A sensitive immunochemical method for detecting 5mC in DNA fragments

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Abstract

Modified nucleotides *Southern transfer* *Biotin–avidin cross-linking* *Gene expression* *X-chromosome inactivation*

1. INTRODUCTION

We report here the production of antibodies highly specific to 5-methyl cytosine (5mC), their characterization, and the development of a sensitive assay system for detecting 5mC in DNA using the biotin–avidin–peroxidase reaction and the method of Southern transfer. The sensitivity of the method is such that it permits the detection of 5mC in nanogram quantities of ϕX174 DNA which contains one 5mC residue in a genome of 5386 nucleotides.

Evidence is accumulating that during transcription of eukaryotic DNA, CpG sites in genes undergoing transcription are often undermethylated [1,2]. In some cases it is known that these undermethylated sites occur towards the 5′-end of the gene being transcribed, either within the coding sequence itself or in the 5′-flanking regions [3,4]. Undermethylation of these particular CpG sites appears to be a necessary but not sufficient condition for gene expression [5]. Incorporation of 5-azacytidine, an analog of cytidine, into DNA of growing cells leads to undermethylation [6]. When azacytidine is incorporated into mammalian cells, one of the consequences is reactivation of genes on the inactive X chromosome [7–9]. These observations lend further support to the idea that gene activity and undermethylation are in some manner interrelated.

Restriction enzymes which recognise the CCGG sequence and cleave DNA, but will not restrict if the internal cytosine is methylated, have been extensively used for characterization of the extent and pattern of cytosine methylation. These enzymes, however, fail to provide information on methylated cytosine occurring in dinucleotides other than CpG. Antibodies to 5mC, on the other hand, are potentially sensitive tools for identifying 5mC in DNA irrespective of the neighbouring nucleotide. Such antibodies, when used on DNA fragments immobilised on nitrocellulose paper by Southern tansfer, also provide a means of characterising the methylation status of DNA sequences of interest [10].

2. METHODS

5-Methyl cytidine was coupled to bovine serum albumin (BSA) using the periodate oxidation procedure [11,12]. The ribonucleoside was oxidised using sodium periodate at room temperature and then coupled to BSA at pH 9–9.5. The reaction product was reduced with sodium borohydride to form a stable nucleoside–protein conjugate. The conjugate was then extensively dialysed and characterised by spectral analysis which showed that, on the average, 21 molecules of 5-methyl cytidine were coupled/BSA molecule.

Rabbits were injected in the foot-pad and back

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muscle, at 10-day intervals, with 1–2 mg conjugate in complete Freunds' adjuvant. The serum was collected and frozen at −10°C after addition of sodium azide (final conc. 0.02%). The immunoglobulins were purified by precipitation with 50% ammonium sulphate followed by DEAE column chromatography [13]. Following Ouchterlony immunodiffusion, the antiserum reacted with 5mC–BSA and BSA but did not show any cross reaction with cytidine–RSA.

3. RESULTS AND DISCUSSION

To assess the specificity of the antigen–antibody (Ag–Ab) reaction, 5-methyl cytidine was labelled using ^{3}H-labelled sodium borohydride (347 mCi/mmol, from New England Nuclear) [14]. The labelled antigen was incubated with antibodies in Tris-buffered saline at 37°C for 10 min and filtered through pre-wetted nitrocellulose paper [12]. The amount of labelled antigen retained as antigen–antibody complex was determined. The pattern of binding of the hapten to antibody is shown in fig. 1 which is a reciprocal plot of concentration of the bound hapten *vs* that of the free hapten. The affinity of the antisera as calculated from the plot in fig. 1 is 1.56×10^9 M^{-1}. In other words, the antibody appears to detect as little as 0.9 pmol

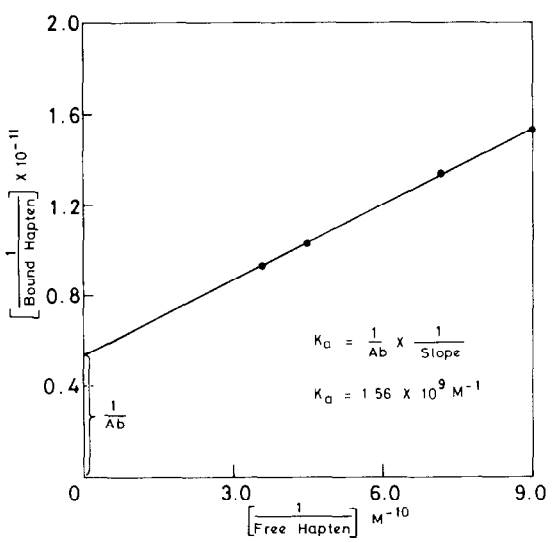

Fig. 1. Reciprocal plot of bound hapten *vs* free hapten. The assays were done as in section 2.

5mC (spec. act. 150 cpm/pmol). The ability of the antibodies to detect even smaller amounts of 5mC could not be verified because of the limitations imposed by the specific activity of labelled 5mC. Competition assays were also carried out using varying amounts of unlabelled nucleotides to determine the extent of cross reaction. Cytosine at 5.28×10^{-5} M does not inhibit the binding of 5mC to antibody. When [^{3}H]cytidine was used as the radioactive hapten no binding was detectable even when cytidine was 3.3×10^{-6} M ([^{3}H]cytidine obtained from BARC, Bombay; 1.5 Ci/mmol).

