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DNA methylase: purification from ascites cells and the effect of various DNA substrates on its activity.

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ABSTRACT

DNA methylase has been purified 405-fold from Krebs II ascites cells. The purified enzyme is homogeneous on SDS-poly acrylamide gel electrophoresis (molecular weight about 80,000) and the only product of the reaction with DNA is 5-methyl cytosine. Both native and denatured DNA are methylated by the enzyme; with calf thymus DNA the double stranded form is the better substrate but the enzyme preferentially methylates single stranded E.coli DNA even in "native" preparations. Our results do not support a mechanism whereby the enzyme methylates DNA by binding irreversibly and "walking" along it. By measuring maximum levels of methylation of DNAs from different sources we have estimated the proportion of unmethylated sites present in them. Homologous ascites DNA can be methylated, but only to about 5% of the level of the best substrate, undermethylated mouse L929 cell DNA. DNA isolated from growing cells or tissues is a better substrate than DNA from normal liver or pancreas, or from stationary cells.

INTRODUCTION

While the methylation of DNA in prokaryotes has been assigned a role in the restriction/modification system,[1] the function of the methylated bases (almost exclusively 5-methyl-cytosine) in eukaryotic DNA is still obscure. The fact that DNA from different tissues of the same animal has differing 5-methylcytosine content [2-4] could conceivably point to a function in gene expression or differentiation. An alternative explanation is that, as DNA is not completely methylated for several hours after synthesis,[5,7] a varying content of 5-methylcytosine may reflect a variation between tissues in the proportion of DNA that is newly synthesised and, as yet, under-methylated.

That isolated nuclei are able to methylate their endogenous DNA [7-9] is evidence for the presence of under-methylated DNA. Moreover nuclei isolated from cells which are making DNA in vivo

show greater incorporation of methyl groups into DNA than nuclei from resting cells[7-9]. Such studies, however, are complicated by variable amounts of DNA methylase and S-adenosyl-L-methionine cleaving activities[9] which leave in doubt to what extent nuclear incorporation depends on enzyme activity or on substrate sites present in the endogenous DNA. To overcome these difficulties and facilitate study of the enzyme, a more highly purified preparation is necessary. Partial purifications of DNA methylase have previously been reported from spleen[10] and rat liver[11], and the mechanism of the latter enzyme has been investigated in some detail[12-14]. While this manuscript was in preparation, reports have appeared of extensive purification of DNA methylase from HeLa cells[15] and Novikoff hepatoma cells[16].

In this paper we report a 405-fold purification of DNA methylase from Krebs II ascites tumour cells. The kinetics and mechanism of action of the enzyme have been studied and compared with the results obtained by Morris and his associates[12-14]. Preliminary reports of this work have already been presented elsewhere[9,17].

MATERIALS AND METHODS

Chromatographic Media

Ultrogel AcA34 was obtained from LKB-Produkter, Sweden. DEAE-cellulose was Whatman DE52, and phosphocellulose Whatman P11, both bought from H.Reeve Angel Ltd., London.

DNA Methylase Assay

The standard assay mixture (140 µl) contained 40 µg DNA (*E.coli* unless otherwise stated), 3.3 µCi S-adenosyl-L-[methyl ^{3}H] methionine (1 µCi/n mole; the Radiochemical Centre, Amersham, England) and 100 µl of buffered enzyme solution. Final concentrations of ingredients were: dithiothreitol and EDTA, each 715 µM, glycerol 7.2%, tris HCl (pH 7.8) 36 mM and S-adenosyl methionine 23.6 µM. After incubation at 37°C for 1 hour the reaction was stopped by adding 2 ml of a solution containing sodium dodecyl sulphate (1%), EDTA (2mM), 4-aminosalicyclic acid (3%) n-butanol (5%), NaCl (0.5 M) and salmon testis DNA (0.5 mg/ml) to act as a carrier. Protein was removed by extraction with phenol (88%)/ m-cresol (12%)/8 hydroxyquinoline (0.1%). After centrifugation at 15°C, the upper (aqueous) layer was removed, and DNA spooled out after over-layering with 2 volumes of absolute ethanol. The DNA was then digested in 0.2 ml of 0.5N NaOH (3 h, 37°) to remove RNA, after which

0.1 ml aliquots were precipitated onto filter discs (Whatman 3MM, 2.5 cm) using 5% trichloroacetic acid, in which the filters were washed 4 times at 0°C. The DNA was dried with ethanol and ether prior to solubilising in hyamine hydroxide, and radioactivity was estimated in a toluene scintillator (0.5% diphenyloxazole in toluene).

