assembly on the transcription from the methylated plasmid. Further, it was thought that there might be a spreading of methylation from a methylated patch to regions of the minichromosome that were transcriptionally inactive.

7.1.1 Micrococcal nuclease digest of transfected plasmid pVHCk

To assess the degree of chromatin assembly of transfected plasmid pVHCk in COS-1 cells, nuclei of transfected cells were isolated 24 and 48 hours after transfection (section 2.2.5.1) and subjected to digestion with micrococcal nuclease as described in section 2.2.5.3. The DNA was isolated and resolved on agarose gels as can be seen in figure 7.1. To reveal digestion patterns of transfected plasmid DNA the gels were

subjected to Southern blot analysis. Figure 7.2 shows that the plasmid is exhibiting the typical pattern of a nucleosomal ladder both, at 24 and at 48 hours after transfection. Although a background of non-nucleosomal DNA is visible, the majority of the transfected plasmid DNA appears to be in a nucleosomal context. No differences can be observed between methylated and unmethylated plasmid, indicating chromatin assembly of similar efficiencies for both plasmids.

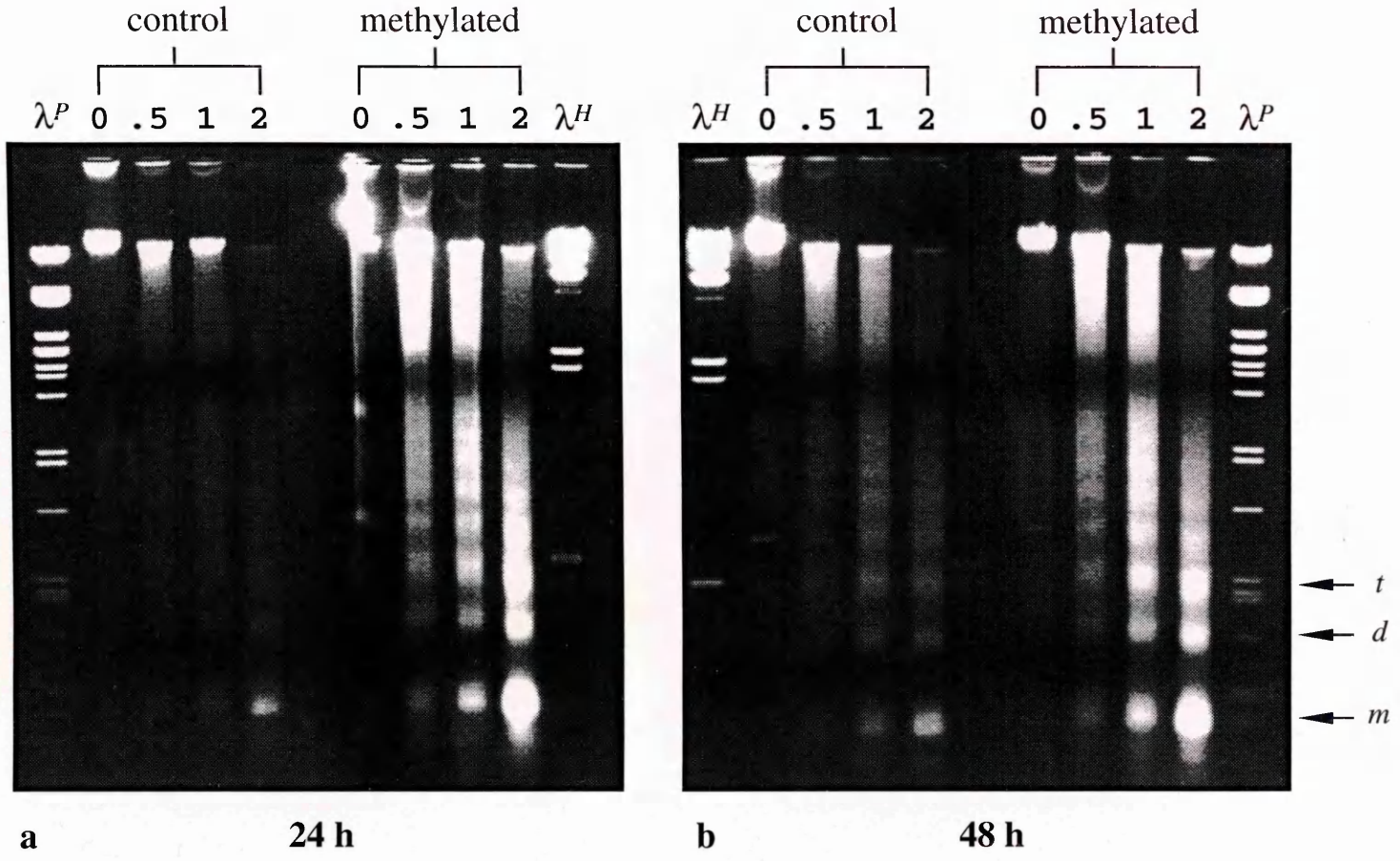


Figure 7.1 Micrococcal nuclease digestion of transfected COS-1 cell nuclei

COS-1 cells were transfected with completely methylated or mock-methylated (control) plasmid pVHCk and nuclei isolated 24 (**a**) or 48 (**b**) hours after transfection as described in section 2.2.4.1 and 2.2.5.1. Nuclei were digested with micrococcal nuclease for 0, 0.5, 1, and 2 min as described in section 2.2.5.3. Total DNA was isolated from the nuclei and separated on 1.5% agarose gels. λ^H and λ^P: λ-DNA digested with *Hind*III or *Pst*I, respectively; *m*, *d*, and *t*: mono, di, and trinucleosomes.

