

DNA methylase from *Pisum sativum*

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DNA methylase activity was detected in nuclei from pea shoots. The enzyme can only be extracted by low-salt treatment if the nuclei are pretreated with micrococcal nuclease. Only a single enzyme was detected, and it was purified to a specific activity of 1620 units/mg of protein. It has an M_r of 160000 on gel filtration and SDS/PAGE. Pea DNA methylase methylates cytosine in all four dinucleotides, and this is interpreted to show that it acts on CNG trinucleotides. Although it shows a strong preference for hemi-methylated double-stranded DNA, it is also capable of methylation *de novo*. Homologous DNA is the best natural substrate. *In vitro* the enzyme interacts with DNA to form a salt-resistant complex with DNA that is stable for at least 4 h.

INTRODUCTION

DNA methylation provides a mechanism for a stable alteration of the local configuration of a gene and could therefore play an important role in the regulation of gene expression (Doerfler, 1983; Adams & Burdon, 1985; Adams, 1990). In plants, 5-methylcytosine can account for up to 30% of the cytosine residues in DNA (Sulimov *et al.*, 1970; Kemp & Sutton, 1976; Wagner & Capesius, 1981). In contrast, in vertebrate DNA the level is of the order of 2–6% (Adams & Burdon, 1985). This difference may be attributed partly to the specificity of methylation. In vertebrate DNA only those cytosine residues in the dinucleotide CG are methylated, and the amount of CpG is lower than expected (Adams *et al.*, 1987). Plant cell DNA shows additional methylation of cytosine residues contained in the trinucleotide CNG (Gruenbaum *et al.*, 1981; Navey-Many & Cedar, 1982; Nick *et al.*, 1986), though there is still a deficiency of CG dinucleotides (Boudraa & Perrin, 1987). In spite of the increased extent of methylation in higher-plant genomes, compared with animal genomes, the extent of modification at methylatable sequences is always less than 100%, and clusters of non-methylated CG groups have been associated with a number of expressing plant genes (Bird, 1980; Antequera & Bird, 1988) similar to the clusters of non-methylated CG islands in expressing vertebrate genes (Bird, 1986). It is possible therefore that the main functions of cytosine methylation in plants and vertebrates are similar.

Chloroplast DNA examined until now does not contain modified bases such as 5-methylcytosine (Kirk & Tilney-Bassett, 1978; van Grisven & Kool, 1988), except for the plastid DNA of the green alga *Chlamydomonas reinhardtii* (Royer & Sager, 1979; Bolen *et al.*, 1982; Feng & Chiang, 1984).

In higher plants and animals, 5-methylcytosine formation is accomplished by a post-replication transfer of a methyl group from *S*-adenosyl-L-methionine to the 5-position on the cytosine ring in DNA. The reaction is catalysed by the enzyme DNA methylase (EC 2.1.1.37), probably through an intermediate formation of an enzyme–DNA complex (Santi *et al.*, 1983). DNA methylase has been purified from a number of animal cells and is found associated with cell nuclei. The first report of DNA methylase activity in higher plants (pea seedlings) was made by Kalousek & Morris (1969). The assay for methylase was identical with that developed for mammalian DNA methylase. However, only low methylase activity was obtained, and the enzyme has not been studied since. More recently the purification of DNA

methylase from wheat embryo has been reported (Theiss *et al.*, 1987). This enzyme is reported to have a native M_r of 50000–55000, in contrast with the high- M_r enzyme from mammalian cells, which has an M_r value of 190000 for the intact enzyme (Pfeifer *et al.*, 1985; Adams *et al.*, 1986; Bestor *et al.*, 1988).

The present paper reports the isolation and characterization of DNA methylase from *Pisum sativum*, the garden pea. Only one enzyme activity was detected, and the M_r of the native enzyme is 160000. A preliminary report of this work has already appeared (Yesufu *et al.*, 1988).

EXPERIMENTAL

Materials

Pea seeds (*Pisum sativum* var. Feltham First) were purchased from Booker Seeds, Sleaford, Lincs., U.K. Radiochemicals were obtained from Amersham International. DNA from salmon testis, calf thymus and *Micrococcus luteus* were obtained from Sigma Chemical Co. Pea DNA was purified as described by Marmur (1961). DNAase I and spleen phosphodiesterase were obtained from Sigma Chemical Co., micrococcal nuclease was from Boehringer Corp. and *Escherichia coli* DNA polymerase I was from Anglian Laboratories.

Pea growth and nuclear preparation

Peas (200 g dry wt.) were swollen in water for 4–6 h and then seeded into a tray (21 cm × 33 cm) under compost. Except for the experiment reported in Table 1, they were grown at 26 °C under continuous white-light illumination and kept moist by watering.

