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Eukaryotic DNA methylases and their use for *in vitro* methylation

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DNA methylases from mouse and pea have been purified and characterized. Both are high molecular mass enzymes that show greater activity with hemimethylated than unmethylated substrate DNA. Both methylate cytosines in CpG preferentially, but not exclusively and show similar kinetics of methylation, which makes it difficult to saturate all possible sites on the DNA, but procedures are described that circumvent this problem.

1. Introduction

In addition to the four major bases, DNA of higher eukaryotes contains a fifth base, 5-methylcytosine. This base, which can replace 2–8% of cytosine residues in vertebrate DNA and up to 30% of cytosine residues in DNA of higher plants, is introduced, after replication of the DNA, by the addition of a methyl group to certain cytosines.

In animals, methylcytosine occurs primarily in the dinucleotide mCG; whereas in plants it also occurs in the sequence mCNG, where N is any base. In neither case are all of the sites methylated. All of these sequences are symmetrical and so, on replication, a methylated di- or trinucleotide is converted, first into a hemimethylated, and then, if methylation does not intervene, into an unmethylated di- or trinucleotide. DNA methylases, however, normally act on hemimethylated sites to preserve the pattern of replication from one generation to the next.

We have been studying the DNA methylase from mouse ascites tumour cells and have raised polyclonal and monoclonal antibodies to the enzyme. This allows us to study the enzyme in crude extracts by using immunoblotting and immunoprecipitation techniques.

DNA methylases have now been purified from a number of different plants and animals and it is probable that all are high molecular mass enzymes, in contrast to the prokaryotic DNA methylases (Adams & Burdon 1985). Where lower molecular mass material is described, this appears to have arisen from the proteolytic degradation of the methylase, which can occur either *in vivo* or *in vitro* (Adams *et al.* 1986, 1989). Table 1 summarizes the situation.

The enzyme present in different species show considerable sequence conservation as is evident from immunological studies that demonstrate the presence in rodents, primates and amphibians of material cross reacting with an antibody raised against the purified DNA methylase from mouse Krebs II ascites tumour cells (Adams *et al.* 1988).

2. Characterization of DNA methylase

(*a*) *Size and substrate preference of mouse DNA methylase*

The mouse DNA methylase is a single subunit enzyme of relative molecular mass, M_r, 185000–190000 most of which is present loosely associated with the nucleus from which it can be extracted with buffers containing 0.2 M NaCl (Turnbull & Adams 1976). The enzyme is a globular structure with an appendix at one side (Spiess *et al.* 1988). Some enzyme remains

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Table 1. The size of eukaryotic DNA methylases

(The enzymes isolated from various sources are reported to have a wide variety of sizes. As described in the text, much of this variation may be caused by proteolysis.)

source of DNA methylase	size (kDa)	reference
wheat	50–55	Theiss *et al.* (1987)
Chlamydomonas	55–58	Sano & Sager (1980)
rat and mouse liver	100–120	Simon *et al.* (1978); Adams *et al.* (1986)
HeLa cells	120	Roy & Weissbach (1975)
bovine thymus	130	Sano *et al.* (1983)
human placenta	126	Zucker *et al.* (1985)
human placenta	135	Pfeifer *et al.* (1983)
Xenopus	140	Adams *et al.* (1981)
mouse plasmacytoma (MPC11) cells	140	Hitt *et al.* (1988)
pea	160	Yesufu *et al.* (1988, 1989)
mouse MEL cells	150, 175	Bestor & Ingram (1983)
mouse ascites cells	160, 185	Adams *et al.* (1986)
mouse P815 mastocytoma cells	190	Pfeifer & Drahovsky (1986)
mouse MEL cells	190	Bestor *et al.* (1988)

firmly bound in the nucleus and cannot be separated from the DNA even by using buffers containing 2 M NaCl (Burdon *et al.* 1985). It is believed that this firmly bound form is the enzyme that carries out maintenance methylation of newly replicated DNA (Davis *et al.* 1985, 1986).

The liver of new-born mice does contain some high molecular mass enzyme tightly bound to chromatin (Adams *et al.* 1989), but this is not detectable in adult liver (nor in many other tissues). Here the enzyme is present as a 100 kDa molecule that shows somewhat different properties from the ascites enzyme. In particular, the liver enzyme shows a marked capacity to methylate duplex DNA *de novo*; a property that can be enhanced in the ascites enzyme by limited treatment with trypsin. Trypsin treatment causes the degradation of the ascites and P815 mastocytoma enzymes through a 165 kDa enzyme and a series of further intermediates to a form of M_r of 100000. Later, smaller, inactive fragments are formed (Adams *et al.* 1986, 1989; Spiess *et al.* 1988). Although, initially, the molecular mass of the native, partially trypsinized enzyme remains unchanged, with further proteolysis the enzyme falls apart (Adams *et al.* 1989). We find the the initial action of trypsin is to bring about an increase in activity and that activity only decreases as the 100 kDa form of the enzyme is degraded (Adams *et al.* 1983, 1986, 1989). This initial increase in activity is not found by all groups (Spiess *et al.* 1988) and we suspect that it may be related to the finding that the high M_r enzyme tends to form large aggregates with DNA, whereas these are not formed with the trypsinized enzyme (Adams *et al.* 1989). Such aggregates may be partially insoluble and hence less active, and are poorly dissociated on phenol extraction leading to loss of DNA at the phenol interface, especially in the presence of excess enzyme. This loss is minimized when a protease step is included after normal incubation and before phenol extraction. However, under these conditions the increased activity of the 'pretrypsinized' enzyme is still apparent. On the other hand, when separated, the 190 kDa enzyme shows the greater activity (figure 1) and the loss of enzymic activity on treatment of cells with azadeoxycytidine is associated with the loss of only the larger band detected by immunoblotting procedures (Adams *et al.* 1986).