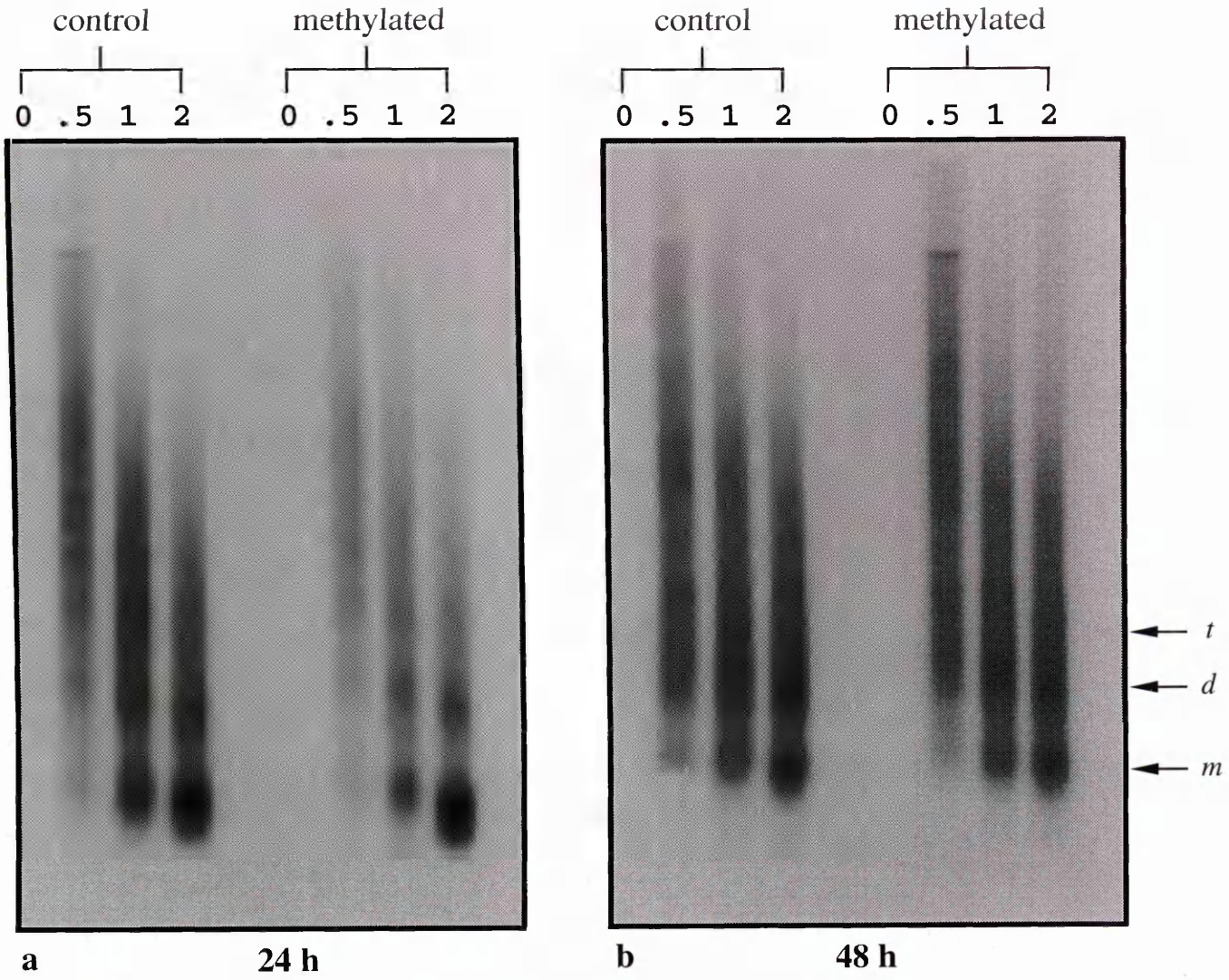


Figure 7.2 Micrococcal nuclease analysis of plasmid pVHCk transfected into COS-1 cells

The gels from figure 7.1 were subjected to Southern blot analysis (section 2.2.1.20) using plasmid pVHCk as a hybridization probe. 0, 0.5, 1, and 2 indicates time of digestion with micrococcal nuclease; *m*, *d*, and *t*: indicates mono, di, and trinucleosomes. **(a)** cells harvested 24 hours after transfection; **(b)** cells harvested 48 hours after transfection. Exposure of the X-ray film was longer for panel (a) to obtain equal band intensities.

7.1.2 Transient expression assay in COS-1 cells with plasmid pVHCk

COS-1 cells were transfected with plasmid pVHCk and CAT activity determined 24 and 48 hours after transfection as described in sections 2.2.4.2 and 2.2.4.3.