The 1.5–2.0 cm apical shoot tips (or other portions where stated) were removed and stored overnight at –20 °C. They were then homogenized in buffer M [50 mM-Mops/NaOH buffer, pH 7.2, containing 1 mM-EDTA, 0.01% (w/v) NaN₃, 1 mM-dithiothreitol, 60 µg of phenylmethanesulphonyl fluoride/ml and 10% (v/v) glycerol] either by grinding in a mortar (100 µl of buffer/g of material) or with a few short bursts in a Philips blender (1.5 ml of buffer/g of material). It is important not to homogenize too vigorously, otherwise excessive nuclear damage occurs. The homogenate was filtered through six layers of muslin and centrifuged at 2000 g for 10 min. The pellet was washed once in buffer M and then resuspended in buffer M containing 1% (v/v) Triton X-100. This causes lysis of plastids while the suspension stands on ice for 15 min. The nuclei were pelleted and

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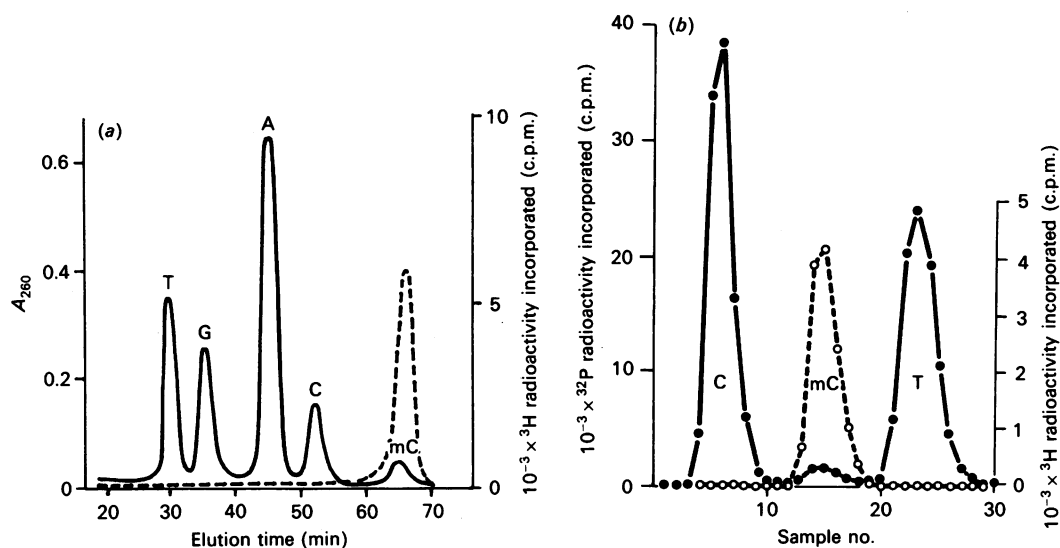


Fig. 1. Sequence-specificity of pea DNA methylase

(a) Pea DNA was methylated by using pea DNA methylase (fraction II) and then hydrolysed to the bases, which were separated on a column of Aminex A6 (Adams *et al.*, 1979). —, A_{260} profile of acid-hydrolysed pea DNA; ----, elution of ^3H radioactivity incorporated from the [^3H]methyl group of *S*-adenosyl-*L*-methionine. (b) Pea DNA that had been nick-translated in the presence of one [$\alpha\text{-}^{32}\text{P}$]dNTP (in this case dATP) was methylated by using pea DNA methylase (fraction III) and hydrolysed to the 3'-monophosphates as described in the Experimental section. These were separated on a Mino RPC h.p.l.c. column and the graph shows the profile after a 4 min delay. ●, ^{32}P radioactivity incorporated; ○, ^3H radioactivity incorporated.

washed in buffer M containing Triton X-100 until no longer green. Nuclei can be stored at -20°C in buffer M containing 50% (v/v) glycerol.

Preparation of soluble DNA methylase

Attempts to obtain a DNA-free preparation of DNA methylase by sonication failed. Brief pulses of sound caused disruption of nuclei and both enzyme and DNA were released into the low-speed supernatant on centrifugation.

