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Vector methylation inhibits transcription from the SV40 early promoter

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Methylation of a plasmid containing the SV40 promoter linked to the chloramphenicol acetyl transferase (CAT) gene, with either murine DNA methylase or methylase *Sss*I results in inhibition of the expression of the reporter gene after transfection into cultured cells. Methylation of the plasmid with the methylases *Hha*I and *Hpa*II has no effect on the expression of this gene. Protein–DNA interactions in the SV40 promoter are not affected by the presence of methylcytosine suggesting that inactivation results from the formation of an inactive chromatin structure that is dependent on the high CG content of the plasmid.

DNA methylation; SV40 promoter; CpG island; Sp1; Chromatin structure

1. INTRODUCTION

Inhibition of transcription has been documented for a large number of genes containing methylcytosine in the promoter region. However, to date there is insufficient evidence for a single mechanism of inactivation. Methylation can interfere directly with the transcriptional machinery by preventing the binding of transcription factors. For example, the major late transcription factor [1], the adenovirus E2 factor [2,3], the cAMP responsive element binding protein [4] and AP2 [5]. However 'methylation sensitive' transcription factors are still in a minority of those studied. Methylation-sensitive genes such as the adenovirus E2a gene [6,7] and the herpes simplex virus tk gene [8] bind transcription factors regardless of the methylation state of their promoters. In such genes the effect of methylation may result from the formation of inactive chromatin structures that are inhibitory to efficient transcription as observed for the herpes simplex virus thymidine kinase gene [9,10]. A protein factor has recently been purified that binds to clusters of methylcytosines in promoter regions resulting in inhibition of transcription [11,12].

On transfection of cultured cells, it has been shown in some cases that methylated DNA assumes an inactive, DNase I insensitive, chromatin structure whereas, unmethylated DNA forms DNase I sensitive chromatin characteristic of expressing genes [13]. In other cases the presence of transcription factors appears to be able to override inhibitory effects of methylation and the binding sites are actively demethylated [14,15].

In this report we study the effect of methylation of the SV40 promoter and its flanking sequences on gene expression. This is a promoter that is not normally methylated in vivo and that is regarded as a model for a housekeeping promoter. We confirm that methylation of CpG dinucleotides within the SV40 promoter sequence does not affect transcription factor binding yet quite low levels of methylation, generated largely in the CpG-rich environment of the prokaryotic vector DNA, significantly reduce transcription when the DNA is introduced into cultured cells. We propose that inactivation most likely occurs via the formation of an inactive chromatin structure that does not involve promoter methylation.

2. MATERIALS AND METHODS

2.1. *Isolation of DNA methylase*

DNA methylase was prepared from mouse Krebs II ascites tumour cells as previously described [16]. Prokaryotic DNA methylases were purchased from New England Biolabs.

2.2. *Construction and organisation of pVHC1*

pVHC1 was constructed by inserting the promoter/enhancer region of SV40 into the promoterless CAT gene containing plasmid p200. p200 was constructed by replacing the promoter sequence of the plasmid pTK3CAT with a polylinker sequence from pIC20H [17]. A map of the plasmid and the location of the CG dinucleotides and CCGG and CGCG sites is shown in Fig. 1a.

2.3. *Methylation of plasmid DNA*

(a) *with murine methylase*: plasmid DNA (5 μg) was methylated in a 70 μl reaction containing 30 μM *S*-adenosyl methionine (in some cases this was tritiated at 0.5 Ci/mmol), 0.1 mg/ml bovine serum albumin, 50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 10% glycerol and 110 U murine DNA methylase (one U of enzyme will incorporate one pmol of methyl group into denatured DNA from *M. luteus* in 1 h). After the appropriate incubation time at 37°C the DNA was reiso-