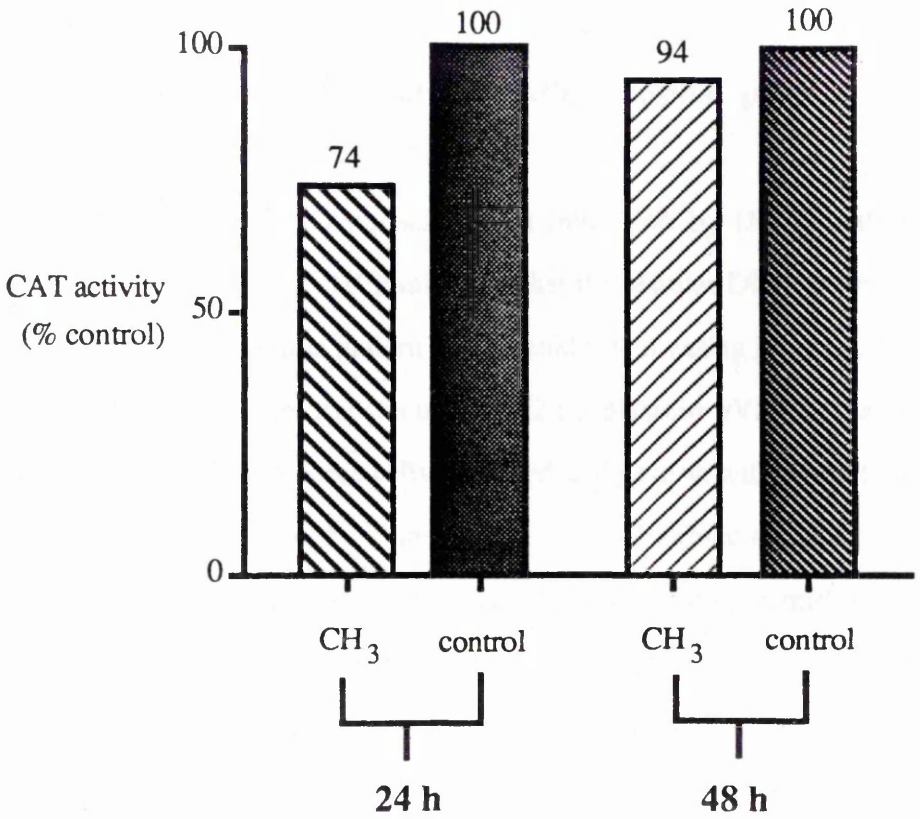


Figure 7.3 Transient expression assay of pVHCk in COS-1 cells

COS-1 cells were transfected with completely methylated or mock-methylated (control) plasmid pVHCk and CAT activity determined 24 and 48 hours after transfection as described in sections 2.2.4.2 and 2.2.4.3. Inhibition of CAT activity by DNA methylation is expressed as percent of the activity of the mock-methylated control.

The result is illustrated in figure 7.3 and shows that transcriptional activity of methylated plasmid pVHCk is only slightly reduced (to around 94%) compared with the mock-methylated control when assayed 48 hours after transfection. 24 hours after

transfection, methylated plasmid pVHCk showed CAT activity of 74% compared to the unmethylated control. Overall CAT activities were about seven times higher at 48 hours than at 24 hours after transfection, consistent with replication of the plasmid in the COS cells. Clearly, inhibition of transcription by DNA methylation in COS-1 cells was much lower than that in L929 mouse fibroblasts. The possibility that this is due to demethylation of the replicating plasmid is considered in the next section.

7.1.3 Methylation status of replicating plasmid pVHCk

One reason for reduced transcriptional inhibition by DNA methylation in COS-1 cells using the vector pVHCk might be that the cellular DNA methylase is not able to maintain the methylation pattern of the rapidly replicating plasmid. To test this hypothesis, COS-1 cells were transfected with 2 μg plasmid pVHCk, methylated or unmethylated, and DNA from these cells subjected to digestion with restriction enzyme *Hpa*II. The DNA was resolved on an agarose gel and subjected to Southern blot analysis to reveal the degree of methylation of the transfected plasmid. Figure 7.4 is the resulting autoradiograph and shows that after transfection the plasmid becomes increasingly demethylated as judged by the appearance of *Hpa*II digestion products. 24 hours after transfection about 30% of the plasmid is completely demethylated (compare final digestion products from lane 3 and 4) and a larger proportion of plasmid molecules appears to be partially methylated. By 48 hours the plasmid DNA is almost completely unmethylated, as judged by the appearance of final digestion products of *Hpa*II, indicating that the DNA methylase of the COS-1 cells is not able to maintain the methylation of the newly synthesized plasmid DNA.

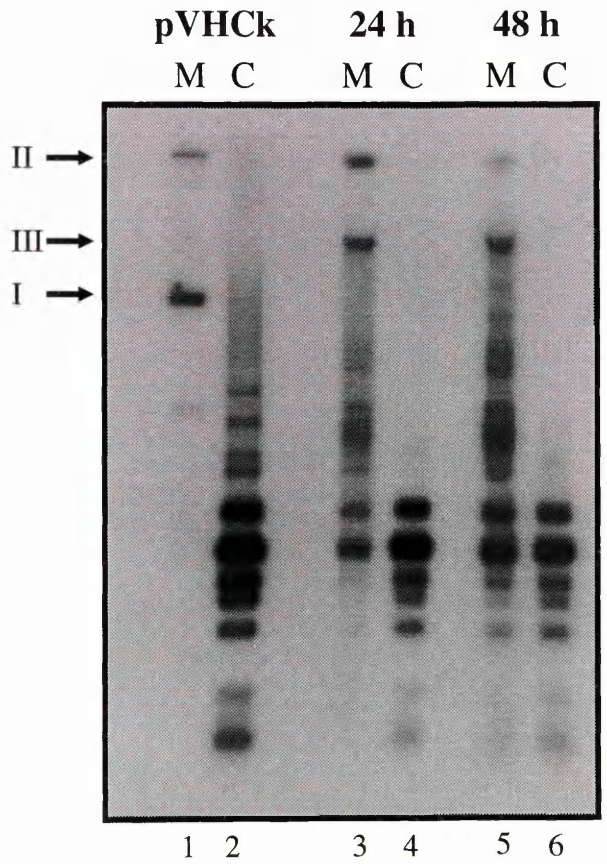


Figure 7.4 Methylation status of replicating plasmid pVHCk

2 µg of completely methylated (M) or mock-methylated (C) plasmid pVHCk was transfected into COS-1 cells as described in section 2.2.4.2. 24 hours (lanes 3 and 4) and 48 hours (lanes 5 and 6) after transfection nuclei were harvested, total DNA isolated, digested with *Hpa*II and separated on a 0.8% agarose gel as described in sections 2.2.5.1 and 2.2.5.2. The gel was subjected to Southern blot analysis using plasmid pVHCk as a hybridization probe. Lanes 1 and 2: plasmid pVHCk before transfection into cells; I, II, and III refer to supercoiled, relaxed circular and linear forms of pVHCk, respectively. X-ray films were exposed for different times in order to obtain equal band intensities for the three pairs of lanes.

