

MAMMALIAN DNA METHYLATION AND A NUCLEAR *S*-ADENOSYL L-METHIONINE-DEPENDENT NUCLEASE ACTIVITY

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Received 29 November 1978

1. Introduction

Studies on bacteriophage–cell interactions have revealed that bacterial cells contain ‘restriction’ nucleases which recognise a specific sequence of nucleotides within DNA and make a double-stranded endonucleolytic cleavage at the sequence, or elsewhere in the DNA. On the other hand ‘modification’ enzymes also exist in these cells which can recognise the same nucleotide sequence but modify it so as to prevent its cleavage [1–6]. The bacterial restriction enzymes can be divided into two classes. Class I-type enzymes require ATP, magnesium ions and *S*-adenosyl L-methionine (SAM) as cofactors and the products of cleavage are heterogeneous. Class II-type enzymes require only Mg^{2+} but give rise to discrete fragments of DNA.

The phenomenon of ‘modification’ is also encountered in eukaryotes. In mammals most of the modification of DNA cytosine residues occurs shortly after DNA synthesis [7] and most of the methylated sequences occur in the sequence m^5C-G , but the distribution of bases on either side of this dinucleotide sequence appears random [8,9].

As yet there has been little positive data to indicate a biological role for DNA modification in mammalian cells. We explore here the possibility that the modification of certain C–G sequences protects DNA from the nucleolytic effects of SAM-dependent deoxyribonucleases, which appear to occur in nuclei of cultured baby hamster kidney fibroblasts (BHK-21 cells).

2. Experimental

2.1. Cell growth

BHK-21/C13 cells were grown as monolayers in 80 oz. rotating bottles. Each bottle was seeded with 3×10^7 cells and the culture allowed to grow for 4 days at 37°C in Eagle’s minimal essential medium supplemented with 10% calf serum, 10% tryptose phosphate broth (Difco, bacto).

2.2. Preparation of nuclei

Cells were harvested after 4 days growth and nuclei were prepared as in [11] using Tween 80.

2.3. Preparation fractionation of soluble nuclear extract

Nuclei were suspended in buffer M (50 mM Tris–HCl, pH 7.8, 6 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol) and made 0.4 M with respect to KCl, stirred gently for 15 min at 4°C, then centrifuged at $12\,000 \times g$ for 60 min to yield a soluble nuclear extract. In initial experiments this was used directly as enzyme source after extensive dialysis against 100 vol. buffer M. In later experiments the dialysed soluble nuclear extract (~14 ml, 5 mg protein/ml) was loaded onto a DEAE-cellulose column (DE-52, 21×1.7 cm pre-equilibrated by the passage of 50 vol. buffer M). The column was then washed with 1 vol. buffer M. The SAM-dependent nuclease activity recovered in this DEAE-cellulose flow through material (~0.6 mg protein/ml) was loaded onto a phosphocellulose column (P11, 12×1.7 cm). After washing

with buffer M (3 column vol.), the SAM-dependent nuclease activity was eluted with 30 ml 0.1 M KCl in buffer M.

2.4. Nuclease assays

The composition of assay mixtures is given in the appropriate figure legends. For electrophoresis the reactions were terminated by the addition of 10 μ l 'stopper' solution comprising 20% (w/v) sucrose, 2% (w/v) SDS, 100 mM EDTA and 0.05% bromophenol blue and applied to 1.5% (w/v) agarose gels in 36 mM Tris–30 mM NaH_2PO_4 –10 mM EDTA (pH 7.5) and electrophoresed at 14 V/cm for 1.75 h. Gels were then stained with ethidium bromide (1 μ g/ml) and DNA visualised with a short wavelength ultraviolet light source.

For zonal ultracentrifugation the reactions were diluted with 100 μ l ice-cold water, layered onto 5–20% (w/v) linear sucrose gradients in 10 mM Tris–HCl (pH 7.9), 1 mM EDTA, 0.1 M NaCl and centrifuged at 40 000 rev./min in an SW50.1 rotor at 20°C. Fractions were collected on Whatman 3MM paper discs and the DNA precipitated thereon by immersion in ice-cold 5% (w/v) trichloroacetic acid. The discs were washed with 5% trichloroacetic acid, absolute ethanol and finally with ether. The DNA on the filters was solubilised by the addition of 0.5 ml hyamine hydroxide solution (1 M in methanol) followed by incubation at 60°C for 20 min. The radioactivity solubilised was determined after addition of toluene scintillator (0.5% (w/v) diphenyl oxazole in toluene).