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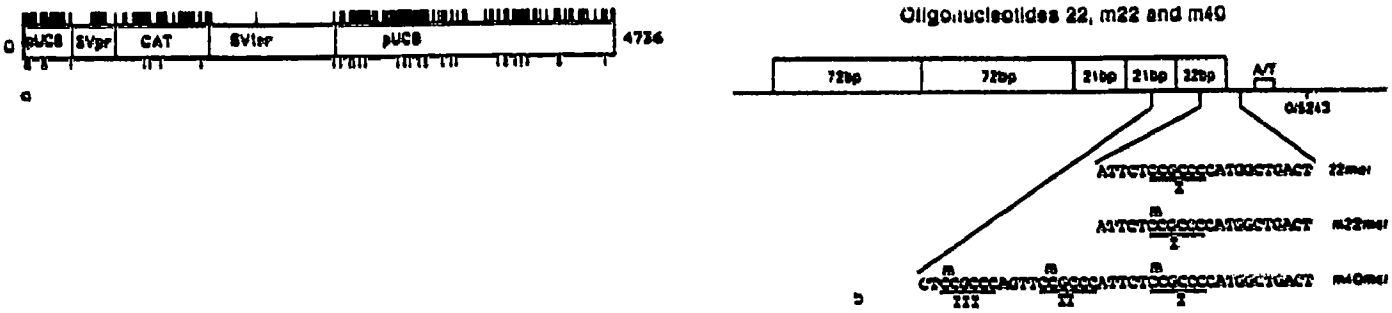


Fig. 1. (a) Linearised map of pVHC1 showing the location of the SV40 promoter and terminator regions and the CAT gene. Above and below the map, short vertical lines show the distribution of CpG dinucleotides and *Hpa*II and *Hha*I sites, respectively. (b) An expanded view of the promoter region showing the location of the three oligonucleotides.

lated. The extent of methylation was determined either by calculating the number of radioactive methyl groups incorporated or by measuring resistance to *Hpa*II and *Cfo*I endonucleases or by Maxam Gilbert sequencing. (b) *with bacterial methylases*: plasmid DNA was methylated as per manufacturers instructions (sometimes using [^{3}H]AdoMet) and completion of methylation assayed as above. Methylases (New England Biolabs) *Sss*I and *Hha*I methylated to completion but M*Hpa*II was incompletely effective.

2.4. *Transfection of tissue cultured cells and analysis of CAT activity*

Methylated or unmethylated plasmid DNA (5 μg) was introduced into mouse L929 (5×10^5) cells by the calcium phosphate co-precipitation method [18]. Cells were grown in 6 cm dishes in Eagle's medium (Glasgow modification), supplemented with 10% newborn calf serum. All transfections were performed in duplicate. Southern blot analysis of transfected cells indicated the presence of comparable amounts of plasmid DNA whether or not it was methylated prior to transfection. Cell extracts were prepared and assayed for CAT activity by following the transfer of butyryl groups to ^{14}C-labelled chloramphenicol according to the method of Seed and Sheen [19]. Protein assays were performed on the same extract using the method of Bradford [20].

2.5. *Gel mobility shift assays*

Oligonucleotides were prepared on an Applied Biosystems apparatus. The sequence and location of oligo 22, m22 and m40 is shown in Fig. 1b. Oligo 62 has the unrelated sequence CAA GCT TGG CGT AAT CA. When required, oligonucleotides were end-labelled with [γ-^{32}P]ATP using polynucleotide kinase (Boehringer) under conditions recommended by the manufacturer. HeLa cell nuclear extracts were prepared according to the method of Dignam et al. [21]. Protein-DNA complexes were formed in a reaction containing 0.5–1.0 ng of end-labelled oligonucleotide, 10 μg of HeLa cell nuclear extract, 2 μg poly dI-dC, 20 mM HEPES pH 7.9, 20% v/v glycerol, 0.1 M KCl, 0.2 mM EDTA, 5 mM DTT in a total volume of 10 μl. Complexes were separated on a 7% non-denaturing polyacrylamide gel and detected by autoradiography.