As an alternative to solubilisation in hyamine hydroxide the DNA was extracted from the filter with perchloric acid (0.5 N; 30 min at 70°C). After hydrolysis in 12N perchloric acid (100°C, 60 min), the liberated bases were chromatographed in n-butanol: HCl: H_2O (65 : 16.7 : 18.3) on Whatman No.1 paper for 66 h. Some 88% of the radioactivity co-chromatographed with 5-methylcytosine marker (Fig.1).

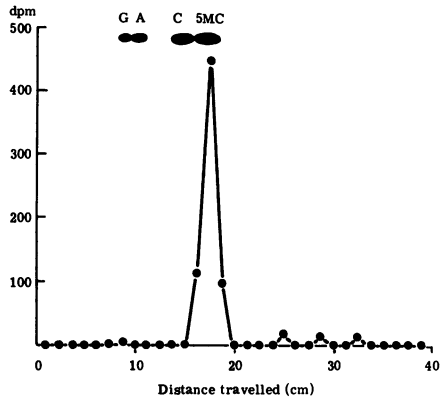

Figure 1 Identification of the product of methylation. Methylated DNA was extracted, hydrolysed to bases and chromatographed as described in Materials and Methods.

DNA Preparation

DNA was prepared from most tissues by the Marmur procedure[1,8] or as described under "DNA Methylase Assay" except that the alkali digestion was replaced by an overnight incubation with pronase and ribonuclease followed by a second phenol extraction.

Undermethylated DNA was prepared by synchronising mouse L929 cells at the beginning of S phase using aminopterin[19]. This block was reversed by addition of thymidine (5×10^{-6}M) in the presence of [^{14}C] deoxycytidine but in the absence of methionine.

DNA synthesis continues normally under these conditions for several hours, but the proportion of 5-methyl cytosine is reduced to 2.3% of the total cytosine as compared with 2.9% in the newly synthesised strand of control DNA isolated from late S-phase cells. (This was shown by hydrolysis of the DNA with perchloric acid and separation of the bases as described above).

On reintroduction of methionine into the culture medium methyl groups were added to the undermethylated DNA over the next 20h. We have evidence to show that over this time and the succeeding 48 h the undermethylated DNA is perfectly stable (no loss of labelled DNA occurs relative to controls) and is not subject to repair (no incorporation into "light" DNA of tritiated 5-bromodeoxyuridine occurs).

E.coli, salmon testis and calf thymus DNA were purchased from Sigma London Chemical Co. SV40 DNA was generously donated by Dr. R. Eason, T4 DNA by Dr. A.D.B. Malcolm, and pseudorabies virus DNA by Dr. W.S. Stevely.

Purification of DNA Methylase from Ascites Cells

Ascites tumour cells were removed from 6 mice 10-14 days after inoculation. Nuclei were prepared by homogenising the cells in 1% Tween 80^{20}. After washing in 0.25M sucrose buffered with 20 mM tris HCl (pH 7.8), the nuclei were suspended in 5-10 volumes of buffer containing dithiothreitol (1 mM), EDTA (1 mM), glycerol (10% v/v) and 50 mM tris HCl, pH 7.8 (buffer M), and an equal volume of buffer M containing 0.8 M NaCl was added slowly with stirring (We are grateful to N.R. Morris for suggesting this extraction method). After centrifugation at 12,000 g for 30 min, the supernatant fluid (Fraction II) was made to 30% saturation with respect to ammonium sulphate. The precipitate was discarded, and the supernatant fluid made to 60% saturation with ammonium sulphate. This second precipitate was redissolved in a minimum volume of Buffer M containing 0.4 M NaCl (Fraction III) and applied to a column of Ultrogel AcA34 (2.5 x 55 cm). This was eluted with buffer M containing 0.4 M NaCl (Figure 2). The pooled peak fractions (34-46, Fraction IV) were diluted with an equal volume of buffer M and adsorbed onto a 10 ml column of phosphocellulose, pre-equilibrated with buffer M containing 0.2 M NaCl and pre-saturated with bovine serum albumin. The column was washed with

the same buffer till no 280 nm-absorbing material was detectable in the eluate, whereupon the enzyme was eluted with buffer M containing 0.5 M NaCl.

The pooled peak of 280 nm-absorbing material from this latter procedure (Fraction V) was dialysed against two changes of buffer M (100 vols) before mixing with an equal volume of a slurry of DEAE-cellulose in buffer M. About 20-30% of the protein present was adsorbed to the cellulose, but DNA methylase activity remained unadsorbed and was recovered by low-speed centrifugation (Fraction V) The pertinent data on the purification are presented in Table 1, which demonstrates that a 405-fold purification was achieved from the isolated nuclei without overall loss of activity.