7.2 *In vitro* transcription of plasmid pVHCk

Previously, it has been reported that transcription in a cell-free extract is inhibited from methylated templates and that this effect is mediated by a methyl-CpG binding protein (Boyes and Bird, 1991). The following experiments were carried out

to investigate whether *in vitro* transcription from plasmid pVHCk is reduced by DNA methylation.

7.2.1 *In vitro* transcription of naked DNA

Nuclear extracts were prepared from HeLa S3 cells according to a method described by Dignam *et al.* (1983), which is regarded as the standard extract for these kind of studies. In pilot experiments, an optimal $MgCl_2$ concentration of 4 mM and a DNA saturating concentration of 500 ng/25 µl assay were established. No differences in the rate of transcription between supercoiled and linear plasmid DNA were observed. A standard *in vitro* transcription assay was carried out as described in section 2.2.6.2 and transcripts were analysed using a primer extension assay (section 2.2.6.3). The resulting cDNA was resolved on a denaturing polyacrylamide gel and visualized by autoradiography. Figure 7.5 shows that no differences in transcriptional activity could be observed between methylated and unmethylated plasmid pVHCk at three different DNA concentrations. Control samples did not give rise to any signals (Fig. 7.5, control, control PI, pBlue, 0). Preincubation of the supercoiled plasmid with the nuclear extract prior to the initiation of the transcription reaction by Mg^{2+} and the four NTPs increased the rate of transcription, but did not result in any differences between methylated and unmethylated vector.

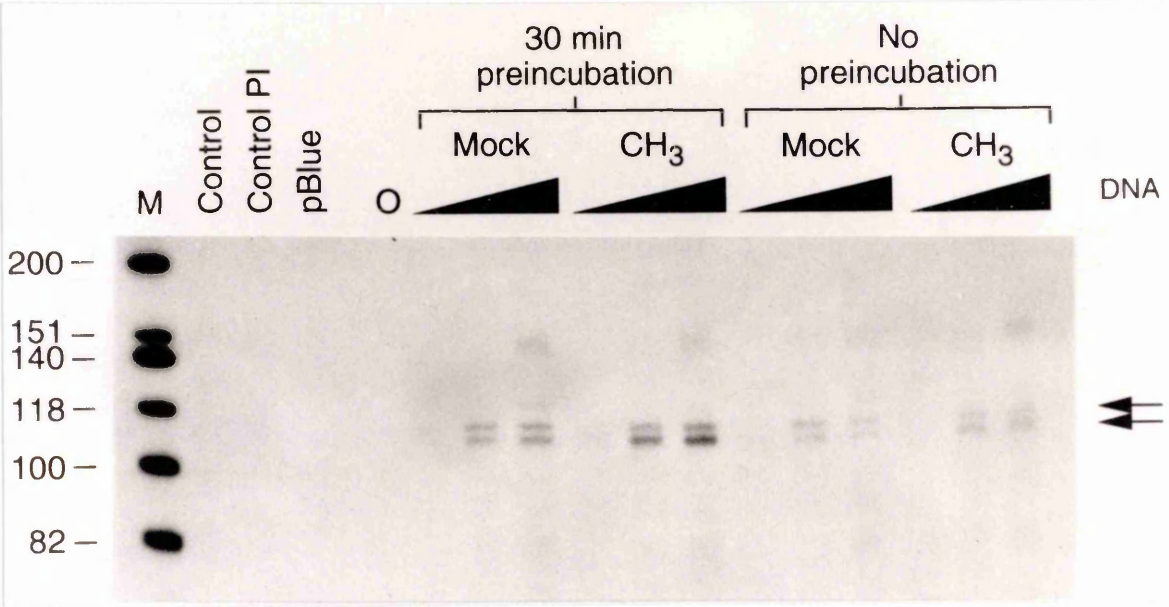


Figure 7.5 ***In vitro*** **transcription assay using plasmid pVHCk**

Completely methylated or mock-methylated (mock) plasmid pVHCk (50, 100, or 300 ng) were used in *in vitro* transcription assays and transcripts were visualized by primer extension analysis as described in section 2.2.6.2 and 2.2.6.3. Preincubation (PI) indicates incubation of the DNA with the HeLa nuclear extract for 30 min at 30°C prior to initiation of the transcription reaction by addition of Mg^{2+} and NTPs. Control: transcription reaction in the presence of stop buffer; pBlue: 250 ng of pBluescript in transcription assay; 0: no DNA in transcription assay; M: ϕX174 DNA digested with *Hin*fI and end-labelled with ^{32}P. Arrows indicate major transcripts of 104 and 110 nucleotides, respectively.

7.2.2 *In vitro* transcription in the presence of histone H1

Many reports strongly suggest that histone H1 is preferentially associated with methylated DNA and might act as a specific repressor of methylated genes (see section 1.5). Therefore, *in vitro* transcription assays were carried out with increasing amounts of histone H1 to reveal any inhibitory effect of DNA methylation on transcription from plasmid pVHCk. Histone H1 from calf thymus was obtained from Boehringer Mannheim. It was reconstituted by stepwise dialysis from 2 M NaCl, 5 M urea, 10 mM Tris-HCl (pH 7.8) to a final 10 mM Tris-HCl as described by Hentzen and Bekhor (1985) and was kindly provided by Colin Johnson. Histone H1 was added to 250 ng plasmid pVHCk in a buffer containing 10 mM HEPES-KOH (pH 7.0) and 1

mM $MgCl_2$ in a total volume of 10 µl. Incubation was for 20 min at 30°C. To the DNA was then added 11 µl HeLa nuclear extract and incubated for a further 30 min at 30°C. *In vitro* transcription assay and detection of transcripts were carried out under the same conditions as described above. The effect of histone H1 on *in vitro* transcription can be seen in figure 7.6. Both, methylated and unmethylated plasmid pVHCk are equally inhibited by increasing amounts of H1. Complete inhibition is observed at a ratio 0.9 µg histone H1/1 µg of DNA. This corresponds to 1 histone H1 molecule per 40 to 60 bp of DNA (Croston *et al.*, 1991; Levine *et al.*, 1993).