3. RESULTS

3.1. *Methylation of pVHC1 with murine DNA methylase reduces expression from the SV40 early promoter*

The plasmid pVHC1, containing the SV40 promoter linked to the chloramphenicol acetyl transferase (CAT) gene, was methylated in vitro using murine DNA methylase. Although occasionally levels of up to 60% CpG methylation were obtained (measured by tritiated methyl group transfer), more typical values after a 5–6 h incubation ranged from 20–30%. This is partly the result of formation of unproductive, covalent complexes between the mouse enzyme and substrate DNA [22]. Moreover, *Hpa*II digestion of the population of methylated plasmid molecules (Fig. 2a) showed some molecules that were largely resistant to digestion, whilst others were completely digested by *Hpa*II. Maxam and Gilbert sequencing on the plasmid which had been methylated in vitro showed little evidence for methylation of promoter or adjacent regions (Fig. 2b). It is not clear from these results 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.

When this DNA was introduced into mouse L929 cells transcription was progressively inhibited (Fig. 3). 80% inhibition was achieved with plasmid that clearly showed less than 30% methylation (Fig. 2). pVHC1 contains a total of 216 CpG pairs (shown in Fig. 1a), 9 of which are contained within the SV40 encoded promoter DNA. If methylation were random, then at this level of saturation fewer than 0.1% of plasmid molecules would have all the 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.

3.2. *Methylation of pVHC1 with bacterial DNA methylases*

The methylase *Sss*I has the same specificity as the murine enzyme, methylating all cytosines in CG dinucleotides, but it acts in a de novo manner which results in a much faster transfer of methyl groups to unmethylated DNA. Complete methylation of pVHC1 by M.*Sss*I (as assessed by resistance to *Hpa*II and by Maxam and Gilbert sequencing; Fig. 2) led to almost complete inhibition of transcription from the SV40 early promoter when the plasmid was introduced into mouse L929 cells (Fig. 3). With the fully methylated plasmid, the percent inhibition of transcription was dependent on the time after transfection (Fig. 4): less than 50% inhibition being