SDS-Polyacrylamide Gel Electrophoresis

This was carried out according to the method of Fairbanks et al[21].

RESULTS

Purification of DNA Methylase from Ascites Nuclei

The purification procedure is described in detail in Materials and Methods, and the purification is summarised in Table 1.

TABLE 1: Purification of Ascites DNA Methylase

Fraction	Total Protein (mg)	Total Enzyme (units)	Specific Activity (units/mg)	Purification (fold)
I Nuclear suspension	450	716	1.59	-
II Salt extract	360	1670	4.64	2.9
IV Ultrogel peak	55.4	1530	27.7	17.4
V Phosphocellulose 0.5 M eluate	3.4	575	169	106
VI DEAE cellulose unbound	3.1	1990	643	405

For details of purification, see text.
1 unit of enzyme activity is defined as that amount which catalyses incorporation of 1 p mole of methyl groups into E.coli DNA in 1 hour under the standard assay conditions. Protein was assayed by the method of Lowry et al[22].

Extraction of nuclei with 0.4 M NaCl leaves 90% of the DNA methylase activity in the supernatant fluid (Fraction II). Methylase activity is precipitated out by a 30–60% ammonium sulphate cut, and gel filtration of this fraction (Fraction III) (not normally assayed) on Ultrogel AcA34 gives the profile shown in Figure 2.

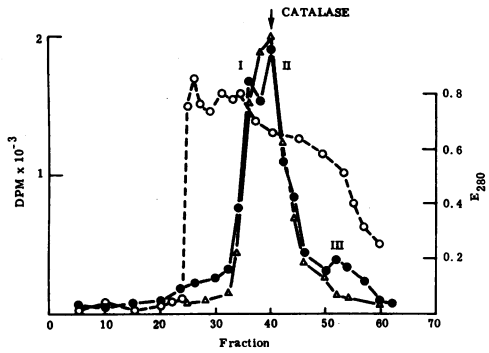

Figure 2 Gel filtration of DNA methylase on Ultrogel AcA34. (—●—●—), (—△-△—), ^{3}H dpm incorporated/h for 2 separate preparations; (--○--○--), A_{280}.

The Ultrogel column is eluted with buffer M containing 0.4M NaCl because at low salt concentrations the methylase aggregates and activity emerges in the void volume, together with most of the protein in the preparation. The peak tubes from this column are pooled (Fraction IV) and applied to a phosphocellulose column pre-saturated with bovine serum albumin. Enzyme activity is eluted at 0.5M NaCl (Fraction V). The final treatment with DEAE-cellulose at low salt removes some protein from the preparation but leaves methylase unbound (Fraction VI). An overall purification of 405-fold is achieved. Fraction VI still shows several bands on SDS-polyacrylamide gel electrophoresis, the most prominent one being at about 80,000 molecular weight (Figure 3). However the peak of enzymic activity (main peak or peak II, Figure 2) elutes from the Ultrogel column in the same fraction as catalase, suggesting an apparent molecular weight for the native enzyme of about 240,000. This may represent a dimer (or possibly a trimer) of the 80,000 dalton species. Peak III from the Ultrogel column (about 180,000 daltons) is not always present and is not included in the material pooled for the next purification stage.

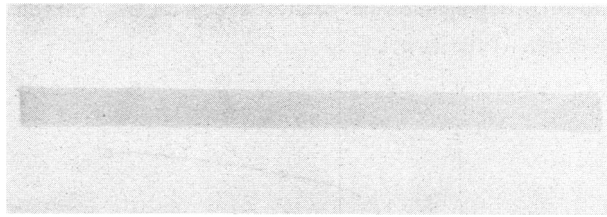

Figure 3 SDS-polyacrylamide gel electrophoresis of purified DNA methylase (Fraction VI). The procedure used was that of Fairbanks et al[21].

DNA Methylase Assay

The assay involves measuring incorporation of radioactivity from S-adenosyl-L- [methyl ^{3}H] methionine into DNA. Special precautions are taken to exclude measuring incorporation into protein or RNA; these contaminants are eliminated by treatment with phenol/m-cresol and alkali digestion respectively. The method of precipitating DNA on filter discs is convenient and gives good reproducibility and low blank values.

The concentration of S-adenosyl methionine used in the assay gives only about 80% of the maximum reaction velocity but was chosen for reasons of economy. The value for the Km (13μM) is intermediate between those reported for the rat liver and HeLa cell enzymes [11,15] and for the enzymes present in isolated sea urchin nuclei[8].

The reaction is linear for at least one hour and depending on the DNA substrate used it may be linear for 8h or more (See Figure 7). Activity is completely dependent on added DNA (Figure 4).