DNAs from the phages ϕX174 and λ, mouse and a mealybug, *Planococcus lilacinus* cockerell (Coccoidea; Homoptera; Insecta), were spotted on BA85 nitrocellulose paper (Schleicher and Schuell) and baked for 4 h and then incubated in the presence of antibodies to 5mC, also for 4 h. For detection of the antigen–antibody complex, anti-antibody (goat anti-rabbit IgG), which had been tagged to biotin, was used. This complex (Ag–Ab–anti Ab) was detected using avidin dH and biotinylated horseradish peroxidase. The peroxidase was visualised by using a substrate solution (H_2O_2, 0.02% and 0.1% diaminobenzidine tetrahydrochloride) [15,16]. The affinity of avidin (M_r 68000) for biotin is of the order of 10^{-15} M which is $>10^6$-times higher than the affinity of antibodies to most antigens. As a consequence, the binding of avidin to biotin is essentially irreversible [15,16].

Avidin has 4 binding sites for biotin, and most proteins can be conjugated with several molecules of biotin. Evidence suggests that many molecules of biotinylated horseradish peroxidase are cross-linked by avidin into three-dimensional arrays. This factor appears to facilitate the detection of very small amounts of antigen.

By use of this assay, 0.005 p 5-methyl deoxycytidine contained in 10 ng ϕX174 DNA could be detected (fig. 2a). Mealybug DNA and mouse DNA also showed a positive reaction (fig. 2b,c). Mouse DNA is known to have ~3 mol% 5mC [17], 95% of which occurs in the dinucleotide CpG. Dinucleotide analysis has shown significant amounts of 5mC (2.33 mol%) in mealybug DNA [18]. DNA from the phage λ, which is essentially devoid of 5mC, was not stained by the antibody at up to 500 ng DNA/assay.

Mouse DNA was digested with the restriction enzyme *Hpa*II, run on 0.8% agarose gel and blotted

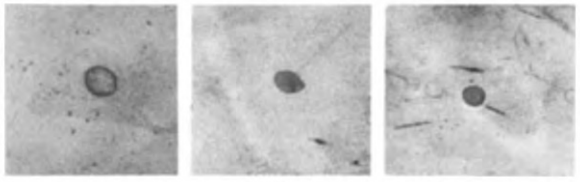

Fig. 2. DNAs from ϕX174 (a), mealybug (b) and mouse (c), spotted on nitrocellulose paper, incubated with antibodies to 5mC and stained by the biotin–avidin–peroxidase method.

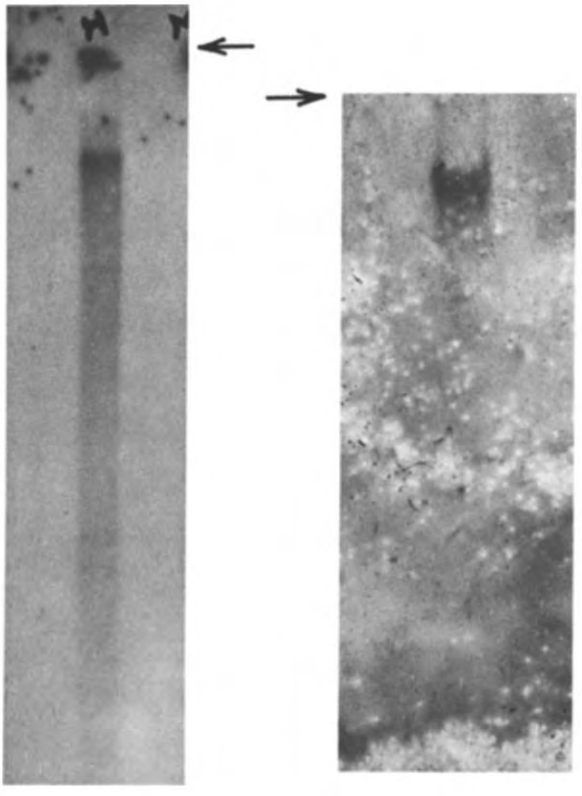

Fig. 3. *Hpa*II digest of mouse DNA labelled with T_4 polymerase (a), and stained with 5mC antibody and biotin–avidin–peroxidase (b). (a) Digest was labelled as described by O'Farrell [20]. Electrophoresis of the digest was carried out in 0.8% agarose (Tris 40 mM, acetate 20 mM, Na-EDTA 2 mM) and an autoradiogram was prepared. The *Hpa*II digestion for both (a,b) was done in 10 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT for 1 h. (b) Electrophoresis was again in 0.8% agarose followed by blotting on nitrocellulose paper [19]. The blot was stained with the antibody as in the text; (→) points of origin.

on BA85 nitrocellulose paper [19]. The blot was treated with the antibody, biotinylated anti-antibody and biotin–avidin peroxidase. A blot stained in this manner is shown in fig. 3. Mouse DNA digested with *Hpa*II was also labelled with ^{32}P using the T_4 polymerase technique [20] and run on 0.8% agarose gel. The T_4 polymerase method enables one to obtain high specific activity labelling without significant alteration in fragment size as would happen during nick translation. This approach was used to detect any low-M_r fragments which might go undetected by ethidium bromide staining because of poor stainability of such small fragments. An autoradiogram so obtained is also shown in fig. 3. DNA uncut by *Hpa*II (by virtue of CpG methylation in the recognition sequence CCGG) remains as a high-M_r band. This band, which is expected to have relatively high concentrations of 5mC, is strongly bound by the antibody and therefore stains darkly whereas the DNA digested by *Hpa*II is not so stained. The clear difference in stainability of those DNA fragments which remain uncut because of CpG methylation and the digested species demonstrates that this is a reliable method for detecting 5mC in DNA. The method thus appears to combine high specificity, sensitivity, low background, and rapid and irreversible staining.

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