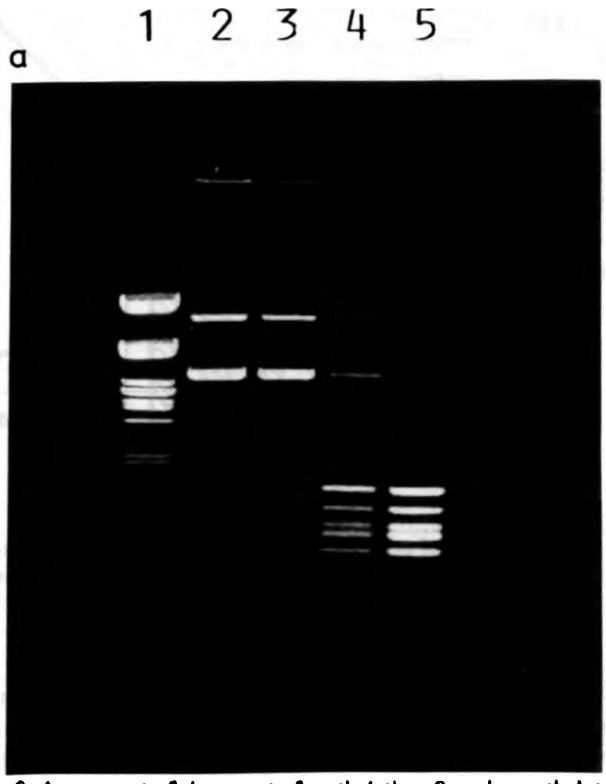
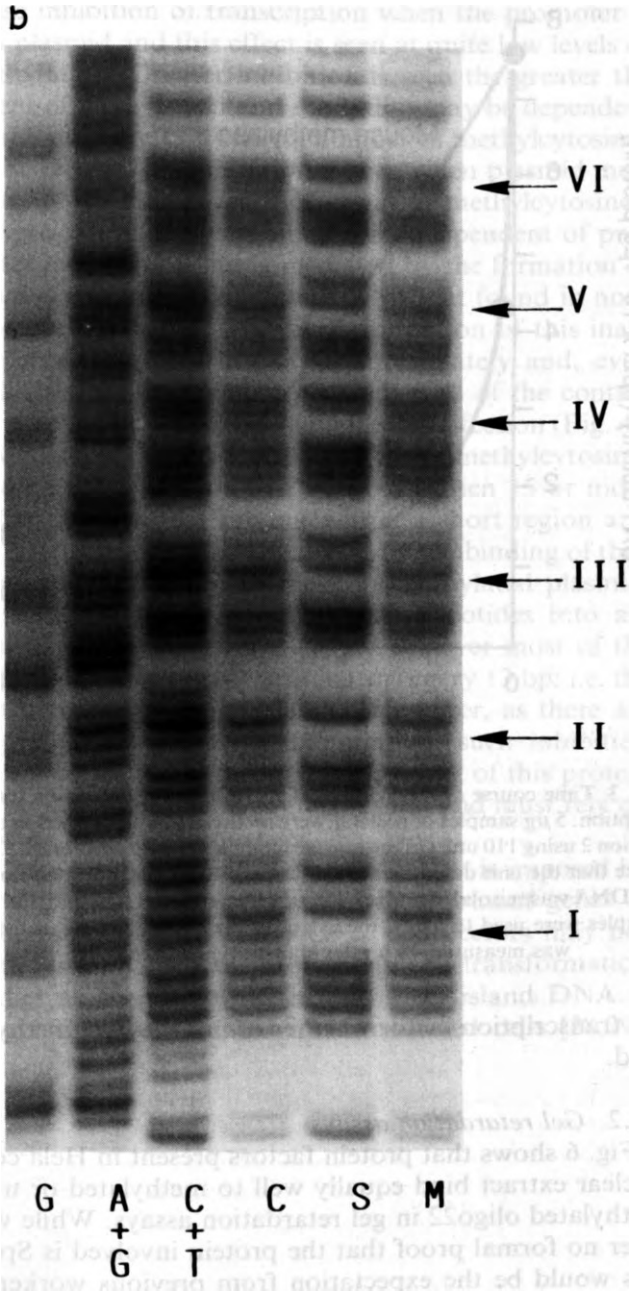


Fig. 2. Assessment of the extent of methylation. Samples methylated for 5 h as described in the legend to Fig. 3 were either digested with *Hpa*II (a) or subjected to partial sequencing by the Maxam Gilbert method (b). In (a) the lanes represent marker (1); undigested plasmid (2); plasmid methylated with M.*Sss*I and digested with *Hpa*II (3); plasmid methylated with murine methylase and digested with *Hpa*II (4); mock methylated plasmid digested with *Hpa*II (5). In (b) the lanes represent the G-reaction; the A+G reaction; the C+T reaction and the C reaction on the *Hin*dIII/*Kpn*I fragment 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). The fragment was endlabelled at the *Hin*dIII end using Klenow polymerase and [α-^{32}P]dCTP. The arrows indicate the position of the cytosines in CG dinucleotides in the six GC boxes of the SV40 promoter.



found at 24 h whilst over 90% inhibition was observed at 48 h. This implies that formation of the inactive structure does not occur immediately following transfection.

In contrast methylation with *Hpa*II and *Hha*I methylases had no effect on transcription (results not shown). There are only 33 sites for these enzymes distributed evenly but sparsely over the prokaryotic section of the plasmid (Fig. 1a). None of these sites lie in the SV40 early promoter region.

