Immunochemical evidence for the presence of 5mC, 6mA and 7mG in human, *Drosophila* and mealybug DNA

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We have reported that production and characterization of antibodies highly specific to 5-methyl-cytosine (5mC) and the development of a sensitive immunochemical method for the detection of 5mC in DNA [FEBS Lett. (1982) 150, 469]. Extension of this method to two other modified bases, 6-methyladenine (6mA) and 7-methylguanine (7mG), is reported here. By use of this immunochemical approach, we are able to detect 5mC, 6mA and 7mG in human and *Drosophila* DNA and confirm their presence in the DNA of two mealybug species.

DNA methylation *Biotin–avidin cross-linking* *DNA–protein interaction* *Gene regulation* *Z-DNA* *X-chromosome inactivation*

1. INTRODUCTION

Several unusual modified bases have been demonstrated in the DNA of bacteriophages, but in general only 5-methylcytosine (5mC) and 6-methyladenine (6mA) are known to occur in the DNA of eukaryotes. 5mC is found in the DNA of many plants and vertebrates [1]. Among higher eukaryotes, 6mA has been reported in the DNA of a mosquito cell line [2] and in a species of mealybug [3]. 7-methylguanine (7mG) occurs in DNA of the Shigella phage DDV1 [4]. These methylated bases introduce into the major groove of DNA a hydrophobic group which may influence DNA–protein interactions [5]. For example, the presence of 5mC in place of cytosine in the operator sequence improves the binding efficiency of the *lac* repressor [6]. Similarly, the binding efficiency of the *lex* A repressor is 10-times greater when the internal cytosine in a CCXGG sequence within the operator is methylated [7, 8]. In constitutive mutants of the arabinose operon in *E. coli*, the presence of 5mC in the promoter reduces the rate of transcription (A. Horwitz and G. Wilcox in [8]). 6mA has similar effects. The presence of 6mA in a GATC sequence appears to be necessary for the expression of the '*mom*' gene in the phage Mu [9,10]. 6mA, when present on one DNA strand, seems to facilitate mismatch repair on the other, unmethylated strand [11]. A reduction in the level of methylation of the internal cytosine in a $CC\overset{A}{\underset{T}{C}}GG$ sequence in bacteriophage λ appears to stimulate genetic recombination [8]. In several prokaryotes, it has been shown by use of appropriate mutagens that stretches of DNA containing 5mC [12] and 7mG are mutational hot-spots [13]. There are, thus, several lines of evidence suggesting a variety of roles for DNA methylation in cellular regulatory processes [5].

Immunochemical methods have been shown to be useful for the detection of modified bases in DNA [14]. We have reported a sensitive immunochemical method which permits detection of femtomole (10^{-15} M) amounts of 5mC in nanogram quantities of DNA [15]. To extend the method to other modified bases, we have raised antibodies to 6mA and 7mG. Using these antibodies we have attempted to detect 5mC, 6mA and 7mG in DNAs from human placenta, rat sperm, gravid females of 2 mealybug species

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(Coccoidea; Homoptera; Insecta) and *Drosophila melanogaster*. The results are reported here.

2. METHODS

2.1. *Preparation and characterization of antibodies*

The periodate oxidation procedure [16,17] was used to covalently link the haptens 6-methyladenosine and 7-methylguanosine to bovine serum albumin. The resulting hapten–protein conjugate was characterized spectrally and injected with complete Freund's adjuvant, at 10-day intervals, into the foot-pad and back muscle of rabbits. Serum from these animals was collected and the immunoglobulin fraction purified by precipitation with 50% ammonium sulfate followed by DEAE-cellulose chromatography [18]. Labelled nucleosides were prepared by reduction with sodium boro-[^{3}H] hydride (756 mCi/mmol; Amersham) after periodate oxidation [19]. The specific activities of the products were 163 cpm/pmol for 6mA and 240 cpm/pmol for 7mG. The procedure for raising antibodies against 5mC was similar [15].