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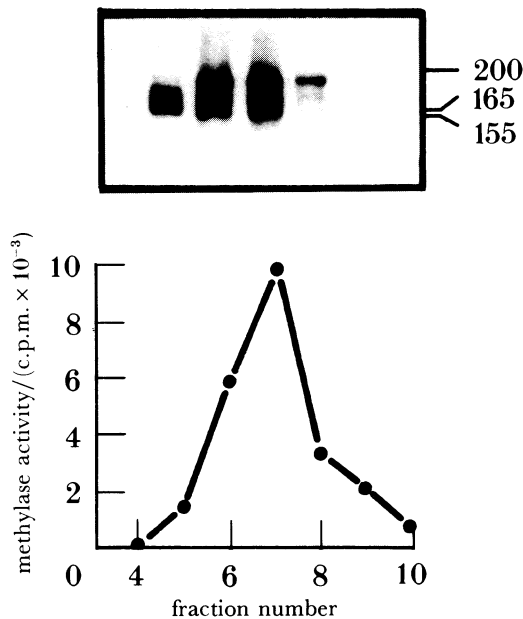

FIGURE 1. Fractionation of mouse DNA methylase on tRNA Sepharose. A sample of partially purified enzyme from mouse ascites cells was applied in low salt buffer to a 2 ml column of tRNA Sepharose and eluted with a linear gradient of 0 to 0.3 M NaCl. The graph shows the activity of each fraction and the autoradiogram above shows the results obtained when the fractions were separated by SDS polyacrylamide gel electrophoresis and immunoblotted against the antimethylase polyclonal antibody. Note the correspondence of the 190 kDa band with activity.

(*b*) *Pea DNA methylase*

The situation is very similar in peas despite the broader range of methylation in plants. There appears to be only one enzyme, which has a native molecular mass of about 160000 (figure 2) and is located in the nucleus (Yesufu *et al.* 1988, 1989).

The enzyme from peas differs from the mouse enzyme in that only about 20% of the activity can be extracted from nuclei with low salt buffers, and the endogenous DNA has to be partially

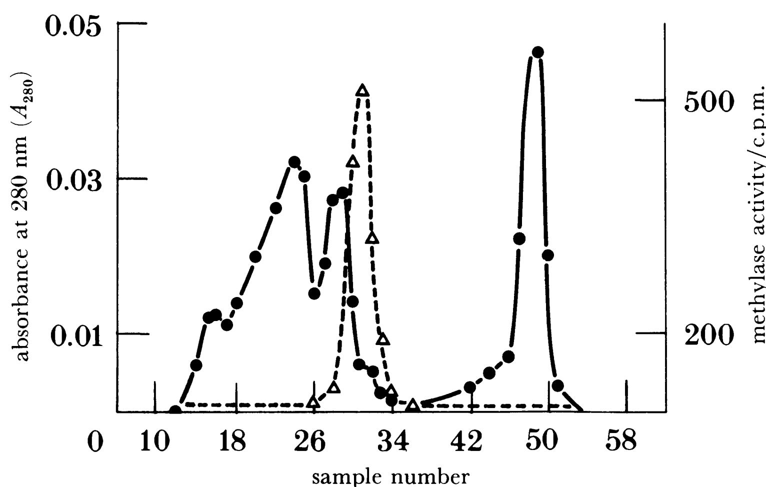

FIGURE 2. Fractionation of pea DNA methylase on Superose 6. A 2 ml sample of crude nuclear extract was applied to a 100 ml Superose 6 column. The graph shows absorbance at 280 nm (●) and the activity of the DNA methylase (△). Yeast alcohol dehydrogenase (M_r = 150000) elutes in fraction 31 showing the native enzyme to have a molecular mass of 150–160 kDa.

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degraded by treatment with micrococcal nuclease before the rest of the enzyme can be extracted, i.e. most of the enzyme is tightly associated with replicating chromatin.

In contrast to the mouse enzyme, which shows little activity on homologous DNA, the best substrate for the pea enzyme is DNA isolated from the tissue that shows the highest enzymic activity i.e. five day old pea-shoot tips. We attribute this preference to the presence in this DNA of a large number of hemimethylated substrate sites and we have shown that the activity on *M. luteus* DNA (which is a very poor substrate) is markedly enhanced following nick translation in the presence of methyl dCTP (Yesufu *et al.* 1989). Although the mammalian enzyme also shows greater activity with hemimethylated DNA it is clear that there are only a limited number of such sites on mammalian DNA.