3.3. *Inhibition of transcription is not mediated through GC box methylation*

The SV40 promoter contains 6 GC box motifs which influence transcription to varying degrees and are capable of binding the transcription factor Sp1 [23]. We confirm here that Sp1 binding is unaffected by methylation.

3.3.1. *Transfection experiments.*

Oligonucleotides 22 or 40 residues long, containing one or three Sp1 binding sites respectively, were synthesized (Fig. 1b). Cotransfection of mouse L929 cells with pVHC1 and the double stranded oligonucleotides resulted in inhibition of gene expression, an effect which was independent of the methylation state of the synthetic oligonucleotides (Fig. 5). Both the methylated and unmethylated 22mers resulted in a 5-fold reduction in gene expression while the methylated 40mer inhibited expression by 90%. A non-specific oligonucleotide (oligo62) or small restriction fragments of pAT153 had no effect. This indicates that oligonucleotides containing Sp1 binding sites are equally effective at sequester-

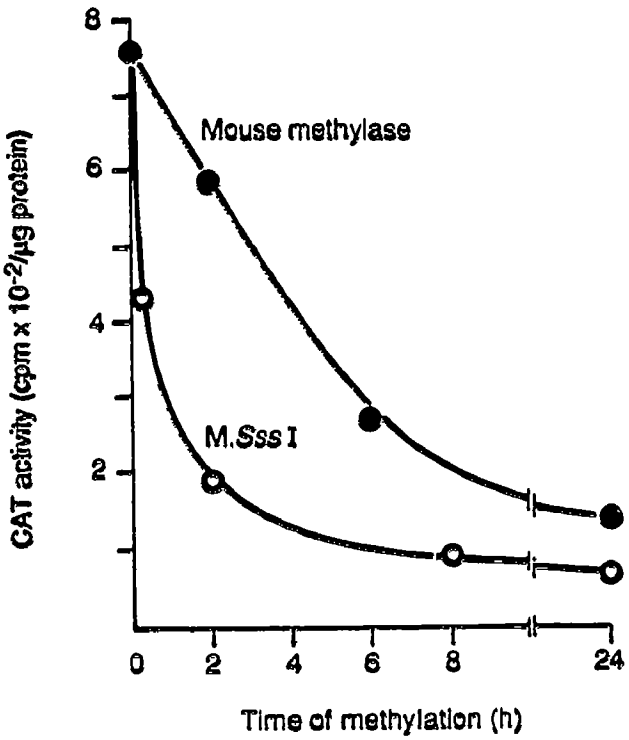


Fig. 3. Time course of methylation of pVHC1 and the effect on transcription. 5 μg samples of pVHC1 were methylated as described in the section 2 using 110 units of the murine methylase or 1.5 units of M.*Sss*I (note that the unit definitions are different). After the indicated time, the DNA was reisolated and used to transfect mouse L929 cells. Other samples were used to assess the extent of methylation. CAT activity was measured 48 h after transfection of the cells.

ing transcription factor whether or not they are methylated.

3.3.2. *Gel retardation assays*

Fig. 6 shows that protein factors present in Hela cell nuclear extract bind equally well to methylated or unmethylated oligo22 in gel retardation assays. While we offer no formal proof that the protein involved is Sp1, this would be the expectation from previous workers' findings [23] and from the competition experiments reported below.

Both the methylated and non-methylated non-labelled oligonucleotides compete equally well for Sp1 binding to labelled probe (Table I and [23,24]). Addition of an excess of the promoterless plasmid, p200, had no effect on binding while oligonucleotides including unrelated promoters had only a small competitive effect. These results confirm that the ubiquitous transcription factor Sp1 can bind to its target sequence independently of the state of methylation of that sequence.