Identification of the Product of Methylation

Before an enzyme can be unequivocally characterised as a eukaryotic DNA methylase, one must be certain that the only product is 5-methyl-cytosine present in DNA. When E.coli DNA is methylated, isolated as in the standard assay, and then hydrolysed to the bases as described in Materials and Methods, about 90% of the incorporated radioactivity co-chromatographs with a marker of 5-methylcytosine (Figure 1). Thus the only target base for methylation by the enzyme appears to be cytosine.

That we are not studying methylation of RNA or protein is also

shown by the complete dependence of methyl group incorporation on the presence of added DNA (Figure 4).

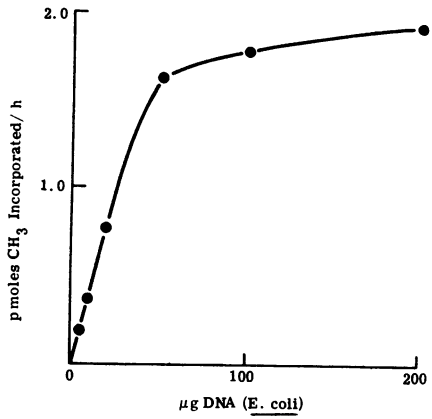

Figure 4 Effect of DNA concentration on methylation of E.coli DNA. Assays were carried out in a total volume of 0.22 ml using 5 µg of enzyme protein (Fraction VI).

Methylation of Single and Double-Stranded DNA

When we investigated the effect of salt on the DNA methylase reaction we were unable to repeat the results of Drahovsky and Morris[12] who showed that the activity with native E.coli DNA was strongly inhibited by 0.2 M NaCl. Further experiments showed, however, that the enzyme preferentially methylates single-stranded regions in "native" E.coli DNA but that with the eukaryotic DNAs tested methylation occurs in double-stranded regions. These experiments were performed as follows: 60 µg of DNA, from either E.coli, calf thymus or methionine-deprived L929 cells (see Materials and Methods) was incubated for 3 hours with enzyme and S-adenosyl-L [methyl ^3H] methionine under the usual conditions. The DNA product was isolated as in the methylase assay and incubated with 10 µl of deoxyribonuclease from N.Crassa (supplied by Boehringer Corporation, London) in a total volume of 600 µl (this enzyme degrades only single-stranded DNA). At intervals, samples were taken, acid-precipitated onto filter discs and prepared for liquid scintillation counting as described under "Methylase Assay".

As shown in Figure 5, this procedure resulted in some 25% of E.coli DNA being rendered acid soluble after 60 min., as measured by absorption at 260 nm. However, when the digestion of incorporated methyl label is examined almost 100% of the methyl groups incorporated into E.coli DNA are found to be acid soluble after nuclease digestion. When native DNA from calf thymus and mouse L929 cells is used as substrate the proportion of methyl label

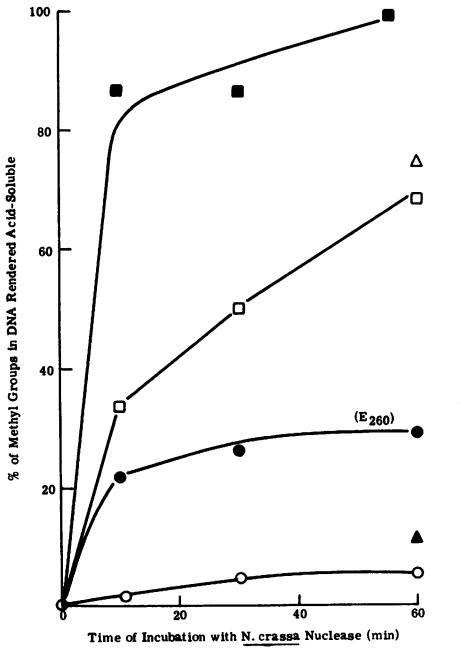

Figure 5 Digestion of methylated DNA with N.Crassa nuclease. 60 µg of native DNA was methylated in vitro under the usual conditions. A portion of the product was denatured and both native and denatured material incubated with N.Crassa nuclease as described in the text.

- ● "native" E.coli DNA (E_{260})
- ■ "native" E.coli DNA (dpm)
- ○ native calf thymus DNA(dpm)
- □ denatured calf thymus DNA (dpm)
- ▲ native L929 cell DNA (dpm)
- △ denatured L929 cell DNA (dpm)

digested does not exceed 10%, while with heat denatured DNA from these sources methylation occurs only in regions susceptible to N.Crassa nuclease. We conclude that the enzyme methylates E.coli DNA almost exclusively in single-stranded regions (about 25% of the molecule). For this reason, most of the kinetic studies which follow - especially those comparing single and double-stranded DNA - use calf thymus DNA as substrate.