(*c*) *Sequence specificity*

The mouse DNA methylase normally methylates cytosines only in the sequence CpG (Adams *et al.* 1986, 1988) although there are reports that the enzyme from rat liver also methylates cytosines in the sequence CpA and CpT (Simon *et al.* 1980) and that the enzyme from mouse P815 cells also methylates certain *Msp* 1 sites in the first cytosine (Pfeifer *et al.* 1985). We have also, on occasion, found evidence of reduced specificity (Adams *et al.* 1988) and figure 3 shows that on methylation of plasmid pVHC1 certain (but not all) CCGG sites become resistant to cleavage by *Msp* I. This may indicate that some methylation has occurred on the

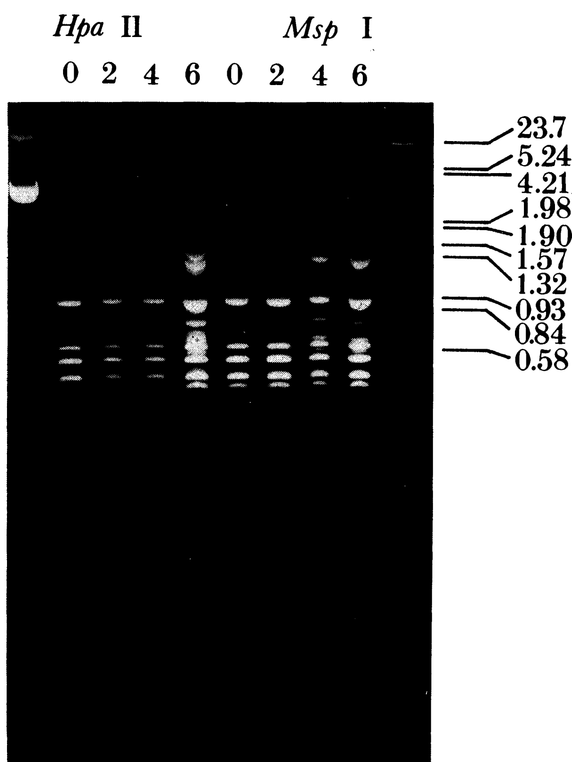

FIGURE 3. Restriction enzyme digestion of pVHC1 DNA methylated *in vitro* with mouse DNA methylase: pVHC1 DNA was incubated with mouse ascites DNA methylase for 0, 2, 4 or 6 hours and the reisolated DNA digested with either *Hpa* II or *Msp* I before separation by electrophoresis on an agarose gel. The figure shows the ethidium-stained gel. The left-hand lane contains undigested plasmid and the right-hand contains lambda DNA digested with *Hin* dIII as marker.

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first cytosine in this sequence (i.e. CC methylation). However, some of these sites (e.g. GCCCGGCG) may be blocked by CG methylation and may represent further examples of the unusual specificity of *Msp* 1.

If we assume that methyl groups are added only to CG dinucleotides, then at least 60 % of the CpGs present in a plasmid DNA can be methylated in a 6 hour incubation at a high enzyme to DNA ratio (figure 4).

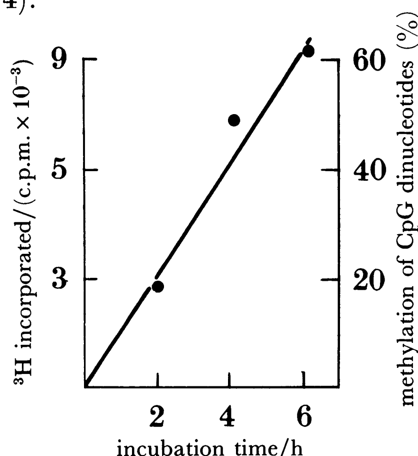

FIGURE 4. Methylation of plasmid DNA. In a series of separate incubations 1 μg of plasmid pVHC1 (Bryans *et al.* 1989) was methylated with ^{3}H AdoMet by using 140 units of mouse ascites DNA methylase in a total volume of 100 μl. The graph shows an approximately linear incorporation of radioactivity for 6 h. Although calculations show that over 60 % of CG dinucleotides are methylated at 6 h, it is clear from figure 3 that little totally resistant DNA is produced and that the original bands are still clearly visible. This indicates that methylation is occurring at dinucleotides other than CG (Adams *et al.* 1988).

Although only one peak of activity is observed with the pea DNA methylase, it is able to transfer methyl groups to CpG and CpNpG sequences. Thus when 20 % of CpG dinucleotides were methylated, about 4 % each of CpA and CpT (and probably CpC) were methylated when either pea or calf DNA was the substrate. As only about one fifth of CpN dinucleotides are in CpNpG this amounts to approximately equal methylation of CpG and CpNpG sequences. As few hemimethylated CpNpG sites exist in calf DNA the enzyme must be able to methylate such sites *de novo*.

(*d*) NaCl *and saturation methylation*

The mouse ascites enzyme methylates single-stranded DNA and native DNA, both unmethylated and hemimethylated, this last being the preferred substrate. The reaction with native DNA is strongly inhibited by 0.1 M NaCl (figure 5; Turnbull & Adams 1976). Activity is greater on supercoiled DNA than on relaxed DNA (Adams *et al.* 1984).

The mouse enzyme was reported to act by a processive mechanism, whereby, once bound to the DNA, it travels along the duplex methylating cytosines in CpGs (Drahovsky & Morris 1971). Binding occurs at the ends of molecules or at sites that are partially denatured (Pfeifer *et al.* 1985; Adams *et al.* 1979). We have taken advantage of this observation and the fact that binding and methylation of duplex DNA is strongly inhibited by 0.1 M NaCl to direct the enzyme to specific regions in gapped duplex molecules. Figure 6 shows how the gapped duplex molecule is constructed and methylated and the location of the methyl groups analysed. The evidence suggests that the tritiated methyl groups are restricted to the single-stranded region of the substrate.