A fraction (about 20%) of the DNA methylase activity can be obtained free of DNA by extracting the nuclei with buffer M containing 0.2 M-NaCl, but no further enzymic activity is released with the use of higher salt concentrations. This is reminiscent of the situation found in animal cells, except that there the proportion of readily solubilized activity is considerably greater (Turnbull & Adams, 1976). Much of the remaining activity can be extracted if the pea nuclei are pretreated with micrococcal nuclease. The following procedure was adopted. Isolated nuclei, suspended in buffer M, were stirred, on ice, for 15 min with an equal volume of buffer M containing 0.4 M-NaCl. After centrifugation at 10000 *g* at 4°C for 10 min, the supernatant was retained. The pellet was resuspended in the original volume of buffer M; CaCl_2 (1 mM) and micrococcal nuclease (400 units/ml) were added, and the suspension was incubated at 37°C for 10 min. EGTA (3 mM) was then added to stop the nuclease action and the nuclei were re-extracted. The two extracts were pooled.

DNA methylase assay

The enzyme was assayed as previously described for the mouse DNA methylase except that the reaction was carried out at pH 7.2 and at 30°C for 2 h (Turnbull & Adams, 1976; Yesufu *et al.*, 1988). One unit of activity is the amount that will catalyse the incorporation of 1 pmol (10^4 d.p.m.) of methyl group into DNA/h at 30°C .

The product of the reaction was confirmed as 5-methylcytosine

by pyrolysis of the methylated DNA and separation of the bases on a column of Aminex A6 (Adams *et al.*, 1979). A typical separation is shown in Fig. 1(a).

Purification

Pea nuclei were prepared from 200 g of 7-day-old pea seedling apical shoot tips, and extracted as described above. The 0.2 M-NaCl extract was applied on to a pre-equilibrated column (1.5 cm \times 5.0 cm) of heparin-Sepharose (Pharmacia) at a flow rate of 5 ml/h. Unbound proteins were washed through with buffer M containing 0.2 M-NaCl and bound protein was eluted with buffer M containing 0.6 M-NaCl at a flow rate of 10 ml/h. The protein peak was pooled and desalted in a Centriprep 30 concentrator (Amicon) centrifuged for four 20 min periods at 2000 *g* at 2°C . The desalted protein peak was injected (1.0 ml/min) into a pre-equilibrated Mono Q f.p.l.c. column (5 mm \times 5 cm) with pre-cooled buffers. The column was washed through with buffer M for 10 min before elution with a 0–1.0 M-NaCl gradient in buffer M (1.0 ml/min). Individual fractions were dialysed against buffer M containing 50% (v/v) glycerol, and assayed for DNA methylase activity. Only one peak of enzyme activity was obtained (Fig. 2).

Sequence-specificity of pea DNA methylase

The sequence-specificity of methyl group incorporation *in vitro* catalysed by the pea enzyme was determined by an adaptation of the method of Rigby *et al.* (1977), as previously described (Adams *et al.*, 1986). A 10 μg portion of pea DNA was nick-translated in a reaction mixture (100 μl) containing 5 mM-Tris/HCl buffer, pH 7.2, 50 μg of BSA, 10 mM-MgCl₂, 1 mM-dithiothreitol, 10 units of *E. coli* DNA polymerase I, 0.1 unit of DNAase I and 4 μM each of all four deoxyribonucleotide triphosphates, one of which was labelled, i.e. with [$\alpha\text{-}^{32}\text{P}$]dNTP (specific radioactivity 3000 Ci/mmol). The reaction mixture was incubated for 60 min at 15°C , and the reaction was stopped with 2 μl of 0.5 M-EDTA. The labelled DNA was re-isolated by ethanol precipitation and methylated with the use of tritiated