Effect of Salt on Methylation

Incubation of native, calf thymus DNA with the enzyme in the presence of NaCl results in inhibition of enzyme activity at all salt concentrations above zero (Figure 6); 50% inhibition is achieved at 40 mM NaCl, and NaCl concentrations above 200 mM reduce activity to less than 10% of its level at zero salt. With denatured calf thymus DNA, on the other hand, low concentrations of NaCl (up to 90 mM) have a marked stimulatory effect on the enzyme activity, and a 50% level of inhibition is not reached until a NaCl concentration of 175 mM. This result is very similar to one obtained by Drahovsky and Morris[13] using E.coli DNA,

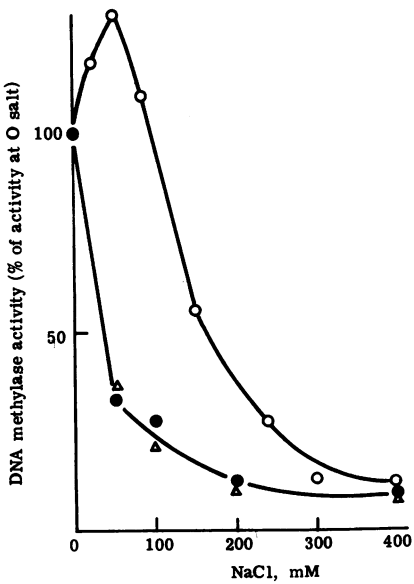

Figure 6 Effect of salt on methylation of calf thymus DNA. Assays were carried out in a total volume of 0.28 ml. (—○—○—) denatured DNA; (—●—●—) native DNA; (Δ Δ), native DNA incubated with enzyme for 5 min before adding salt.

although they observed stimulation at rather higher salt concentrations with the denatured DNA.

In view of the evidence already presented that the methylase will methylate only single-stranded E.coli DNA, an experiment was carried out to examine the effect of salt on this reaction (Table 2). As might be expected, fully denatured DNA was a better substrate than "native" DNA (which we know to have some 15% single-stranded material); moreover, the denatured DNA shows stimulation at 80 mM salt (compare Figure 6) while salt has a slight inhibitory effect on the reaction with "native" DNA. Prior treatment of the "native" DNA with N.Crassa nuclease, leaving only double-stranded material, greatly reduces the rate of methylation, and also leads to a salt effect very similar to that on native calf thymus DNA (some 78% inhibition at 80 mM salt). This experiment lends support to our finding that "native" E.coli DNA has a proportion of single-stranded regions, and that the enzyme methylates this DNA primarily in these regions.

TABLE 2: Effect of Salt on Methylation of E.Coli DNA

Type of DNA	Salt Concentration	P moles methyl incorporated/h
Denatured	80 mM	2.24
Denatured	10 mM	1.13
Native	80 mM	0.81
Native	10 mM	0.86
Native*	80 mM	0.04
Native*	10 mM	0.18

Denatured and Native assays were carried out with 50 µg DNA. Native*: 50 µg native E.coli DNA was treated with N.Crassa nuclease before use in the assay (15% of this DNA was rendered acid soluble in 8 h by the N.Crassa nuclease).

In an earlier paper[12], Drahovsky and Morris presented evidence that their enzyme formed a tight complex with native DNA, and once this complex was formed the enzyme remained bound to DNA for the duration of the reaction. One of their reasons for this conclusion was the fact that, if their enzyme and DNA were preincubated in the

absence of salt, the subsequent methylation reaction was markedly more resistant to salt than the reaction where salt was present from the beginning; they argued that it is the initial binding reaction which is affected by salt, not the actual methylation. When a pre-incubation experiment was carried out with our enzyme (Figure 6) the inhibition by salt was exactly the same as it had been without pre-incubation. This does not support the idea of an initial tight binding reaction for the purified ascites methylase.

Competition between methyl accepting and non-accepting DNAs as substrate

Another approach to the question of complex formation between methylase and DNA is to examine the effect of adding to the reaction mixture another species of DNA which is known not to be methylated by the enzyme, but may still be able to interact with it. Table 3

TABLE 3: Binding of Methylase to DNA

DNA added at		Activity
0 min.	5 min.	(p moles "CH_3" incorporated/2h)
L929	-	0.03
E.coli	-	0.83
L929 + E.coli	-	0.40
L929	E.coli	0.42
E.coli	L929	0.44
E.coli	E.coli	0.81

Each addition of DNA was of 70 µg (L929) and/or 80 µg (E.coli).