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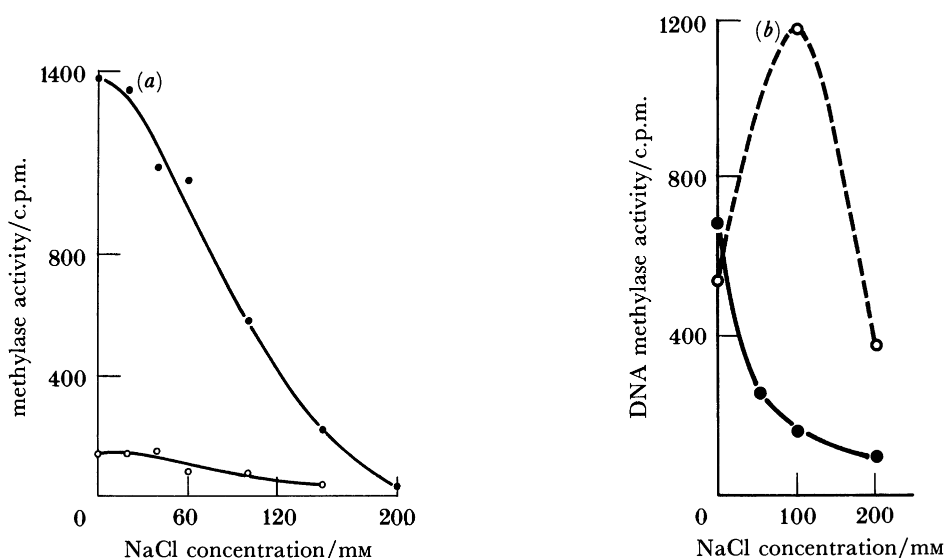

FIGURE 5. Effect of NaCl on methylation. The graphs show the effect of NaCl on the methylation of unmethylated (○) and hemimethylated (●) duplex DNA, (*a*); or on single stranded (--) and duplex (——) M13mp9 DNA (*b*). In the presence of 0.1 M NaCl, single-stranded DNA is the preferred substrate.

When the hemimethylated or fully methylated $tRNA^{Glu}$ gene (Goddard *et al.* 1983) is incubated in an *in vitro* transcription assay with an S100 fraction from HeLa cells, methylation has been shown to have a dramatic effect on this expression (Smart *et al.*, unpublished results).

The enzyme in pea *nuclei* shows only a limited salt sensitivity in comparison with the solubilised enzyme or with the mouse enzyme. This is a result of the nuclear enzyme being already firmly associated with the endogenous DNA substrate. Initial incubation of the *soluble* pea DNA methylase with DNA in the absence of NaCl for 10 minutes allows formation of a salt-resistant complex that remains stable in the presence of 0.1 M NaCl for up to 4 h at 30 °C (Yesufu *et al.* 1989). Thus the pea enzyme has a greater potential than the mouse enzyme for *in vitro* methylation and we have been using it to methylate plasmid and viral DNAs (figure 7).

3. Effect of *in vitro* methylation on DNA expression

By using a plasmid (pVHC1) containing the SV40 promoter linked to the chloroamphenicol acetyltransferase (CAT) gene as a reporter we have shown that even a limited degree of methylation by using the mouse ascites DNA methylase leads to almost complete inhibition of CAT expression when the plasmid is transfected into mouse L929 cells (Bryans *et al.*, unpublished results). Although there are six GC boxes in the SV40 promoter, each containing one CpG, it is unlikely that the effect we see can be mediated through methylation of these sequences. We arrive at this conclusion because, on average, only one of the six will be methylated in plasmids that show extensive inhibition of gene expression. Secondly, methylated and unmethylated oligonucleotides are equally effective at blocking transcription when cotransfected with the SV40CAT plasmid, showing that they are equally effective at sequestering essential transcription factors. Thirdly, in gel retardation experiments that used constructs containing one, three or six GC boxes, cytosine methylation fails to affect binding of the transcription factor, Sp1, to the DNA (Bryans *et al.*, unpublished results; Harrington *et al.* 1988; Höller *et al.* 1988).