2.5. DNA preparations

[^3H]DNA was isolated as in [13] from cultures of mouse (L-929), human (HeLa) and hamster (BHK-21/C13) cells grown to stationary phase in 80 oz. rotating bottles in the presence of 2 mCi [^3H]thymidine (23 Ci/mmol, Radiochemical Centre, Amersham). L-929 cell DNA thus produced [12] was found to be methylated to the fullest *in vivo* extent, there being a considerable time lag between DNA synthesis and its complete modification [12].

To produce undermethylated DNA the method in [12] was followed which yields L-929 cell DNA with ~60% of its normal level of 5-methyl cytosine [12].

2.6. Methylation of λ phage DNA

λ phage DNA (10 μ g) was incubated in 25 μ l final

vol. for 72 h with 8.8 μ g BHK-21/PyY cell DNA methylase [10] and dithiothreitol (72 μ M), EDTA (72 μ M), glycerol (7.2% v/v) Tris–HCl (pH 7.8) (36 mM), *S*-adenosyl L-[methyl- ^3H]methionine (33 μ M, 0.82 μ Ci). The reaction was stopped by the addition of a solution containing SDS (1%), EDTA (2 mM), 4-aminosalicylic acid (3% w/v), *n*-butanol (5% v/v), NaCl (0.5 M). Protein was removed by extraction with phenol (88%)/*m*-cresol (12%)/8-hydroxyquinoline (0.1%) and the DNA precipitated from the aqueous layer by addition of ethanol. From the level of ^3H radioactivity in the product the procedure resulted in the incorporation of 4.5 pmol $\text{CH}_3/\mu\text{g}$ λ phage DNA (this is equivalent to 1 $\text{CH}_3/725$ bases).

3. Results

In initial experiments, a soluble extract was prepared from BHK-cell nuclei using 0.4 M KCl as in section 2. When incubated along with SAM at > 55 μ M and < 10 mM, this preparation was capable of bringing about the degradation of λ phage DNA, however no discrete fragments were observable as judged by agarose gel electrophoresis of the products (fig.1). On the other hand this degradation of λ phage DNA did not require ATP, or Mg^{2+} , but was inhibited by *N*-methyl maleimide as well as by *S*-adenosyl L-homocysteine (fig.1) a known inhibitor of DNA methylases. Whilst other DNAs such as T7 phage DNA, herpes virus DNA, adenovirus 2 DNA were also sensitive to this SAM-dependent activity the DNA from SV40 was surprisingly resistant to degradation. This is of particular interest as SV40 is not a substrate for mammalian DNA methylases [12].

The possibility of a relationship between eukaryotic DNA modification and this degradative activity is further supported by the fact that it is possible to protect λ phage DNA from degradation by the BHK-cell enzyme activity by prior methylation using a partially-purified DNA methylase from BHK-21 cells (fig.1). Previous experiments had indicated that the partially-purified methylase preparation used [10] did not contain any SAM-dependent nuclease activity.

With regard to the effects of this BHK-cell nucleolytic activity on heterologous and homologous mammalian DNAs, these appear in part at least to be related to the extent to which the DNAs have been