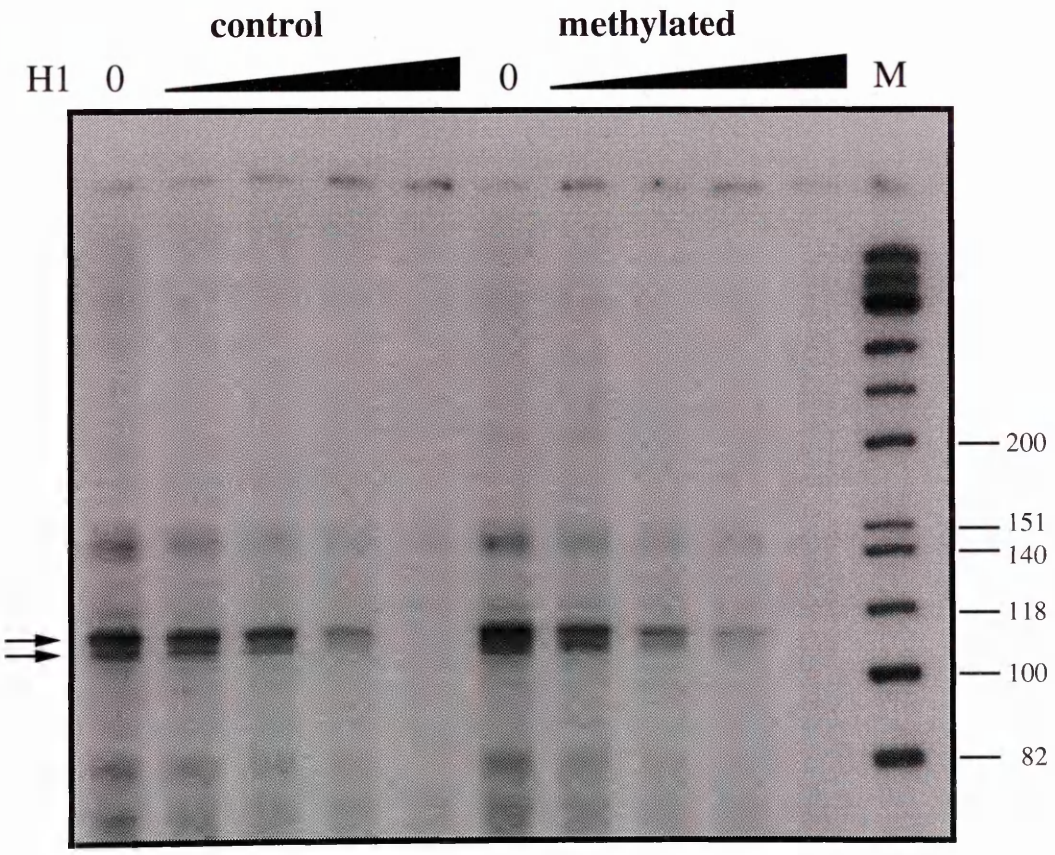


Figure 7.6 ***In vitro*** **transcription assay using plasmid pVHCk in the presence of histone H1**

250 ng of methylated or mock-methylated (control) plasmid pVHCk were used in *in vitro* transcription assays in the presence of increasing amounts (0, 28, 56, 112, or 224 ng) of histone H1 as described in section 2.2.6.2 and 7.2.2. Transcripts were analyzed by primer extension as described in section 2.2.6.3. M: ϕX174 DNA digested with *Hin*fI and end-labelled with ^{32}P. Arrows indicate major transcripts of 104 and 110 nucleotides, respectively.

7.3 Discussion

This chapter describes the use of two different systems, COS-1 cells and expression assays in cell-free extracts, to investigate the effect of methylation on transcription from plasmid pVHCk.

Plasmid pVHCk replicates in COS-1 cells as it contains the SV40 origin of replication (Cereghini and Yaniv, 1984; Reeves *et al.*, 1985). It forms nucleosomal chromatin (Fig. 7.2) and different amounts of plasmid transfected into COS-1 cells result in similar CAT activities 48 hours after transfection (data not shown), indicating that the rate of replication of the plasmid has reached a plateau level two days after transfection. Replication of the plasmid appears not to be inhibited by DNA methylation as equal amounts of methylated and unmethylated plasmids are recovered from transfected cells, both at 24 and 48 hours after transfection (Fig. 7.2 and 7.4). This is in concordance with results by Hsieh and Lieber (1992), who could not detect any differences in replication efficiencies between a methylated and unmethylated polyoma replicon.