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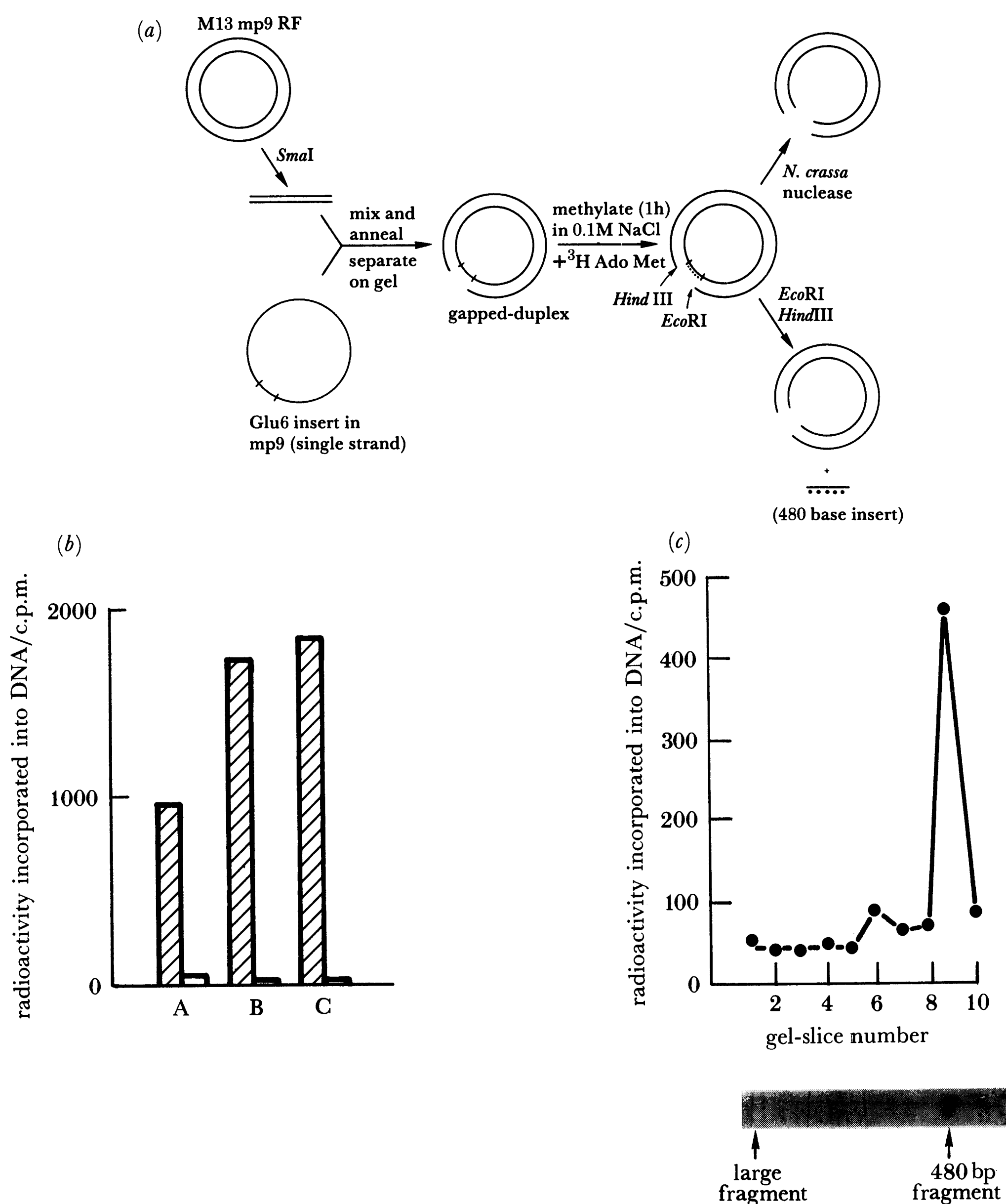

FIGURE 6. Construction and methylation of a gapped duplex DNA molecule: (*a*), the construction of a gapped duplex molecule from a construct containing a 480 b.p. insert in the *Sma* 1 site of M13mp9 (Goddard *et al.* 1983) and single stranded mp9. Following methylation *in vitro* with ^{3}H AdoMet the location of the methyl groups was established in two ways; (*b*), in three different experiments (A, B and C the methylated gapped duplex was treated with *N. crassa* nuclease that selectively digests single-stranded DNA. The figure shows the radioactivity present in acid insoluble material before and after digestion; (*c*), following treatment of the gapped-duplex DNA with *Eco* R1 and *Hin* dIII the insert is released. The digest was separated by agarose gel electrophoresis and subjected to fluorography (lower panel) and then sliced and counted to establish the location of the tritiated methyl groups. The figure shows the methylation is restricted to the single-stranded region in the gapped duplex.

These results contrast with those from using SV40 viral DNA where it has been shown that methylation has no effect on early gene expression when the DNA is introduced into cells (Grässmann *et al.* 1983; Bryans *et al.*, unpublished results). We believe this apparent contradiction is explained by the fact that the SV40CAT plasmid is well endowed throughout

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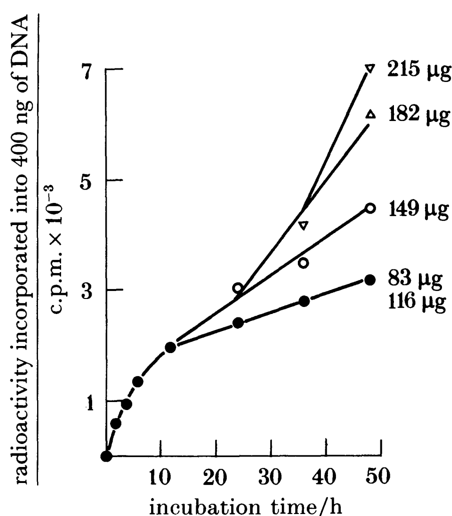

FIGURE 7. Methylation of ϕX174RF DNA with pea DNA methylase. In five separate tubes, 4 μgϕX174RF DNA was methylated at 30 °C with pea DNA methylase (66 units/mg). Further additions of methylase were made at 6 h (tubes 2–5), 12 h (tubes 3–5), 24 h (tubes 4 and 5) and 36 h (tube 5) and samples containing 400 ng DNA were removed from each tube at the indicated times for analysis. The figures on the right indicate the total amount of protein added to the tubes.

its length with CpG dinucleotides (there are 215 CpG dinucleotide pairs out of 4736 base pairs), whereas SV40 viral DNA contains only 27 pairs of CpG dinucleotides in its 5342 nucleotide pairs and these are clustered in the promoter region (Buchman *et al.* 1980). Methylation of 20% of the CpGs in the plasmid would lead to a methylCpG every 56 bases and these methyl groups may lead such DNA to assume an inactive chromatin conformation as suggested by Keshet *et al.* (1986) and Murray & Grosveld (1987). Viral DNA, deficient in CpG and hence in methyl groups, may not readily form such inactive chromatin.

4. Conclusion

DNA methylase is a high molecular mass, protease-sensitive enzyme that carries out maintenance methylation of newly replicated DNA with which it is firmly associated. It also has the ability to methylate DNA *de novo*, although this reaction occurs at a lower rate. The enzyme from plants will methylate cytosines in CpG and CpNpG sequences and this may also be true for the animal DNA methylase under certain circumstances.