The specificity of the antigen–antibody reaction was assessed in a nitrocellulose filter binding assay by use of the appropriate radioactive antigen [17]. The K_a for 6mA was $8.7 \times 10^9\ M^{-1}$ (fig. 1a) and that for 7mG, $3.9 \times 10^{10}\ M^{-1}$ (fig. 1b). As described in [15], the K_a for 5mC, was $1.56 \times 10^9\ M^{-1}$. Competition assays were carried out by incubating the labelled antigen and antibody in the presence of competing unlabelled molecules. Adenosine and 2-methyladenine at 3.3×10^{-5} M did not inhibit the binding of 6mA antibodies to labelled 6mA. 1-Methyladenine showed 18% inhibition at the same concentration. Similarly the binding of 7mG antibodies was not inhibited by guanosine at 10^{-5} M. Both antibodies thus appear to have high specificity. Data on the specificity of the 5mC antibodies and detailed experimental procedures have been published [15].

2.2 *Preparation of DNA*

Drosophila melanogaster DNA was isolated from an Oregon-K stock maintained by Dr. N.N. Godbole (Zoology Department, Pune University). Flies were allowed to lay eggs on yeast-free agar medium and DNA was isolated from eggs, embryos and larvae. Dr Ken Jones (University of Edinburgh) kindly provided a sample of DNA isolated as in [20]. This DNA had been purified by isopycnic centrifugation on CsCl gradients [21]. This step largely eliminates 'contamination' of *D. melanogaster* DNA by DNA–RNA complexes and by DNA from other sources such as yeast which is a common constituent of the laboratory medium in which these flies are grown. Because it is generally believed that *D. melanogaster* DNA lacks detectable levels of methylation, and a recent set of experiments seems to confirm this view [22], it was necessary to take the above steps to obtain from this species samples of DNA whose purity was not in doubt. If low levels of DNA methylation are observed, it becomes necessary to be sure that the methyl groups recognized by the antibodies are not

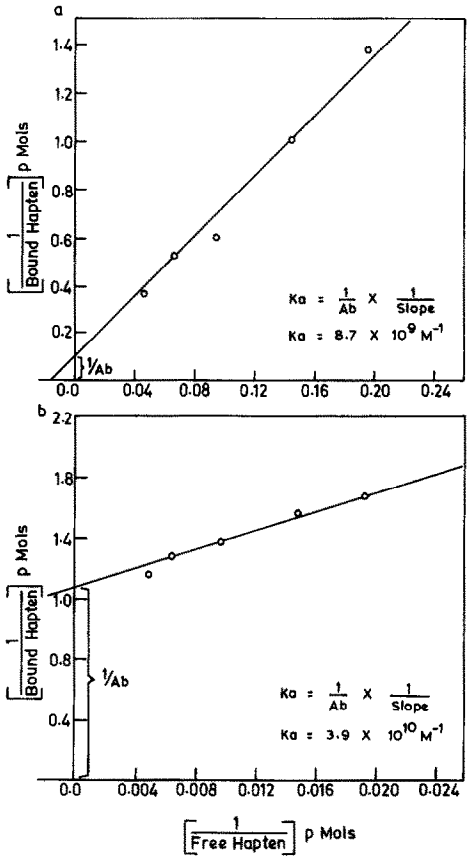

Fig. 1.(a) Reciprocal plot of bound hapten *vs* free hapten (6mA). (b) Reciprocal plot of bound hapten *vs* free hapten (7mG). Assays were done as in section 2.

from residual RNA some of which may be covalently bound to the RNA under investigation. To rule out this possibility extensive RNase treatment and alkali treatment were carried out (see below).

Mealybug DNA was isolated as in [3]. DNA from two species of mealybug were studied. One of them was *Planococcus citri* (Risso) and the other was provisionally identified as *Planococcus lilacinus* (Cockerell). The source of these organisms and the culture conditions have been described [3]. The taxonomic identification was kindly provided by Dr. B.K. Rajagopal (Department of Entomology, University of Agricultural Sciences, Bangalore).