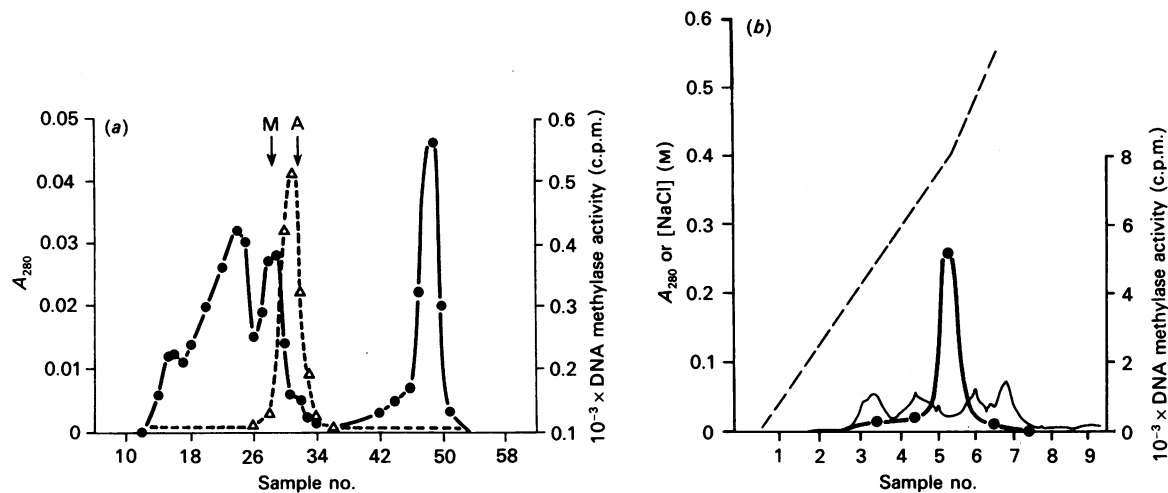


Fig. 2. Detection of only one pea DNA methylase

(a) Gel filtration of the nuclear extract on a calibrated f.p.l.c. column of Superose 6 (Pharmacia). —, A_{280} ; ----, DNA methylase activity. The positions of elution of two of the markers [mouse ascites DNA methylase (M; M_r 190000) and alcohol dehydrogenase (A; M_r 150000)] are indicated. (b) Ion-exchange chromatography on a Mono Q (Pharmacia) f.p.l.c. column. —, A_{280} ; ●, DNA methylase activity; ----, NaCl gradient.

S-adenosyl-L-methionine as described. The double-labelled DNA product was re-isolated and digested with micrococcal nuclease (10 units/ μ g) and spleen phosphodiesterase (2 units/ μ g) in buffer A (10 mM-Tris/HCl buffer, pH 7.0, containing 1.0 mM- CaCl_2 and 2 mM- MgCl_2) for two 2 h periods. The deoxynucleotide 3'-monophosphates were separated on a Mino RPC h.p.l.c. column (5 mm \times 20 cm) in buffer B (25 mM-tetrabutylammonium phosphate buffer, pH 6.0, containing 80 mM-NaCl). The ^{32}P and ^3H radioactivities were monitored by liquid-scintillation counting. A separation profile is shown in Fig. 1(b) for the early part of the elution profile from an experiment with [α - ^{32}P]dATP-labelled DNA. Labelling with each of the four [^{32}P]dNTPs allows the estimation of methylation at all possible dinucleotide sequences by comparing the ^{32}P in the dCMP and methyl-dCMP peaks. It is noted that, after labelling with [α - ^{32}P]dCTP, anomalously high radioactivities are always found in the methyl-dCMP peak, and this is attributed to a small amount of hydrolysis of the DNA to 5'-deoxynucleotides. For this reason the values for methylation of CC dinucleotides are unreliable.

RESULTS

Distribution of DNA methylase activity

As shown in Table 1, nuclei isolated from pea shoot tips contain all the recoverable DNA methylase activity. Essentially

no activity is found in plastids from plants grown either in continuous light (chloroplasts) or in continuous dark (etioplasts).

The nuclei with the highest specific activity are obtained from 5-day-old shoot tips (Table 2). However, at this stage only about half the seedlings have emerged sufficiently through the compost to be harvested, and most of the subsequent work has been done with 7-day-old pea shoots, as they provide the greatest yield.

At all ages the most active nuclei are those obtained from the apical shoot tips, i.e. the top 1.5–2.0 cm. A lower level of activity

Table 2. Variation in pea nuclear DNA methylase activity with location and age

DNA methylase activity was assayed in nuclei isolated from the indicated part of pea seedlings, harvested at the indicated age from the time of swelling.

| Age (days) | DNA methylase activity (units/mg of protein) | | | |
|------------|--|----------------------|------|----------|
| | Shoot (apex) | Shoot (sub-terminal) | Leaf | Root tip |
| 5 | 93 | — | — | 23 |
| 7 | 64 | 25 | — | 4 |
| 9 | 24 | 10 | 10 | 2 |
| 11 | 15 | 13 | 5 | 1 |

Table 1. Location of pea DNA methylase activity

Plants were grown, either in continuous light or in continuous dark, for 7 days before being harvested. The nuclei were prepared from one tray of plants as described in the Experimental section. The plastids were the material solubilized in the wash with Triton X-100.

| Source of enzyme | Light | | Dark | |
|------------------|------------------------|------------------------------|------------------------|------------------------------|
| | Total activity (units) | Specific activity (units/mg) | Total activity (units) | Specific activity (units/mg) |
| Homogenate | 112.8 | 0.09 | 30.0 | 0.04 |
| Nuclei | 76.4 | 28.6 | 28.8 | 12.7 |
| Plastids | 0.1 | 0.04 | 0.2 | 0.06 |

Table 3. Purification of pea DNA methylase

| Step | Fraction | Total activity (units) | Protein (mg) | Specific activity (units/mg) | Purification (fold) |
|---------------------------------------|----------|------------------------|--------------|------------------------------|---------------------|
| Pea nuclei | I | 337 | 10.20 | 33 | 1 |
| 0.2 M-NaCl extract | II | 330 | 4.00 | 75 | 2.3 |
| Heparin-Sephacel (0.6 M-NaCl peak) | III | 279 | 0.60 | 465 | 14 |
| Mono Q (0.35 M-NaCl peak) | IV | 16 | 0.01 | 1620 | 49 |

is found in root tips (1.5–2.0 cm), in the first expanded leaves and in sub-terminal shoot portions (regions 2.0–4.0 cm from the growing tip) of the same age (Table 2). Those regions of stem further than 4 cm from the shoot tip show little or no activity.