Transfected plasmids were analysed 24 and 48 hours post-transfection by micrococcal nuclease digestion of isolated nuclei and both methylated and unmethylated plasmids were found in typical nucleosomal ladders (Fig. 7.2). No differences in the degree of nucleosomal order or in the repeat length could be observed between methylated and unmethylated vector indicating similar assembly efficiencies for both plasmids. The degree of inhibition of transcription by DNA methylation was lower at 48 hours (6%) than at 24 hours after transfection (26%) (Fig. 7.3). This is caused by demethylation of the transfected plasmids in the COS cell nuclei (Fig. 7.4). Approximate levels of completely demethylated plasmids are about 30% at 24 hours and 80% at 48 hours after transfection, respectively (as estimated from the degree of complete digestion by *Hpa*II, lanes 3 and 4, Fig. 7.4). The use of restriction enzyme *Hpa*II does not allow one to distinguish between hemimethylated and symmetrically methylated DNA and hence it is difficult to make estimations about

the degree of methylation of the partially methylated plasmids. However, it can be assumed that the level of hemimethylated sites on the plasmids increases with the time after transfection. Thus, it appears that the maintenance methylase activity of the COS cells is not able to maintain the methylation pattern of the rapidly replicating plasmids, which become increasingly demethylated. Interestingly, this is not the case for the replication of plasmids carrying the polyoma origin of replication after transfection into a murine pre-B lymphoid cell line (Hsieh and Lieber, 1992). Only 6 to 16% of the replicating plasmids were reported to lose their methylation and, most surprisingly, no partially methylated plasmids were detected. The apparent differences in the level of demethylation may be due to a lower rate of replication of the polyoma replicon, thus allowing the cells methylase to maintain the methylation pattern of almost all transfected plasmids. Replicating plasmid pVHCk becomes increasingly demethylated in COS-1 cells and it can be assumed that this is the reason for the relative high transcriptional activity of methylated plasmid (94% of the unmethylated control, 48 hours after transfection, Fig. 7.3) compared to the levels observed in mouse L929 fibroblasts (5% of the unmethylated control, Chapter 3). However, not all plasmids were completely demethylated 48 hours after transfection and yet, transcription was only marginally reduced. The high copy number of the replicating plasmid (transcriptional activity in the COS cells was about 10 times higher than in the mouse fibroblasts) means, that, in the case of the methylated plasmid, there are unusually high amounts of methylated DNA in the nuclei of the COS cells. This may well result in the pool of factors responsible for transcriptional repression (e.g. methylated-DNA binding proteins) being completely bound to the methylated DNA and, thereby, allowing newly synthesized (methylated or hemimethylated) DNA to escape transcriptional inhibition.

In recent years many studies have used *in vitro* transcription assays to dissect the different components which contribute to transcriptional activation or silencing of a particular gene. The functional requirements of a gene can be assessed by deleting

factors (via immunoprecipitation or affinity columns) or adding components (histones, potential repressors) to extracts of "known" composition. HeLa nuclear extracts prepared to a recipe published by Dignam *et al.* (1983) efficiently support the *in vitro* transcription by RNA polymerase II and III.

Plasmid pVHCk, completely methylated or unmethylated, was used to establish the effect of DNA methylation on transcription in HeLa nuclear extracts. A preincubation step of the DNA with the nuclear extract was introduced to allow the binding of any potential repressors to the DNA, before the transcription reaction was initiated. However, the preincubation turned out to enhance the transcription from both methylated and unmethylated template in a similar fashion (Fig. 7.5). Presumably, this involves the formation of preinitiation complexes over the promoter region (Roeder, 1991). Levels of transcription were the same for both templates over a range of 50 - 300 ng of plasmid DNA per assay. No increase in transcription was observed at concentrations of over 500 ng per assay under the conditions described in section 2.2.6.2 (data not shown). The failure to detect any differences in transcriptional activity between methylated and unmethylated plasmid may be due to the relative high template concentrations used in the assays. No signals were obtained at 30 ng vector DNA per assay using the primer extension method. Boyes and Bird (1991, 1992) observed reduced transcriptional activities from methylated templates only at very low DNA concentrations (3 - 30 ng/assay) and linked the inhibitory effects to the presence of a methylated-DNA binding protein (MeCP-1). Using a mouse metallothionein promoter construct, Levine *et al.* (1992) did find effects of M.*Sss*I methylation on transcription *in vivo*, but not *in vitro* and ascribed that to the lack of methylation-specific repressors in the nuclear extracts, which may also apply to the experiment described in Fig. 7.5.

Several studies suggest a role for histone H1 in the specific repression of methylated genes (Ball *et al.*, 1983; Higurashi and Cole, 1991; Jost and Hofsteenge, 1992; Levine *et al.*, 1993; see also section 1.5). Increasing amounts of histone H1 were incubated with plasmid pVHCk at low ionic strength before performing standard

transcription assays (Fig. 7.6). Methylated and unmethylated templates were inhibited equally by histone H1 at a ratio 0.9 µg histone H1/µg DNA. This is in concordance with the results obtained by Shimamura *et al.* (1989) and Croston *et al.* (1991) who could demonstrate that histone H1 represses transcription from minichromosomes and from naked DNA templates, respectively. However, in a recent study by Levine *et al.* (1993) histone H1 inhibited not only transcription from an unmethylated template, but was found to be an even more efficient repressor for transcription from a methylated template (at a ratio of 0.6 µg H1/µg DNA). This was consistent with their findings that H1 has a higher binding affinity to methylated than to unmethylated DNA. This is in contrast to results obtained by Higurashi and Cole (1991) and Bird and colleagues (personal communication) who could not demonstrate preferential binding of histone H1 to methylated DNA. The conflicting data about the role of histone H1 as a methylated-DNA binding protein is reflecting the different methods used by the authors to purify H1, to assemble it on DNA and to assay its binding capabilities. Association of histone H1 with DNA is highly salt-dependent and varies among the different H1 subtypes (Clark and Thomas, 1986, 1988). Although it appears that some aspects of H1 binding to naked DNA resemble its interaction with chromatin (Croston *et al.*, 1991; Layboum and Kadonaga, 1992) a more extensive approach needs to be undertaken to conclusively answer the question whether histone H1 does selectively inhibit transcription of methylated genes.