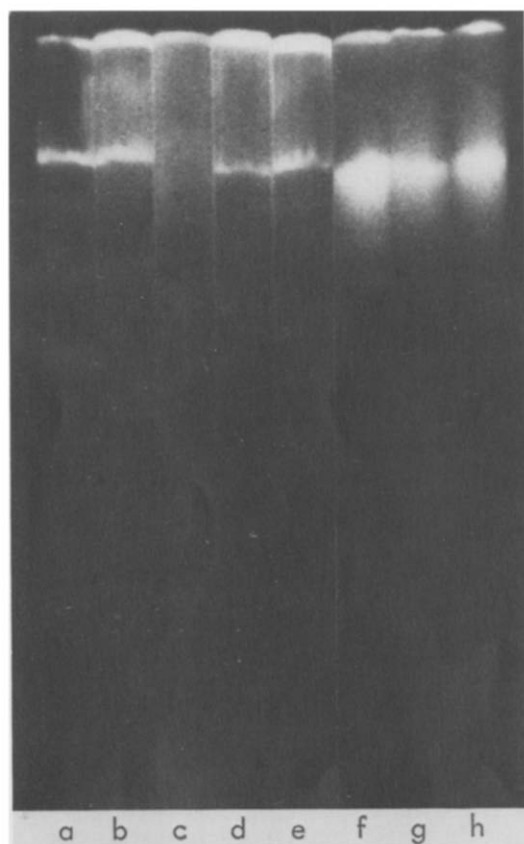


Fig. 1. Degradation of phage λ DNA by a soluble extract prepared from stationary BHK-21/C13 cell nuclei. The reaction mixtures (20 μ l) contain 1 μ g or 1.5 μ g (see below) λ phage DNA, 35 μ g extract protein, 6 mM EDTA 30 mM Tris-HCl (pH 7.8) 0.6 mM dithiothreitol and 6% glycerol. After incubation for 60 min at 37°C the reactions were stopped and the products analysed by electrophoresis as in section 2. (a) 1 μ g λ phage DNA alone; (b) 1 μ g λ phage DNA + nuclear extract; (c) 1 μ g λ phage DNA + nuclear extract + 1 mM SAM; (d) 1 μ g λ phage DNA + nuclear extract + 1 mM SAM + 10 mM *S*-adenosyl-L-homocysteine (SAH); (e) 1 μ g λ phage DNA + nuclear extract + 10 mM SAH; (f) 1.5 μ g methylated λ phage DNA (methylated for 76 h using a partially purified BHK-21 cell methylase as in section 2); (g) 1.5 μ g methylated λ phage DNA + nuclear extract; (h) 1.5 μ g methylated λ phage DNA + nuclear extract + 1 mM SAM.

modified *in vivo*. DNA from either mouse (L-929), human (HeLa) or hamster (BHK-21) cells cultured in the absence of L-methionine [12] is both deficient in modified cytosine residues (~60% of normal) and

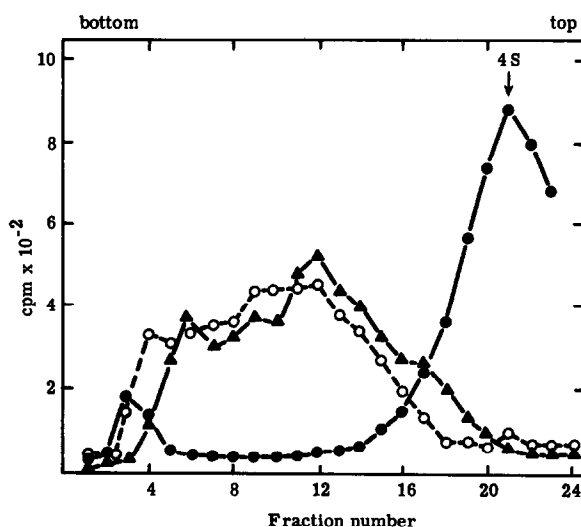


Fig. 2. Degradation of ^{14}C -undermethylated L929 cell DNA. The ^{14}C -undermethylated DNA was prepared as in section 2. 5 μ g [^{14}C]DNA was incubated with 69 μ g BHK-21 cell nuclear extract protein in the presence of 6 mM EDTA 6 mM dithiothreitol, 30 mM Tris-HCl (pH 7.8) and 6% glycerol. Incubation was for 3 h at 37°C. Analysis was by zonal ultracentrifugation as in section 2. (▲—▲) Unincubated labelled L929 DNA alone; (○—○) labelled L929 cell DNA incubated with nuclear extract; (●—●) labelled L929 cell DNA incubated with nuclear extract plus 10 mM SAM.

sensitive to the nucleolytic effect of the BHK-21 cell SAM-dependent activity (e.g., fig. 2). However culture of these cell types to stationary growth phase in the presence of L-methionine yields DNA preparations which are almost fully modified [12] and which are much more resistant to the BHK-cell enzyme activity (e.g., fig. 3).

Another feature of the BHK-cell SAM-dependent activity is its ability to cleave 'denatured' methyl-deficient DNA (fig. 4). This suggests that whatever the cleaving enzyme recognises it would not appear to be a DNA duplex sequence with 2-fold rotational symmetry.