Purification

The purification procedure starting with whole nuclei is summarized in Table 3 and detailed in the Experimental section. Only one peak of enzyme activity was obtained by gel filtration on Superose 6 (Pharmacia) of the initial nuclear extract (Fig. 2a), corresponding to a protein of M_r 160000. This procedure is not included in the scheme in Table 3, as the incorporation of more than two chromatographic steps in the purification leads to loss of enzyme activity and was found to be unnecessary. The enzyme was also eluted as a single peak from DEAE-Sephacel and Mono Q (Pharmacia), at 0.2 M-NaCl and 0.35 M-NaCl respectively (Fig. 2b), giving a protein fraction of a specific activity of 1620, and a 49-fold purification over the nuclear fraction. This represents an 18000-fold purification with respect to the original homogenate. When this peak fraction was subjected to SDS/7%-PAGE, one major protein band, of M_r about 160000, is seen on silver staining (Fig. 3). However, the enzyme is unstable and,

when subjected to a second gel electrophoresis (within 48 h), the high- M_r protein was replaced by bands of lower M_r .

Optimal assay conditions

DNA methylase was assayed in isolated nuclei with or without added DNA, or in soluble extracts in the presence of exogenous DNA. Greater activity is found at 30 °C than at 25 °C or 37 °C, and the optimum pH is 7.2 for both the nuclear and the solubilized enzyme (Fig. 4b). A DNA concentration of 170 $\mu\text{g}/\text{ml}$ is sufficient to half-saturate the soluble enzyme (Fig. 4c and derivative curves, not shown). At low protein concentrations activity is not directly proportional to the amount of enzyme in the assay, and for efficient methylation a protein concentration of about 1 mg/ml is required (Fig. 4d). The reaction is approximately linear for at least 4 h (Fig. 4a), but with a high enzyme

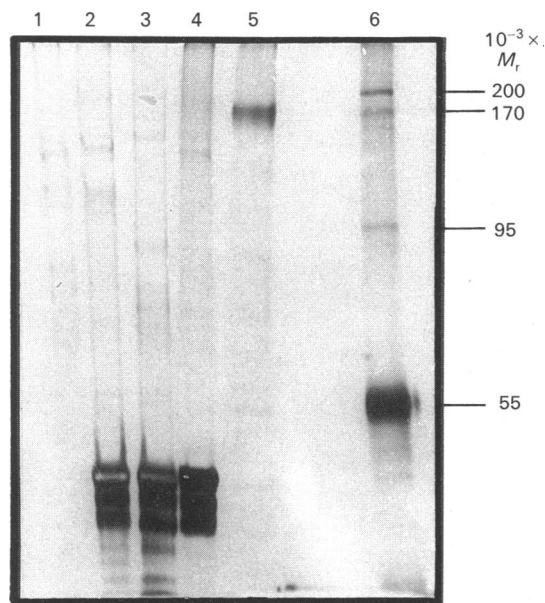


Fig. 3. SDS/PAGE of pea DNA methylase

The Figure shows the silver-stained SDS/7%-PAGE gel on which samples from various stages in the purification were separated. Lane 1, first 0.2 M-NaCl extract (no nuclease treatment); lane 2, 0.2 M-NaCl extract after micrococcal-nuclease digestion; lane 3, pooled 0.2 M-NaCl extract; lane 4, 0.6 M-NaCl fraction from heparin-Sephacel; lane 5, 0.2 M-NaCl peak fraction from DEAE-Sephacel; lane 6, M_r markers.