CHAPTER EIGHT

Discussion

Early SV40 gene expression is not inhibited by DNA methylation (Fradin *et al.*, 1982; Grässmann *et al.*, 1983) and, consistent with these findings, protein-DNA interactions in the SV40 early promoter region are not affected by DNA methylation (Harrington *et al.*, 1988; Höller *et al.*, 1988; Bryans *et al.*, 1992). However, when the SV40 early promoter is placed in a CpG-rich, methylated environment, transcription is considerably reduced in transient transfection assays (Bryans, 1989; Bryans *et al.*, 1992; Chapter 3 and 4). Greater inhibition is seen the greater the extent of methylation and, furthermore, methylation with the prokaryotic methylases *Hpa*II and *Hha*I also results in transcriptional inhibition (section 3.4), although no sites for these enzymes are present in the promoter region. For these reasons, it was proposed that methylation of the vector sequences, which surround the SV40 promoter and are, unlike the SV40 DNA, rich in CpGs, is responsible for transcriptional silencing by DNA methylation.

To test this hypothesis, a new method was developed to methylate specific regions in plasmid DNA (section 4.2.2). This method yields plasmids with methylated regions, i.e. patches of DNA in which the CpGs are methylated. The methylation is confined to the patched region and is stable in the preimposed pattern for at least two days *in vivo*. About ten years ago, a different method had been described to generate regionally methylated DNA. However, the stable integration of *in vitro* hemimethylated constructs was required to yield regions of symmetrically methylated DNA (Stein *et al.*, 1982; Keshet *et al.*, 1985). In contrast, regionally methylated plasmids generated as described in section 4.2.2 do not require integration and thus, are suitable for use in transient expression assays.

Several regionally methylated constructs were generated to assess the effect of methylation of non-promoter sequences on transient expression. The results, summarized in Table 2, are unambiguous: methylation of vector sequences leads to transcriptional inhibition of the SV40 promoter and the inhibition is independent of the position of the methylated region within the plasmid. Rather, inhibition is dependent on the length or the CpG content of the methylated patch on the plasmid. This is in stark contrast to the general belief that DNA methylation is inhibitory only in the promoter region, but not in sequences flanking the promoter (Razin and Cedar, 1991; Bird, 1992). However, there are examples where it has been demonstrated that methylation of non-promoter sequences effects gene expression. Methylation of the 3′ region of the hamster adenine phosphoribosyltransferase (*aprt*) gene reduced transcription by 17%, and methylation of the 3′ regions of the herpes thymidine kinase (*tk*) gene completely abolished transcription (Keshet *et al.*, 1985). In the case of the human β-globin gene, Yisraeli *et al.* (1988) identified a methylation-sensitive region at the 3′ end of the gene and for the well-documented example of the human glucose-6-phosphate dehydrogenase gene, it was shown that a CpG island in the 3′ coding region (i.e., a non-promoter region) on the active X chromosome is hypomethylated and hypersensitive to nucleases (Wolf *et al.*, 1984; Wolf and Migeon, 1985). Other reports, however, clearly relate transcriptional repression to promoter methylation (Busslinger *et al.*, 1983; Murray and Grosveld, 1987; Levine *et al.*, 1992). As stated earlier, the reasons for these discrepancies are unknown but may be based on the fact that 80% of the sequence of plasmid pVHCk is CpG-rich and thus, represents a rather large CpG island of 4 kb. One might argue that CpG island methylation does not reflect the *in vivo* situation although island methylation on the inactive X chromosome is thought to play a role in transcriptional repression (reviewed by Singer-Sam and Riggs, 1993; see also section 1.6). The downstream CpG island of the imprinted *Igf2r* gene, which is methylated on the active maternal allele and might constitute the oocyte-specific imprint (Stöger *et al.*, 1993; see also section 1.7) represents an example of CpG island methylation outwith the inactive X chromosome. Bird (1992) suggests a

model, whereby promoter methylation is dependent on the proximity of methyl-CpGs to the promoter, the strength of the promoter and the density of methyl-CpGs. Certainly, CpG density of the regionally methylated constructs is abnormally high and thus, might be able to override the distance effect normally associated with methylation-mediated gene repression. It would be interesting to see whether regional methylation is still effective using a larger plasmid, where patches of methylation are further away from the promoter than 2.5 kb.

As methylated DNA can adopt a nuclease-resistant chromatin structure, which is a characteristic of inactive chromatin (reviewed by Lewis and Bird, (1991); see also section 1.5), the chromatin formation of transiently transfected plasmids was investigated (Chapter 5). Consistent with reports by Reeves *et al.* (1985) and Archer *et al.* (1992), no nucleosomal repeat pattern was found for the non-replicating plasmid. However, resistance to digestion with restriction enzyme *Msp*I increased post transfection, indicating the binding of nuclear factors to the plasmid. In addition, methylated DNA was found to be more resistant to digestion by *Msp*I than unmethylated DNA, a phenomenon previously described for both integrated and transiently transfected DNA sequences (Antequera *et al.*, 1989; Levine *et al.*, 1991). The most surprising observation is that unmethylated regions in regionally methylated plasmids showed reduced cleavage by *Msp*I compared to mock-methylated constructs, indicating that methylation in one region can influence the nuclease sensitivity in an unmethylated region on the same plasmid. The spreading of inactive chromatin structure was previously observed for integrated sequences, however, it was concluded that the range of spreading is limited to a few hundred nucleotides (Keshet *et al.*, 1986). It may well be that this effect is more efficient in circular plasmid molecules than in cellular DNA.

What is the molecular basis of this spreading process? The assembly of ubiquitous nuclear proteins, such as histones, HMG or matrix proteins on the plasmid templates may be responsible for the observed differences in *Msp*I sensitivity of methylated and unmethylated DNA. Buschhausen *et al.* (1987) demonstrated that

chromatin formation was required for transcriptional silencing of the methylated *tk* gene, however as mentioned above, nucleosomal assembly was not observed on the non-replicating plasmid and yet, it is transcriptionally silenced by DNA methylation. In addition, differences between nucleosomal arrangement on methylated and unmethylated DNA have not been reported (Buschhausen *et al.*, 1987; Weih *et al.*, 1991). Interestingly, Englander *et al.* (1993) observed differences for histone tetramer, but not octamer assembly on differentially methylated templates *in vitro*.