shows the result of such a competition experiment, using E.coli DNA as substrate and DNA from stationary mouse L929 cells as the non-methylatable competitor. The relative amounts of the two DNAs are adjusted so that, when both are present from the start of the incubation, incorporation of methyl groups after 2 hours is reduced to half that attained with E.coli DNA alone. Addition of E.coli DNA after 5 min. incubation of enzyme with L929 cell DNA, or of L929 cell DNA after 5 min incubation with E.coli DNA both reduced incorporation to half the original value for E.coli. If irreversible binding of enzyme to DNA took place at the start of the incubation, one would expect no significant incorporation in the first of these two cases and incorporation to the full level of unaccompanied E.coli DNA in the second case. An analogous experiment using calf thymus DNA as substrate and T4 DNA as the competitor gave similar

results.

Time Course of Methylation

Figure 7A shows the time course of methylation of calf thymus DNA, both native and denatured. In both cases, methylation continues for 50 h without the addition of further enzyme, although addition of more enzyme at the times shown does produce a stimulation. Native DNA is a better substrate than denatured. This is in accordance with the results of Drahovsky and Morris[13], but not those of Roy and

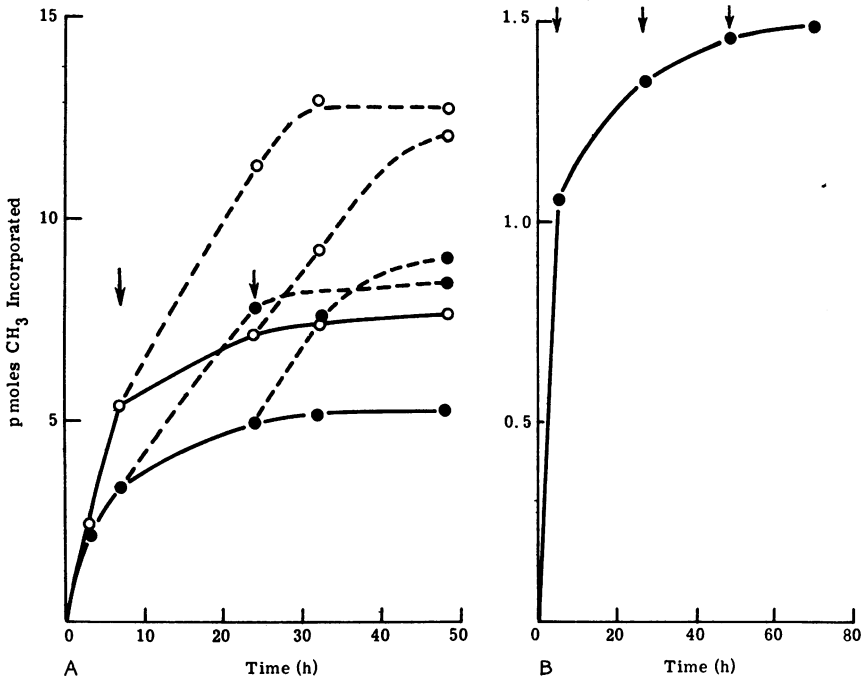

Figure 7A Time course of incorporation of methyl groups into calf thymus DNA (40 µg/assay). (—●-●—), denatured DNA; (—o-o—), native DNA. Dotted lines show increased incorporation resulting from addition of fresh enzyme (20 µl Fraction III) and S-adenosyl-L [^{3}H methyl] methionine (20 µl) at the times shown by the arrows.

Figure 7B Long-term methylation of native calf thymus DNA (4 µg) in an initial volume of 12 µl. Additional enzyme (Fraction III) and S-adenosyl-L [methyl ^{3}H] methionine (5 µl each) were added at times indicated by arrows.

Weissbach[15]. The stimulation on adding more enzyme indicates that the DNA is not saturated with methyl groups, even after 50 h. An experiment to saturate native calf thymus DNA was carried out using a limiting amoung (4 µg) of DNA with 64 µg of enzyme protein (Figure 7B). Saturation is reached after 70 h, at a level of 1.48 p moles of methyl groups per 4 µg of DNA, representing approximately one methyl group per 10^4 bases. This would suggest that only one in every 1760 cytosine residues in calf thymus DNA is susceptible to methylation by the enzyme. Figures in the literature[2] show that about 6% of cytosines are already methylated in calf thymus DNA. Taking this to be the 100% value for methylatable cytosines, the in vitro methylation reported here is of the order of 1.7%. Even at this low value, calf thymus DNA is one of the better substrates for the enzyme, as shown in Figure 8.