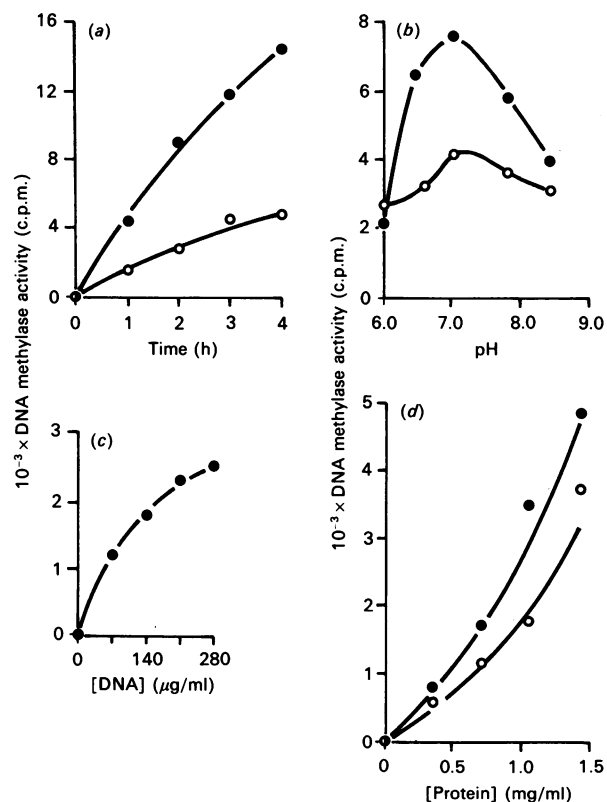


Fig. 4. Optimum reaction conditions for pea DNA methylase

(a) Time-dependence of the nuclear pea DNA methylase activity (●, control) and the effect of 200 mM-NaCl (○). (b) pH of optimum activity for the nuclear (●) and solubilized (○) pea DNA methylase. (c) Dependence of soluble pea DNA methylase activity (●) on DNA substrate concentration. (d) Effect of protein concentration on solubilized pea DNA methylase activity with native (●) or denatured (○) pea DNA as substrate.

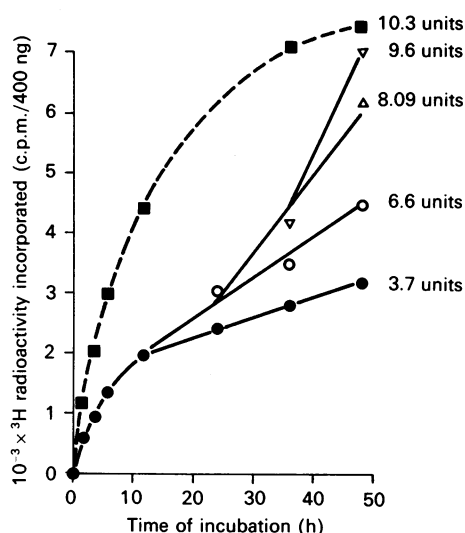


Fig. 5. Time course for methylation *de novo* of ϕ X174 RF DNA

The graph shows two incubations initiated with 3.7 units or 10.3 units of pea DNA methylase (fraction III) in a volume of 100 μ l or 200 μ l respectively, each containing 4 μ g of ϕ X174 RF DNA. To the former, additional enzyme (20 μ l; 1.5 units) and *S*-adenosyl-L-[3 H] methionine (5 μ l) were added at 6, 12, 24 and 36 h. Portions containing 400 ng of DNA were removed at the indicated times and the extent of methylation was analysed.

concentration, even with limiting DNA, methylation continues for much longer (Fig. 5). As with the mouse enzyme (Turnbull & Adams, 1976), addition of more enzyme to an incubation whose rate is slowing leads to further incorporation, but the initial rate of reaction depends on the initial enzyme concentration and the rate can remain linear for up to 12 h (Fig. 5).

DNA substrate requirements

The best substrate discovered so far for the pea enzyme is native DNA isolated from 5-day-old pea shoot tips. DNA from salmon or bovine sources is intermediate as a methyl-group acceptor, and DNA from *M. luteus* is a poor acceptor (Figs. 4d and 6a). This is in contrast with the situation with the vertebrate DNA methylases, where homologous DNA is usually a very poor substrate and DNA from *M. luteus* is commonly used (Adams *et al.*, 1986). Hemi-methylated DNA from *M. luteus* or calf thymus (nick-translated in the presence of methyl-dCTP) is a much better substrate than the same DNA nick-translated with dCTP (Fig. 6b). As less than 1% of the DNA was nick-translated in this reaction, the preference for the hemi-methylated regions must be of the order of 300 times that for the unmethylated DNA. Both these findings support the proposal that the presence of some methyl groups on the DNA substrate is important for activity, but it is too soon to conclude that the enzyme will preferentially methylate a hemi-methylated di- or tri-nucleotide.