Human placental DNA, wild-type λ DNA and other DNAs were isolated by standard procedures. Non-methylated λ DNA and calf-thymus DNA were obtained from Sigma (St. Louis MO). Rat sperm DNA was kindly provided by Dr M.R.S. Rao of this Institute.

2.3. *Spot assay*

DNA was spotted on BA85 nitrocellulose paper (Schleicher and Schuell), baked for 4 h and processed as in [15]. The blot was incubated for 4–6 h with one of the three antibodies, washed in Tris-buffered saline (TBS; 10 mM Tris–HCl (pH 7.5), 0.14 M NaCl) and treated with biotinylated anti-antibody. This was followed by extensive washing with TBS prior to the addition of a complex of biotinylated horseradish peroxidase H and avidin DH. This complex was prepared by mixing the 2 components 10 min before use. The blot was then washed and stained for the peroxidase reaction using 0.1% diaminobenzidine tetrahydrochloride and 0.02% H_2O_2 [23,24].

To avoid binding to normal bases or other cross-reacting bases, in all assays the competing nucleotide, nucleoside or base was present at 10^{-5} M at the time of incubation of the DNA with the antibodies. At pH ≥ 8.5, 7mG in DNA undergoes spontaneous hydrolysis and even at neutral pH there is hydrolysis to a significant extent [25]. In initial experiments we observed that poor staining was obtained with 7mG antibodies when DNA samples which had been stored for long periods were used as substrates. For this reason, freshly isolated samples of DNA were used in assays for this modified base. As a further precaution, all DNAs were freshly dissolved before spotting on nitrocellulose paper and incubation of DNA blots with the antibodies carried out at pH 7.5, 4°C.

2.4. *NaOH treatment*

In some test assays, DNA was incubated in 0.3 N NaOH at 70°C for 4 h. After neutralization, DNA was spotted and the assay carried out.

3. RESULTS AND DISCUSSION

In all spot assays, non-methylated λ DNA was used as negative control. DNA does not react with any of the 3 antibodies (fig 2). *Mycobacterium smegmatis* DNA, which is known to contain 3 mol% of 6mA [26], shows a strong reaction against 6mA antibodies and a very weak reaction with 5mC and 7mG antibodies. DNAs from the mealybug *P. lilacinus* and *P. citri* react very strongly with all 3 antibodies, suggesting the presence of 5 mC, 6mA and 7mG. Dinucleotide analysis of mealybug DNA has shown that it contains high levels of all 3 modified bases (in approximately amounts: 5mC, 2.3 mol %; 6mA, 4 mol%; 7mG, 2 mol%) [3].

Human and calf-thymus DNAs contain 5mC [27,28]. Therefore, as expected, both react strongly with 5mC antibodies (fig. 2). The reaction of these two DNAs against 6mA and 7mG antibodies is faint but clearly positive. Similar results were obtained with rat sperm DNA, suggesting the presence of small amounts of 6mA and 7mG in all 3 samples of mammalian DNA. HeLa cell and bovine sperm DNA have been shown to contain small amounts of 7mG, 1-methylguanine, 2-methylguanine and 2-dimethylguanine [29,30]. These rare modified bases were identified on the basis of the R_f values of labelled products following growth of these cells in the presence of labelled methyl methionine.

Drosophila DNA showed a weak but clearly positive reaction with the 5mC antibodies. A reaction with 6mA and 7mG antibodies was visible only when a high concentration (100 ng) DNA was used. All DNA samples had been extensively treated with RNase before blots were prepared. As a further precaution several DNA samples were subjected to alkali treatment (NaOH) before blot preparation. A few test blots were also incubated with RNase (50 μg/ml; 37°C for 4 h) before assay.