In contrast however to the DNA methylases of mammalian cells whose level is highest at mid log phase of cell growth [14] the activity of the BHK-cell SAM-dependent nuclease was barely detectable in nuclei of mid-log phase cells (~2 days growth under the conditions in section 2). The activity is readily found in nuclei of cells entering the stationary phase of growth

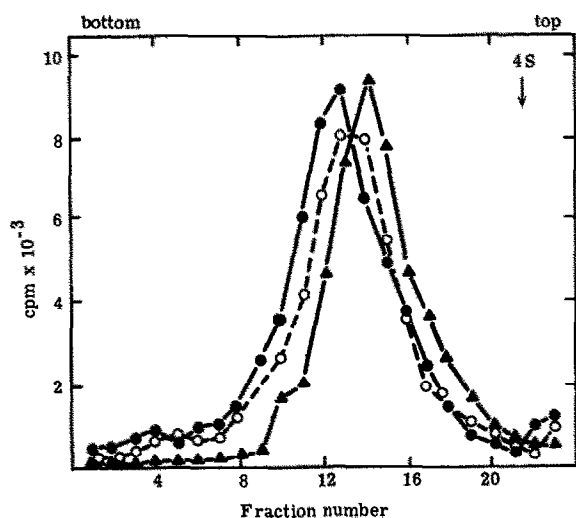


Fig. 3. Degradation of L929 mouse cell $[^3\text{H}]$ DNA from cells grown to stationary phase (see section 2 for preparation). Symbols and conditions of incubation and analysis were as indicated in legend to fig. 2.

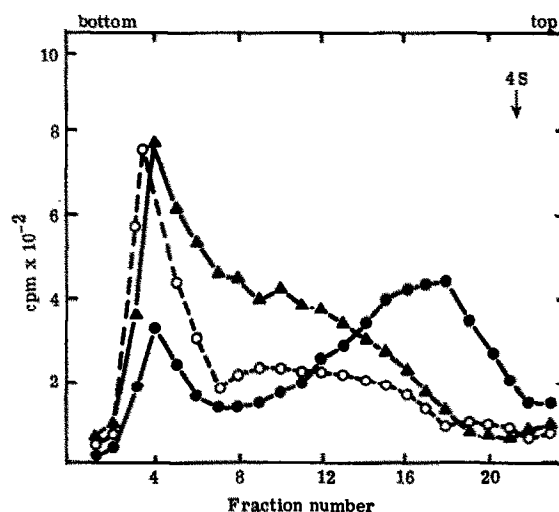


Fig. 4. Degradation of heat-denatured ^{14}C -undermethylated L929 cell DNA. 5 μg samples of ^{14}C -undermethylated L929 cell DNA were heat-denatured by heating to 100°C for 5 min followed by rapid cooling then incubated with nuclear extract, with or without 10 mM SAM, as in fig. 2. Symbols and analysis are as in fig. 2.

(i.e., after 4 days growth under the conditions in section 2).

In these initial experiments using the dialysed soluble nuclear extract quite a high concentration of EDTA (6 mM) was included in the assay mixtures. This suppressed the activity towards λ phage DNA of endonucleases in the preparation which did not require SAM. However the lack of specific DNA fragments amongst the end-products of the SAM-dependent activity might be attributable to the activity of other contaminating nucleases (e.g., exonucleases). In the experiments using the ^3H -undermethylated L-929 cell DNA only 5% of the original ^3H radioactivity was converted to an acid-soluble form upon incubation with the nuclear extract alone for 3 h. In the presence of SAM however the conversion was around 15%. Another difficulty with the high level of EDTA was that it tended to inhibit the basic SAM-dependent activity to some extent. To answer these questions a fractionation of the soluble nuclear extract was attempted. This involved the use of DEAE-cellulose and phosphocellulose columns (see section 2). As mentioned in section 2 the SAM-dependent activity was present in the flow-through fraction from the DEAE-cellulose column and can be further subjected to chromatog-

raphy on phosphocellulose to yield a preparation which can be assayed at much lower concentrations of EDTA (0.6 mM). Figure 5 shows that using this preparation and λ phage $[^3\text{H}]$ DNA as substrate a population of DNA fragments is produced (between 10–20 S). Examination of the levels of acid-precipitable $[^3\text{H}]$ DNA radioactivity recovered before and after incubation indicated that exonuclease activity is absent and thus cannot explain the heterogeneous size range of the fragments. The pattern however is very similar to that obtained upon digestion of λ DNA by the *E. coli* K12, class I restriction enzyme [1,15]. Also the pattern in fig. 5 would appear to represent a 'limit' digest as there is no difference even after a further period of incubation with fresh SAM and enzyme.