Effect of NaCl

The soluble enzyme is very strongly inhibited by salt: 100 mM-NaCl causes 85% inhibition of DNA methylase activity (Figs. 4a and 7). In contrast, the nuclear enzyme is only 45% inhibited by 100 mM-NaCl. This is also true for the enzyme that remains bound in nuclei after the first extraction with 0.2 M-NaCl (see the Experimental section; results not shown). This observation may result from the nuclear enzyme being associated with DNA before the addition of salt. To test this hypothesis, 200 mM-NaCl was added to the soluble enzyme after 10 min incubation with DNA. In this situation only 72% inhibition was obtained

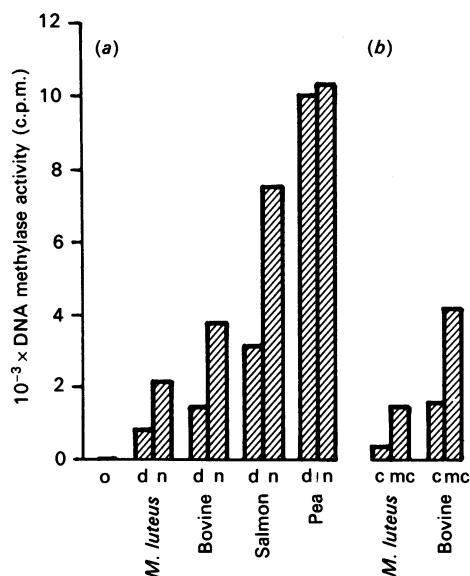


Fig. 6. Substrate-specificity of pea DNA methylase

(a) Fraction II (1 unit) was incubated with the indicated DNAs at 70 μ g/ml. Key: d, heat-denatured single-stranded DNA; n, native double-stranded DNA. (b) Hemi-methylated DNA (see the Experimental section) was compared with control unmethylated DNA as substrate for pea DNA methylase (fraction II). The DNA concentration was 42 μ g/ml. Key: mc, hemi-methylated DNA; c, control DNA.

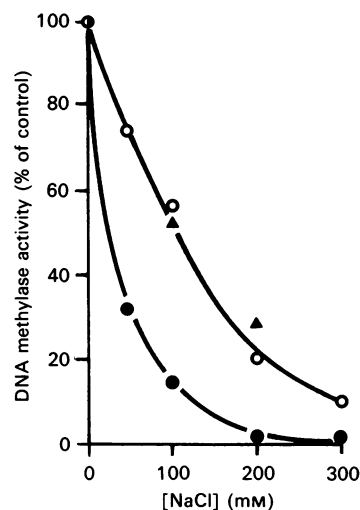


Fig. 7. Effect of NaCl on nuclear and solubilized pea DNA methylase

Nuclei (66 μ g of protein) (\circ) or solubilized enzyme (25 μ g of fraction II) (\bullet and \blacktriangle) were incubated with the indicated concentration of NaCl. The NaCl was added at zero time (\circ and \bullet) or after 10 min of incubation (\blacktriangle).

compared with the 98% inhibition obtained when the NaCl was added at zero time (Fig. 7). We interpret this to mean that the binding of the enzyme to DNA is prevented by NaCl concentrations in excess of 100 mM.

This, however, can be only a partial explanation. Fig. 4(a) shows the effect of 200 mM-NaCl on the time course of nuclear methylase activity. In the presence of salt the rate is lower by a constant fraction throughout the 4 h incubation, implying either (a) that the methyl transfer reaction itself or enzyme translocation is inhibited by salt, or (b) that only about 34% of the nuclear enzyme is initially bound to DNA. In either event the result

Table 4. Sequence-specificity of pea DNA methylase

Pea DNA and calf thymus DNA were nick-translated with one [α - 32 P]dNTP and, after methylation, were hydrolysed to the 3'-monophosphates, which were separated on a Mino RPC h.p.l.c. column (Fig. 1a). The experiment was performed twice, in duplicate, for pea DNA and once, in duplicate, for calf DNA.

| [α - 32 P]dNTP | Methylation of DNA | | | |
|-----------------------------|--|----------|---------------------------------------|----------|
| | [100 \times (c.p.m. in mC)/ (c.p.m. in mC+C)] | | (% of expected if CNG methylated)* | |
| | Pea DNA | Calf DNA | Pea DNA | Calf DNA |
| dATP | 3.5 | 2.2 | 17.5 | 11 |
| dCTP | 1.3 | 1.9 | 13 † | 38 † |
| dGTP | 5.7 | 5.2 | 28.5 | 26 |
| dTTP | 3.8 | 3.4 | 19 | 17 |

* This assumes a 40% G+C base composition and a 50% deficiency of CG for pea DNA and a 75% deficiency for calf DNA.