Various DNAs as Methyl Acceptors

Figure 8 shows the initial rate of incorporation of methyl groups

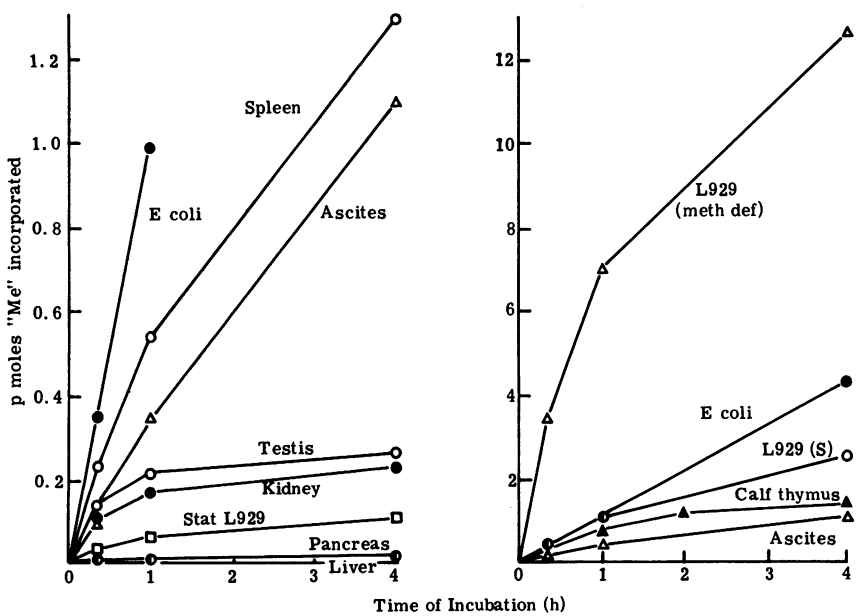

Figure 8 Initial rate of methylation of DNAs from various cells and tissues. Each assay contained 20 µg of the appropriate DNA, prepared as described in Materials and Methods, and 100 µl of Fraction III enzyme.

into DNA isolated from various mouse tissues, from L929 cells gorwn under different conditions, from E.coli and from calf thymus. E.coli DNA is the best substrate apart from L929 cell DNA artificially rendered methyl deficient (see Materials and Methods). It will be noted that DNA from rapidly-growing tissues (e.g. spleen) is generally a better substrate than DNA from slow-growing tissues (e.g. pancreas). This is consistent with the finding[4,6,7] of a distinct time lag between synthesis and methylation of DNA; presumably rapidly dividing cells will contain a higher proportion of newly synthesised, and therefore undermethylated, DNA. Also, the ascites enzyme is able to methylate homologous DNA at a significant level. In this respect it resembles the cell-free preparation of Kalousek and Morris[23], but not the crude nuclear fraction of Sheid et al[24].

A long-term incubation was carried out over 70 hours to saturate these DNAs with methyl groups in the same way as calf thymus DNA in the previous section, and Table 4 shows the results. As might be expected, the extent of undermethylation calculated on this basis is generally greatest in DNA from rapidly growing cells.

SV40 DNA apparently has no methylatable sites (one per 40 molecules). Native pseudorabies virus DNA accepts about 1.6 methyl groups per molecule and on denaturation its accepting ability rises. No form of SV40 DNA would accept methyl groups (we tried native and denatured, form I and form II). In addition, the supercoiled form I of SV40 DNA is not nicked during a 3 hour incubation with the enzyme, suggesting that no endonuclease activity has co-purified with the enzyme. No degradation of pseudorabies virus DNA occurs on incubation with the enzyme for 70 h.

DISCUSSION

A 405-fold purification of DNA methylase from Krebs II ascites cell nuclei has been achieved. The specific activity of the purified enzyme is 643 units/mg protein using E.coli DNA and one band is particularly prominent on SDS-polyacrylamide gel electrophoresis. Although we have no direct evidence that this 80,000 dalton band is produced by the enzyme, it appears prominent only in the purified fraction. The results of gel filtration suggest that the active form of the enzyme must be at least a dimer of this species.

In the standard assay an excess of E.coli DNA is used, but in the presence of excess enzyme the source of the DNA substrate affects both

TABLE 4: Saturation of Various DNAs with methyl groups

DNA	p moles CH_3/ 5 µg DNA	"Normal" % 5-methylcytosine	Undermethyl-ation %
Methyl-def L929	9.46	3.2	9.1
Late S-phase L929	1.02	3.2	1.1
Stat L929	0.26	3.2	0.25
Ascites	0.80	5.0	0.49
Spleen	1.88	5.0	1.15
Kidney	0.47	5.0	0.29
Liver	0.22	5.0	0.14
Bull sperm	0.30	3.4	0.27
Calf thymus	2.8	6.1,	1.41
SV40	0.05	-	-
Pseudorabies	0.13*	-	-