4. Discussion

A possible relationship between modification of DNA and its protection from the nucleolytic effects of a SAM-dependent nuclease activity is indicated. At

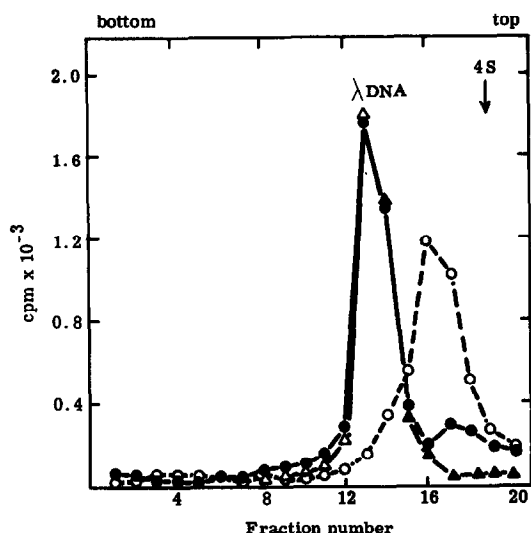


Fig.5. Degradation of λ phage [^3H]DNA by the BHK-21 cell SAM-dependent nucleolytic activity. The assay mixture (38 μl) contained 2.5 μg λ phage [^3H]DNA (3.4×10^4 dpm/ μg) 30 mM Tris-HCl (pH 7.8), 0.6 mM EDTA, 0.6 mM dithiothreitol, 6% (v/v) glycerol and 6 μg protein (0.1 M KCl eluate from phosphocellulose column). The reaction mixtures were incubated with or without SAM at 37°C for 6 h. The products were analysed by zonal ultracentrifugation through 5–20% sucrose gradients as in section 2 but for 75 min at 39 000 rev./min in an SW56 rotor at 20°C. (\blacktriangle — \blacktriangle) λ phage [^3H]DNA uncultured; (\bullet — \bullet) λ phage [^3H]DNA incubated with enzyme preparation; (\circ — \circ) λ phage [^3H]DNA incubated with enzyme preparation plus 5 mM SAM.

present it is not known whether 'restriction' in the bacterial sense exists in higher organisms. In fact most of the available data would argue to the contrary. For instance it is possible to insert portions of λ phage DNA into the SV40 genome from which a portion of the late genes have been removed and then propagate this recombinant in eukaryotic cells with the aid of helper virus, the λ phage segment retaining its normal bacterial restriction enzyme cleavage sites [16]. Additionally SV40 DNA has been successfully used to transduce suppressor tRNA genes of *E. coli* into monkey cells [17]. Microinjection of 'cloned' (hence 'unmodified') amphibian DNA into *Xenopus* oocytes results in their transcription rather than destruction [18,19]. Purified human chromosomes have been used to transfer certain human genes to recipient mouse cells [20]. Purified DNA from human lympho-

blastoid cells is taken up by human skin fibroblasts in culture and retained in a relatively undegraded form for over 20 h [21]. T7 phage DNA however undergoes extensive degradation after entry into the fibroblasts [23]. On the other hand in cell fusion experiments in which mouse-human hybrid lines are produced some human chromosomes tend to be eliminated but only after several cell generations.

Our data would indicate that provided the DNA from human or mouse sources is modified to its fullest in vitro extent then it is quite resistant to the hamster SAM-dependent nuclease. On the other hand DNAs from stationary cultures of nonmammalian cells such as amphibian (*Xenopus laevis*) or insect (*Aedes albopictus*) are completely sensitive to the enzyme activity, as are *E. coli* DNA and *M. luteus* DNA. It remains to be seen however whether or not a recombinant of SV40 DNA (normally resistant) with a segment of λ phage or *E. coli* DNA will be resistant or not.

In any evaluation of these in vivo and in vitro data a great deal will depend on whether or not a foreign DNA is incorporated into a cell at a time when it is likely to become modified rather than cleaved. Growing cells will be likely to modify whereas non-growing cells appear to be more equipped to cleave. Successful use of naked herpes virus DNA [22] or adenovirus DNA [23] as infectious agents appears to have involved the use of growing monolayer cell cultures, however there have been no definitive experiments to answer this problem. Clearly this will be of relevance in any recombinant DNA techniques involving mammalian cells.

Recently a possible alternative role for bacterial 'restriction' enzymes has emerged. The *EcoR*1 enzyme has been shown to be involved in site specific recombination events [24]. In mammalian cells the occurrence of nucleases related to the specific methylation of C—G sequences may be of relevance in possible recombination events.

Acknowledgements

We are indebted to Drs R. L. P. Adams and I. S. Villadsen for helpful advice and criticism and to Miss J. Douglas for skilled technical assistance. Thanks are due to the Medical Research Council for a grant to R.H.B.

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