4. DISCUSSION

DNA methylation controls the level of expression of different genes by different mechanisms. In some cases,

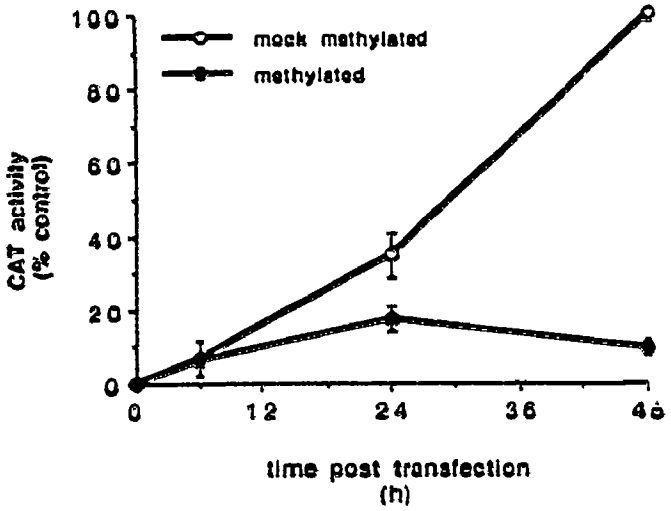


Fig. 4. Time course of expression of CAT activity. Cells were transfected with the plasmid, methylated (or mock methylated) using M.*Sss*I and CAT activity was determined at 6, 24 and 48 h.

single, key methylcytosines in the promoter of a gene may prevent the binding of an essential transcription factor and hence reduce transcription.

However, it has been shown previously [24,25] and we have confirmed here that Sp1 can bind to its target sequence independently of its methylation status. This is despite the fact that each Sp1 binding site contains a CG dinucleotide. 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, however infrequently this were to occur. On such grounds one would not expect promoters of housekeeping genes to be controlled via cytosine methylation. It is also pertinent that the SV40 enhancer is free of CG dinucleotides and so is not susceptible to inactivation by methylation. A previous study on the effect of methylation on the SV40 early promoter in its natural environment, showed that methylation of the 27 pairs of CpG dinucleotides in the SV40 genome had no effect on early gene expression when the viral DNA was introduced into cells [26]. However, the SV40 genome is very deficient in CpGs and their distribution is very uneven; 19

Table I

Competition in bandshift experiments. 2 ng end-labelled 22mer (double-stranded) was incubated with 10 μg nuclear extract (as for Fig. 6), in the presence of increasing concentrations of competing oligonucleotides as indicated. The retarded band was excised and counted

Competitor DNA	Amount (ng) required to give 50% inhibition of binding
p200	500
22mer	8
22mer-methylated	6
40mer-methylated	3

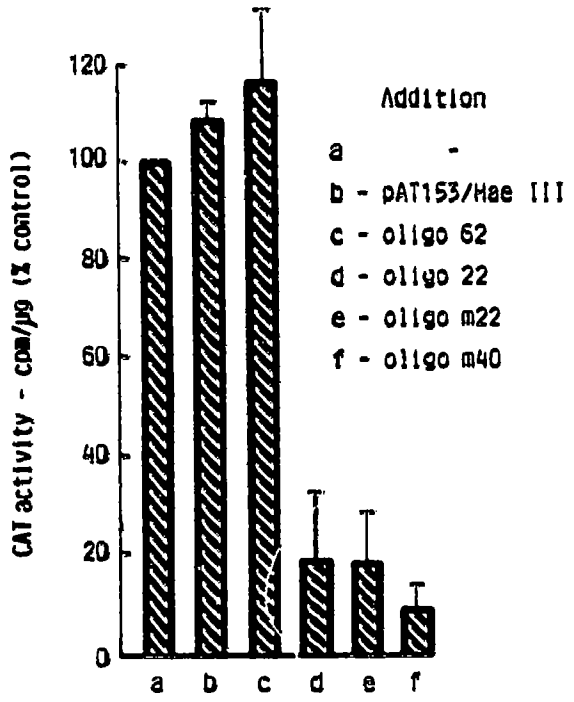


Fig. 5. Cotransfection of pVHC1 with oligonucleotides. Cells were transfected with 5 μg pVHC1 in the presence of 5 μg of the indicated oligonucleotide and CAT activity measured 48 h later.

of the 27 CpGs occurring within a region of 350 bp spanning the promoter region and the origin of replication [27]. These CpGs may be functionally important (e.g. 6 are present in Sp1 binding sites) but are not normally methylated in vivo.