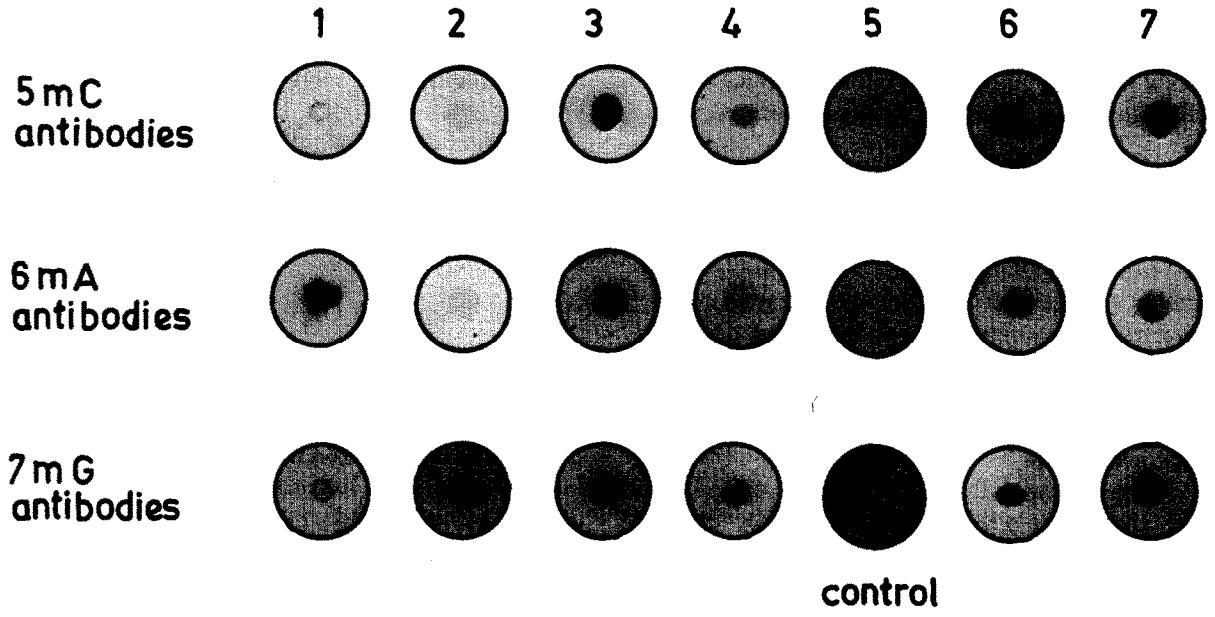

Fig. 2. DNAs from *Mycobacterium smegmatis* (1), *Drosophila melanogaster* (2), mealybug (gravid females) (3), lambda (methylated) (4), lambda (non-methylated) (5), calf-thymus (6) and human placenta (7) were spotted on nitrocellulose paper, incubated with antibodies to 5mC (first row), 6mA (second row), 7mG (third row) and stained as in section 2. Non-methylated DNA, isolated from λ grown in *E. coli* (dam^-, dcm^-) served as negative control in these assays (see text). Even a 6-fold higher concentration of this DNA (upto 200 ng) showed no staining in any of the experiments. Mealybug and *M. smegmatis* DNAs served as positive controls. Mealybug DNA is known to contain high levels of 5mC, 6mA and 7mG and therefore shows a strong staining reaction against all 3 antibodies. *M. smegmatis* DNA contains high levels of 6mA and, as expected, shows as intense a staining as mealybug DNA when tested against antibodies to 6mA. *D. melanogaster* DNA shows a weak but clearly positive staining with all 3 antibodies.

All blots of *D. melanogaster* DNA gave a faint but clearly positive reaction as described. On the other hand, 'non-methylated' DNA from λ grown in dam^- and dcm^- hosts did not show any staining. The products of dam^+ and dcm^+ genes are known to methylate, respectively, adenine and cytosine residues in DNA. The reasons for our failure to obtain, with 7mG antibodies, staining of λ-DNA isolated from phages grown in dam^-, dcm^- hosts is therefore not clear.