*16 h incubation only

Various amounts of DNA (up to 5 µg) were used in the assays but the figures have been corrected to 5 µg. Fresh enzyme and S-adenosyl-L [^{3}H methyl] methionine were added at 0, 19, 27 and 52 h, and the reactions terminated at 70 h. The figures for the normal % 5-methylcytosine (other than those for L929 cell DNA) are taken from the literature[2].

the rate and extent of the reaction. The best substrate is under-methylated DNA isolated from a heterologous source (mouse L929 cells), but a comparison of the effectiveness of DNA isolated from various mouse tissues shows a correlation with the in vivo rate of DNA synthesis. Thus the extent of methylation shown (varying from zero for SV40 DNA to over 9% of the normal 5-methylcytosine content for DNA from methionine deprived L929 cells) may be interpreted as reflecting the completion of a process left unfinished in vivo. This would occur because of the time lag between synthesis and methylation, which is experimentally lengthened by depriving cells of methionine. An alternative explanation is that the varying acceptor abilities of the different DNAs represent their varying content of specific unmethylated recognition sites. The number of such sites might differ between DNAs from different tissues

depending on the presence in the cell of regulatory proteins which could thereby play a role in differentiation. Clearly this explanation does not by itself account for the high acceptor ability of DNA from methionine deprived cells, which would presumably be subject to the same regulatory processes as normal S-phase L929 cells, and in which the number of unmethylated sites is increased by experimental manipulation and not by biological regulation.

The enzyme will also methylate homologous ascites DNA at a significant rate. We are confident, however, that there is no ascites DNA in the enzyme preparation which might interfere with the assays, since there is no detectable incorporation of methyl groups into DNA in the absence of added substrate DNA.

It is remarkable that the ascites methylase acts almost exclusively on single-stranded regions of "native" E.coli DNA while double-stranded calf thymus and L929 cell DNA are methylated. Clearly the enzyme can recognise single-stranded DNA as a substrate; it must also be able to recognise some sort of double-stranded site which is present in calf thymus and L929 DNA but lacking in E.coli. As DNA from stationary L929 cells appears fully methylated we can assume that undermethylated DNA will be completely methylated in one strand but undermethylated in the other. Base composition analysis of the undermethylated DNA synthesised in the presence of ^{14}C-deoxycytidine shows it to be about 19% under-methylated. All these undermethylated cytosines will be in the newly synthesised strand. Table 4 shows that this DNA can accept methyl groups on 9.1% of its methylatable cytosines, calculated on its overall (G+C) content. If we assume that these are all in the newly synthesised strand the proportion rises to 18.2% which is almost identical to the deficiency calculated by base analysis. This demonstrates that the enzyme is capable of methylating the available sites and that these are the sites left unmethylated in vivo.

It has been postulated by Drahovsky and Morris[12,13] that DNA methylase binds to native DNA at or near one end and "walks" along the molecule methylating sites as it goes, rather like E.coli RNA polymerase. They based this conclusion on the proposed formation, in a temperature dependent reaction, of a strong DNA-enzyme complex which, once formed, is resistant to dissociation by salt or competing DNAs. Our results contradict some of their observations and support the idea that the purified ascites enzyme binds loosely and reversibly to DNA - perhaps at random - and that

methylation occurs only when the enzyme binds to a methylatable site. This would explain the extended time course of reaction (Figure 7) and the lack of affinity of the enzyme for DNA cellulose (Turnbull and Adams, unpublished data). The difference between our results and those of Drahovsky and Morris may be accounted for by the presence in their much less pure preparation of accessory factors upon whose physiological significance we can only theorise.

Our finding that the ascites DNA methylase is able to methylate denatured DNA is in agreement with a number of earlier reports,[10,13,24]. Salt has differing effects on the reactions with single- and double-stranded DNA; while methylation of double-stranded DNA is inhibited even by low concentrations of NaCl, the reaction with single-stranded DNA shows a marked stimulation by salt at concentrations as high as 90 mM. The behaviour of "native" E.coli DNA as substrate in the presence of salt provides incidental confirmation of our conclusion that this DNA is methylated in single-stranged regions. Drahovsky and Morris[13] sought to explain this differential salt effect on the assumption that salt stabilises the DNA double helix, thereby preventing local unwinding which (they argue) is a prerequisite for methylation. This explanation, while plausible, is not the only possibility. It seems feasible that the action of salt might be to dissociate a tetrameric form of the enzyme (molecular weight about 400,000) to the putative dimeric form isolated from the Ultrogel column. The tetrameric enzyme would methylate native DNA whereas the dimer would methylate single stranded DNA in a salt stimulated reaction. These possibilities are at present under investigation.

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