† The values for CC methylation are believed to be at least partly an artifact (see the Experimental section).

shows that all enzyme bound to the endogenous DNA at the beginning of the incubation remains bound throughout the 4 h incubation period. In light of the extraction data (see the Experimental section) we consider that at least 80% of the enzyme is bound within nuclei, and we therefore favour the first proposal above.

Sequence-specificity of the enzyme

Our results show incorporation of [3 H]methyl group into cytosine only (Fig. 1). Nearest-neighbour transfer occurs for CG, CA, CT and CC dinucleotides, although incorporation into all four dinucleotides is not equal (Table 4).

DISCUSSION

The tissue showing the greatest activity of DNA methylase is the rapidly growing shoot tip. This is as expected for an activity whose presumed substrate (hemi-methylated DNA) is formed largely in the S-phase of the cell cycle. The enzyme appears to be located entirely within the nuclear fraction, there being no evidence of enzymic activity associated with chloroplasts or plastids from etiolated seedlings. The absence of enzymic activity from the chloroplasts is consistent with the finding that these organelles contain no methylcytosine. There has been some controversy over whether or not plastid DNA is methylated in dark-grown plants (Ngernprasirtsiri *et al.*, 1988; van Grisen & Kool, 1988), but our failure to detect enzyme in plastids under such conditions does not support the original observation.

The pea shoot DNA methylase is firmly bound within the cell nucleus, and only a small fraction can be extracted with low-salt buffers. Only after brief digestion with nuclease could the bulk of the enzyme be extracted. In vertebrates most of the DNA methylase is readily solubilized, with only a small fraction remaining tightly bound. This difference may reflect the rate of growth of the cells used, or the higher proportion of cytosines methylated in plant DNA. Thus, to methylate up to 30% of the cytosine residues in the DNA may require a greater fraction of the enzyme to be associated with the replicating DNA than is the case when only 3% of cytosine residues are to be methylated.

Another difference from the vertebrate enzyme is the finding that native homologous DNA is the best natural substrate found for the pea enzyme, and this is also the case for the enzyme from wheat embryo (Theiss *et al.*, 1987). Native mouse DNA is a poor substrate for the mouse DNA methylase, but its effectiveness is improved when the DNA is isolated from rapidly dividing cells,

and particularly from S-phase cells growing in the absence of methionine (Turnbull & Adams, 1976; Adams, 1990). As pea DNA is not a particularly good substrate for the mouse enzyme, we presume that it is rich in hemi-methylated sites that can be used only by the pea enzyme, i.e. CAG and CTG.

Although the initial association of the enzyme with DNA is sensitive to inhibition by low concentrations of NaCl, a salt-resistant complex quickly forms that may be analogous to the tightly bound nuclear enzyme. The complex, once formed, is long-lived and does not dissociate during a 4 h incubation. From this we conclude that NaCl, as well as interfering with the initial binding of the enzyme to DNA, also has an effect on the methylation reaction, either by slowing the rate of movement of the enzyme along the DNA or by interfering with the actual transfer of the methyl group.

The specific activity of the nuclear extract is higher than the comparable value for the vertebrate enzyme (Adams *et al.*, 1986), yet we are only able to obtain a modest purification relative to nuclear extract and the final specific activity obtained is low. The purification relative to whole cells is, however, 18000-fold and the final preparation shows the one major band of M_r 160000 on a silver-stained SDS/PAGE gel. This corresponds to the native M_r of the enzyme as estimated by gel filtration. The enzyme is quite unstable and breaks down on storage to lower- M_r polypeptides that still retain activity. It is these products that may have given the impression that the enzyme from wheat germ is of M_r 40000 (Theiss *et al.*, 1987).

Although it has been widely reported that plant DNA is methylated at CG dinucleotides and CNG trinucleotides (Gruenbaum *et al.*, 1981), we have only been able to find evidence of one enzymic activity. The results presented in Table 4 show that the enzyme is able to methylate all four dinucleotides, though methylation of CCG may be an artifact, i.e. an extremely small amount of hydrolysis of [α - 32 P]dCTP-labelled DNA to 5'-nucleotides rather than 3'-nucleotides can be confused with transfer of a phosphate to the 5'-nucleotide in a CC dinucleotide. (This seems a particularly likely explanation with the calf DNA substrate, where it appears that 38% of the 5'-cytosine residues in CCG are methylated.) Assuming that the third base is guanine, 11–19% of cytosine residues in CAG and CTG have been methylated in this experiment. This may also be true for CGG methylation. However, the extent to which methylation of the CG dinucleotide in other contexts occurs is not known; in this experiment it may vary up to a maximum value of 5.7%, which is clearly less than the methylation of CAG and CTG. On the

other hand, the enzyme may be adept at methylating CGG (26–29%) and may only methylate CGs in this trinucleotide.

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