When antibodies were assayed in competition experiments involving 5-methylcytosine (against 5mC antibodies) 6-methyladenine (6mA antibodies) and 7-methylguanine (7mG antibodies) each at a concentration of 10^{-5} M, near complete inhibition was seen in all three cases as judged by the staining intensity. Results of the other competition assays (also at 10^{-5} M and as judged by the staining intensity of the spot) were as follows.

(1) *5mC antibodies*: cytosine, 6-methyladenine and 7-methylguanosine did not show inhibition.
(2) *6mA antibodies*: Adenine, 5-methylcytosine, 7-methylguanosine, 1-methyladenine and 2-methyladenine did not inhibit the reaction.
(3) *7mG antibodies*: Guanosine, 5-methylcytosine, 6-methyladenine, deoxyguanylic acid and thymidine did not show any inhibition, while 1-methylguanine, 2-methylguanine and hydrolysed 7-methylguanosine all showed slight inhibition.

The extent of inhibition was greater with hydrolysed 7-methylguanosine than with either 1-methylguanine or 2-methylguanine. However, in view of the clear inhibition observed when DNA is competed with 7mG, it appears that this population of antibodies, while possibly heterogenous, is largely specific to 7mG in DNA.

3.1. *Sensitivity of the antibody method*

The sensitivity of the methods used in the earlier attempts to detect DNA methylation in *D. melanogaster*. *Drosophila* DNA does not show any difference in its susceptibility to digestion by the restriction enzymes *Msp*I and *Hpa*II [31]. However, these isoschizomers would not permit recognition of methylation in sequences other then CCGG. HPLC permits the detection of at least 0.001 A_{260} units of a single nucleotide component [32]. By use of a fluorescence detector 0.00005 A_{260} units (about 5 pmol) can apparently be detected. Nearest neighbour analysis of *D. melanogaster* DNA also did not show evidence of 5mC or 6mA. This method is said to detect one 5mC residue in 10000 nucleotides [22]. The antibody method described here appears to provide a significantly higher level of sensitivity. For example, if *D. melanogaster* DNA contains at least one 5mC residue in 50000 nucleotides a positive reaction would be seen in this immunochemical method if the total amount of DNA in the spot is ≥ 100 ng. This conclusion follows from our earlier finding that these antibodies are capable of detecting 0.005 pmol 5mC in 10 ng $\phi X174$ DNA [15]. This level of methylation, it appears, can be missed when less sensitive methods are employed.

Our data confirm the results [3] that mealybug DNA contains high amounts of 7mG. Detection of 7mG in human DNA as well as in *Drosophila* DNA was unexpected and raises questions about the possible role of this unusual DNA modification in eukaryotes. 7mG, 3-methyladenine (3mA) and O^6-methylguanine are the major purine products formed when cells are exposed to mutagenic and carcinogenic alkylating agents [33]. This is because the major sites of alkylation in DNA are the strongly nucleophilic nitrogen atoms of the purine rings. Whereas a correlation appears to exist between carcinogenesis and the concentration of O^6-methylguanine in DNA, no such correlation is apparent in the case of 7mG and 3mA [34]. 7mG is known to undergo hydrolysis in isolated DNA [35]. However, within cells it appears to have no deleterious effect even though it is the most abundant product of DNA alkylation. For example, it does not appear to lead to significant levels of miscoding in DNA as happens with O^6-methylguanine [34]. In *E. coli*, a glycosylase rapidly removes 3mA through a repair pathway and similar mechanisms seems to exist for scavenging O^6-methylguanine [36]. Evidence suggests that between 3mA and O^6-methylguanine, the latter is more harmful because it appears to be a major factor in tumor formation following exposure of laboratory mammals to alkylating agents. Methylation of guanine, like methylation of cytosine, is known to induce the transition of DNA from the B to Z conformation [37]. If all the cytosines or guanines in poly (dG–dC)·poly(dG–dC) are methylated, such B to Z transition occurs even under physiological salt concentrations [37]. It is not unlikely, therefore, that even within the cell such B to Z transitions are regulated by methylation of cytosine and guanine.

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