In vitro, methylation of DNA takes place over many hours, yet the evidence shows that a stable enzyme–DNA complex is rapidly formed at 37 °C. It appears either that the enzyme processes only slowly along the DNA duplex or, more likely, that it is very inefficient at transferring methyl groups. This may be a result of disruption of the *in vivo* organization.

There are thus severe limitations to the use of eukaryotic DNA methylase for extensive *in vitro* methylation of cloned DNA. We have demonstrated how these limitations can be turned to advantage by directing the enzyme to specific, single-stranded regions of DNA engineered into duplex molecules, and we are studying the effects of methylation on the expression of several human tRNA genes.

Certain effects of *in vitro* methylation are quite dramatic and occur when as few as one in six CpG dinucleotides are methylated. We conclude that such effects are mediated, not by an effect on transcription factor binding, but rather an an affect on chromatin structure.

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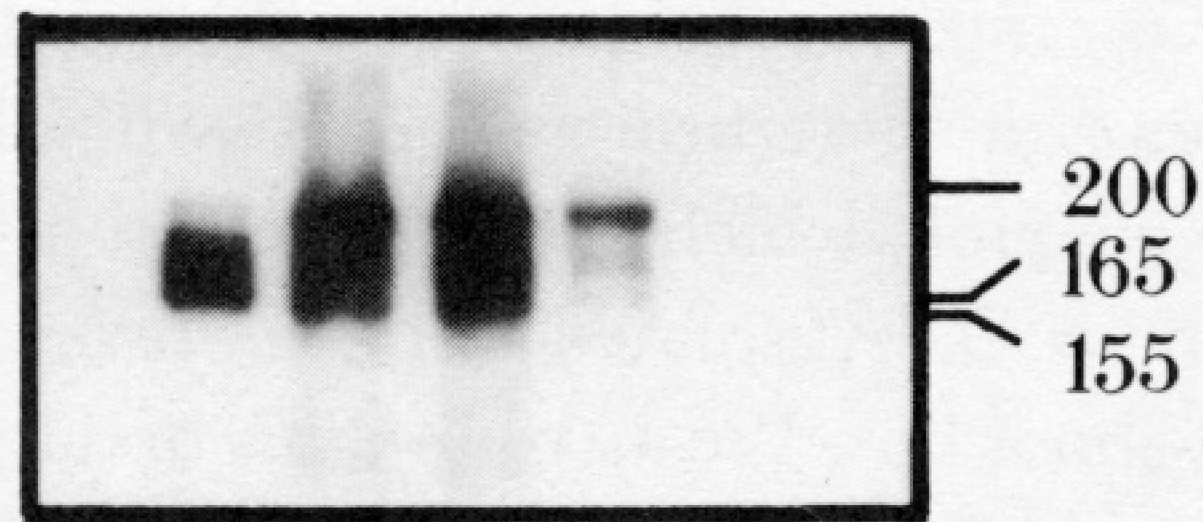