Highly abundant nuclear proteins, which bind preferentially to methylated DNA seem tailor-made to mediate the effects of DNA methylation on chromatin structure and transcription. Several of these proteins have been identified (recently reviewed by Ehrlich and Ehrlich, (1993) and Tate and Bird, (1993)) and have been shown to be involved in some of the functions required for methylation-mediated gene silencing. MeCP1 (methyl CpG-binding protein 1; Meehan *et al.*, 1989) and MDBP-2 (methylated DNA-binding protein 2; Pawlak *et al.*, 1991) do inhibit transcription from methylated templates *in vivo* and *in vitro* (Boyes and Bird, 1991; Boyes and Bird, 1992; Jost and Hofsteenge, 1992). For two of these proteins, MDBP-2 and MeCP2, a direct correlation with chromatin has been established, suggesting a functioning of these factors within a chromatin context. MDBP-2 shows sequence homologies to histone H1 (Jost and Hofsteenge, 1992) and MeCP2 is located in pericentromeric heterochromatin (Lewis *et al.*, 1992). It may be the ability of a second domain in MeCP2 to bind to unmethylated DNA (Meehan *et al.*, 1992; Nan *et al.*, 1993), which is important in the spreading of the nuclease-resistant chromatin structure from methylated to unmethylated regions. The preferential association of these or similar, as yet unknown proteins to methylated DNA, together with the interaction of other chromatin factors may cause the spreading of nuclease-insensitivity of regionally methylated plasmids.

An alternative explanation is that cooperative binding of histone H1 mediates the spreading of nuclease-resistant chromatin structure. Although it is not clear whether histone H1 binds preferentially to methylated DNA (Higurashi and Cole,

1991; Levine *et al.*, 1993; see also section 1.5) it remains a prime candidate for a mediator of methylation-induced gene repression. Higurashi and Cole (1991) found, that when DNA was associated with histone H1 *in vitro*, digestion with *Msp*I, but not with other restriction enzymes was specifically inhibited on the methylated DNA substrate. They concluded that binding of H1 to methylated DNA changes DNA conformation, thus rendering the DNA resistant to digestion by *Msp*I. It has been shown that histone H1 acts as a general repressor of transcription (Croston *et al.*, 1991; Laybourn and Kadonaga, 1992), but does it preferentially inhibit transcription from methylated templates? Levine *et al.* (1993) gave a first answer to that question in performing *in vitro* transcription assays and found that H1 acts as a more potent repressor of transcription on methylated than on unmethylated templates. This however, is not the case for transcription from plasmid pVHCk (section 7.2.2). The reasons for these contrary findings are probably similar to those quoted earlier to explain the varying reports about the affinity of histone H1 to methylated DNA: the different sources and methods used to purify H1 and the different ways to assemble it on DNA and to assay its effect on *in vitro* transcription. A similar approach as that described by Laybourn and Kadonaga (1992) needs to be undertaken to be able to conclusively define the role of histone H1 in methylation-mediated gene repression.

Although both the methylated and unmethylated plasmids form nucleosomal chromatin after transfection into COS-1 cells, the experiments described in section 7.1 did not deliver the anticipated results due to the increasing demethylation of the rapidly replicating plasmids (Fig. 7.4). However, if modified, the system has the potential to be a powerful tool to investigate the structural differences between chromatin assembled on methylated and unmethylated templates. By careful mutation of the origin of replication region of the SV40 promoter (see Fig. 1) it might be possible to achieve a replication level of plasmid pVHCk in COS-1 cells whereby the cellular methylase is able to maintain the preimposed methylation pattern. By isolating the resulting minichromosomes one would then be able to analyse the protein structure of

the chromatin on the differentially methylated plasmids or possibly even on regionally methylated plasmids.

The approach used throughout this study utilizes the CpG density of vector sequences in connection with a methylation-insensitive promoter (Fradin *et al.*, 1982; Grässmann *et al.*, 1983; Bryans *et al.*, 1992) to investigate the effects of regional DNA methylation on transcription and chromatin structure. A strong correlation between the methylation of non-promoter sequences and transcriptional inhibition is observed, and the data presented in Chapter 5 suggest that regional methylation of CpG-rich sequences can drive plasmids into nuclease-insensitive chromatin formation.

It has been reported that DNA methylation gradually spreads from integrated DNA sequences into the genome (Toth *et al.*, 1989; Orend *et al.*, 1991). The reverse case, spreading of DNA methylation from host sequences to an integrated marker gene, has been observed in *Petunia* and it was concluded that the methylation status of the integration region is critical for the transcriptional activity of the transgene (Pröls and Meyer, 1992). An interesting proposal is made by Doerfler (1992; 1993) who interprets this spreading of DNA methylation as a defense mechanism against the realisation of foreign gene expression. Furthermore, it has been demonstrated that DNA methylation follows X-chromosome inactivation (Lock *et al.*, 1987), indicating that methylation is not a prerequisite for the formation of inactive chromatin. Taking these data together with the results described above, a two-stage process can be proposed. First, DNA methylation supports the formation of inactive chromatin, possibly via the binding of proteins which bind specifically to methylcytosine, and, in addition, has a function in maintaining this inactive state, which then can spread to adjacent regions. Second, following replication, DNA in inactive chromatin will become methylated. The evidence presented here supports the first step, and the results quoted above support the second step. Thus, it appears that DNA methylation "is both dancing to the piper and calling the tune" (Bird, 1992).

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