Nevertheless, we show here that methylation does cause inhibition of transcription when the promoter is in a plasmid and this effect is seen at quite low levels of methylation. Greater inhibition is seen the greater the extent of methylation and inhibition may be dependent on the presence of a critical number of methylcytosines in the DNA. 70% inhibition is seen when plasmid molecules contain an average of about 50 methylcytosines. We propose that this inhibition is independent of promoter methylation but is mediated by the formation of inactive chromatin characteristic of that found in non-expressing cells [13]. However, formation of this inactive structure does not occur immediately and, even with the fully methylated plasmid, 50% of the control enzymic activity is found 24 h after transfection (Fig. 4).

Meehan et al. [11] have shown that a methylcytosine-binding protein binds to DNA only when 15 or more methylcytosines are present within a short region and the effect we see may be mediated by the binding of this, or a similar, protein. In the fully methylated plasmid, taking the distribution of CG dinucleotides into account, methyl groups will be present over most of the molecule (excluding the terminator) every 13 bp; i.e. the vector resembles a CpG island. However, as there are only 9 CGs in the promoter region, such inhibition cannot be mediated through the binding of this protein to the promoter region of the plasmid and must rely on binding to vector sequences.

These results indicate a constraint that is imposed by the vector on the expression of recombinant genes in higher eukaryotes. Clearly G+C rich vectors may not be the most satisfactory for use in cell transformation studies as there is evidence that CpG island DNA is progressively methylated in transformed cells [28,29].

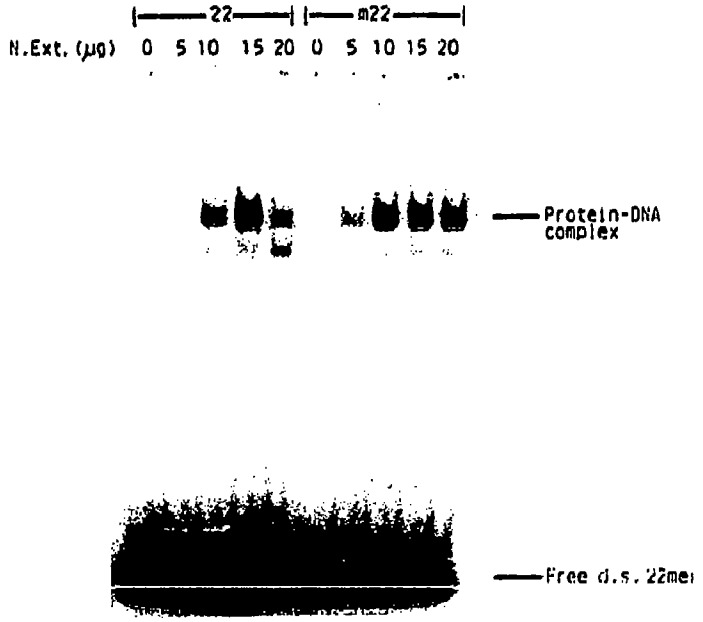


Fig. 6. Gel retardation analysis: 2 ng of end-labelled 22mer or m22mer (double-stranded) were incubated with 0 to 20 μg nuclear extract as described in the section 2. The products were separated on a 7% polyacrylamide gel which was dried and autoradiographed.

Furthermore, following gene transfer, an introduced gene can integrate at random into the host chromosome and may become located in a G+C-rich environment. The present study shows that methylation of flanking sequences can have a dramatic effect on subsequent gene expression.

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