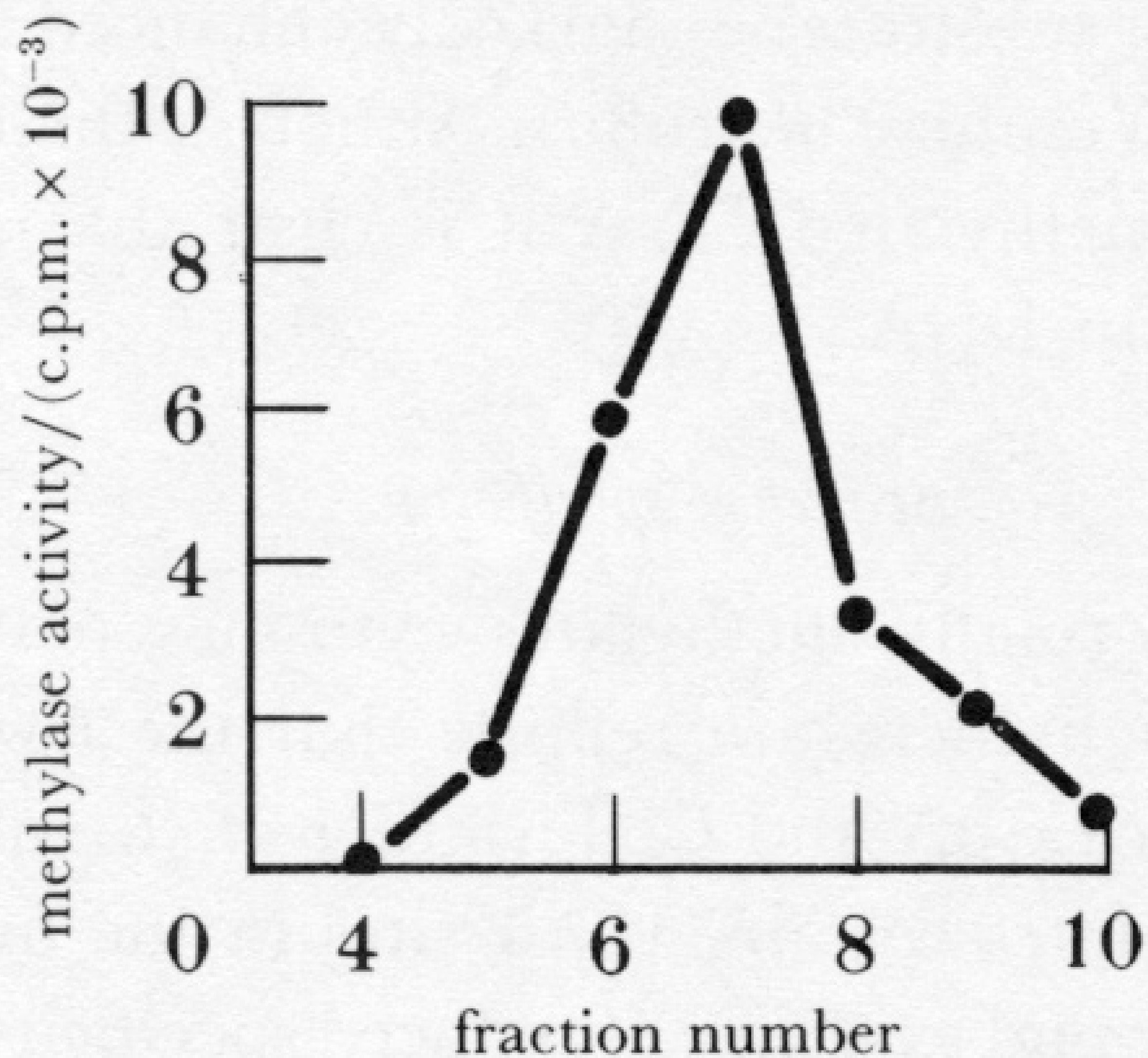

FIGURE 1. Fractionation of mouse DNA methylase on tRNA Sepharose. A sample of partially purified enzyme from mouse ascites cells was applied in low salt buffer to a 2 ml column of tRNA Sepharose and eluted with a linear gradient of 0 to 0.3 M NaCl. The graph shows the activity of each fraction and the autoradiogram above shows the results obtained when the fractions were separated by SDS polyacrylamide gel electrophoresis and immunoblotted against the antimethylase polyclonal antibody. Note the correspondence of the 190 kDa band with activity.

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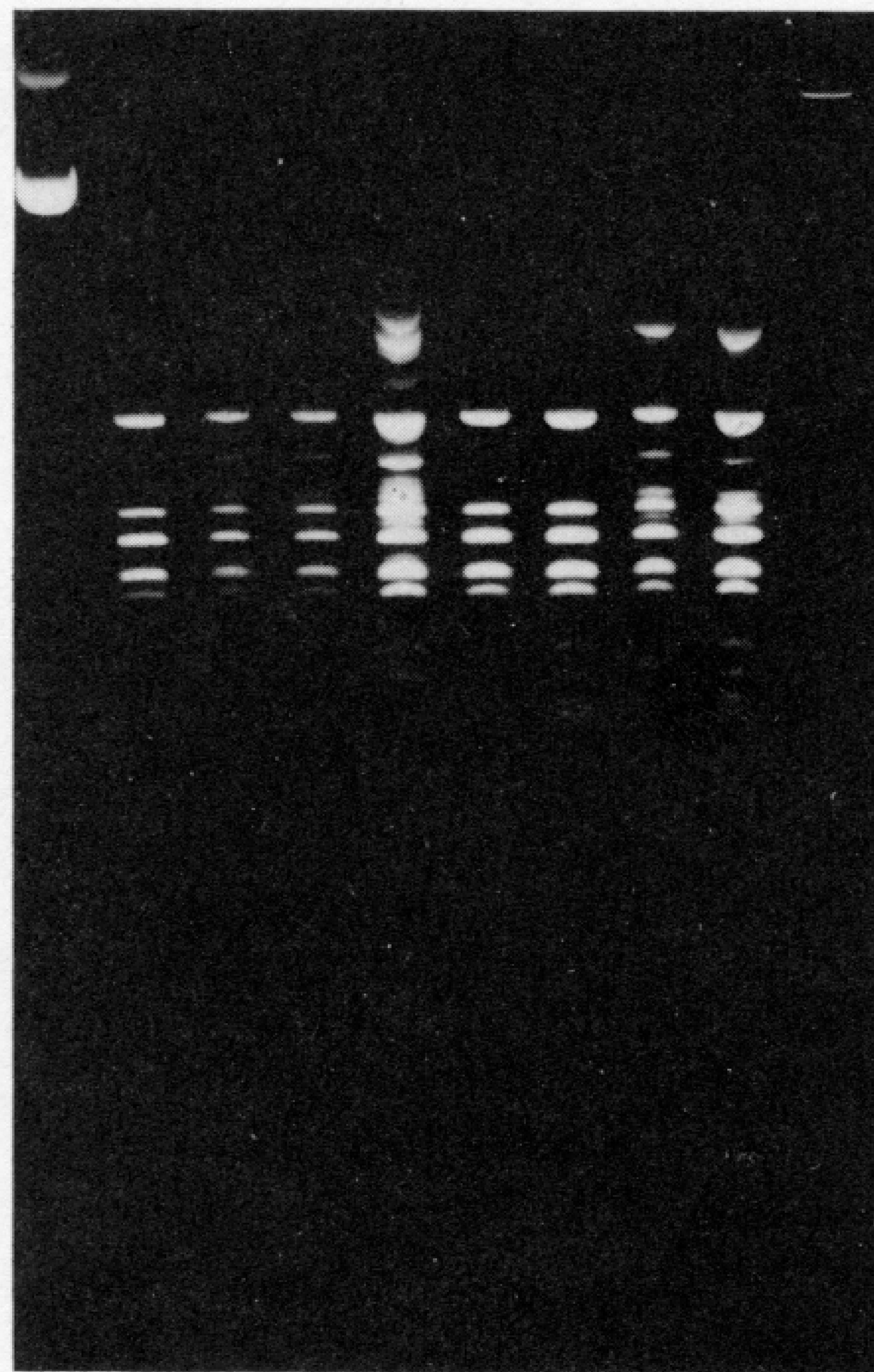

FIGURE 3. Restriction enzyme digestion of pVHC1 DNA methylated *in vitro* with mouse DNA methylase: pVHC1 DNA was incubated with mouse ascites DNA methylase for 0, 2, 4 or 6 hours and the reisolated DNA digested with either *Hpa* II or *Msp* I before separation by electrophoresis on an agarose gel. The figure shows the ethidium-stained gel. The left-hand lane contains undigested plasmid and the right-hand contains lambda DNA digested with *Hin* dIII as marker.

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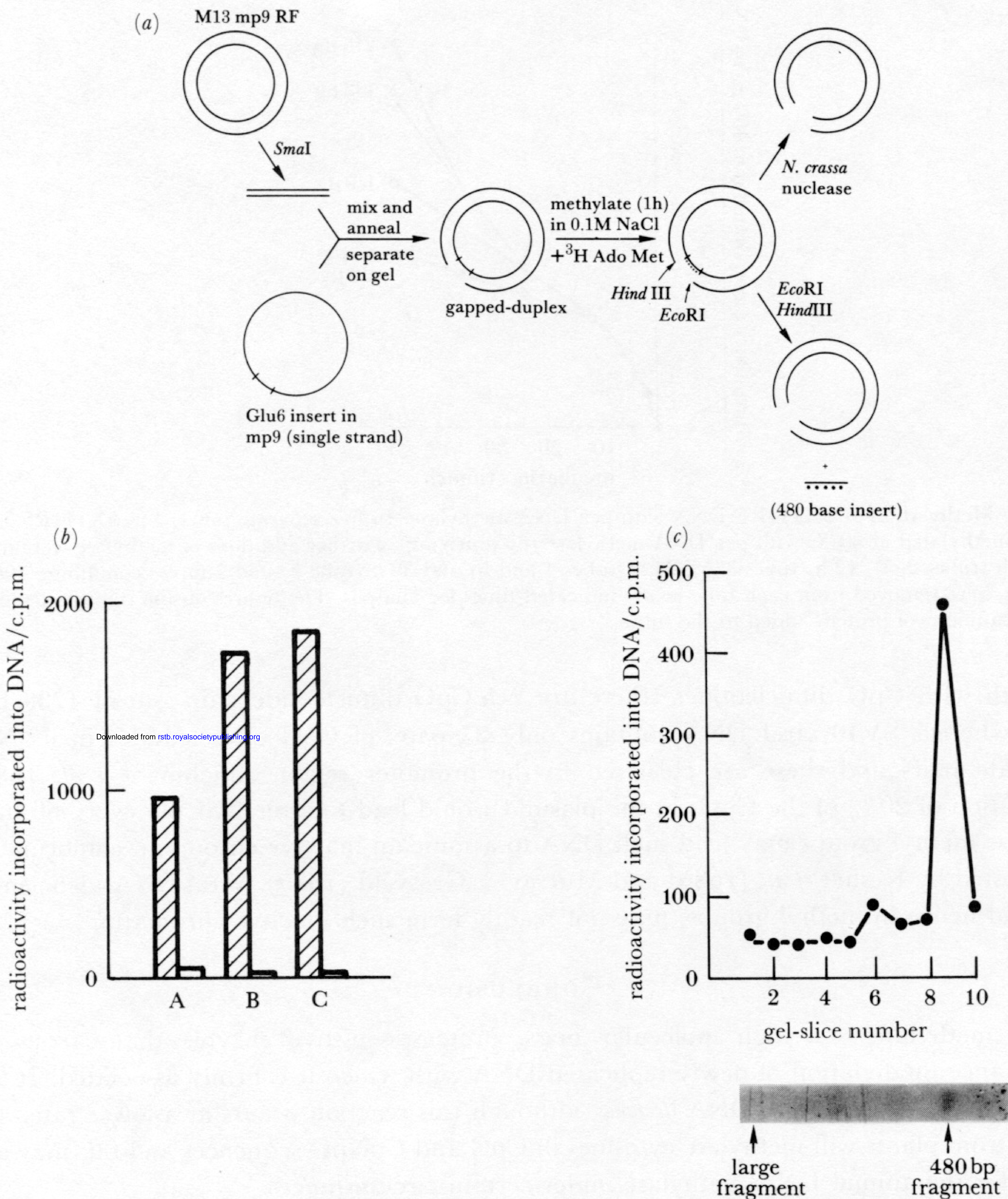

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FIGURE 6. Construction and methylation of a gapped duplex DNA molecule: (*a*), the construction of a gapped duplex molecule from a construct containing a 480 b.p. insert in the *Sma* 1 site of M13mp9 (Goddard *et al.* 1983) and single stranded mp9. Following methylation *in vitro* with ^{3}H AdoMet the location of the methyl groups was established in two ways; (*b*), in three different experiments (A, B and C the methylated gapped duplex was treated with *N. crassa* nuclease that selectively digests single-stranded DNA. The figure shows the radioactivity present in acid insoluble material before and after digestion; (*c*), following treatment of the gapped-duplex DNA with *Eco* R1 and *Hin* dIII the insert is released. The digest was separated by agarose gel electrophoresis and subjected to fluorography (lower panel) and then sliced and counted to establish the location of the tritiated methyl groups. The figure shows the methylation is restricted to the single-stranded region in the gapped duplex.