Biomethylation of Deoxyribonucleic Acid in Cultured Human Tumour Cells (HeLa)

METHYLATED BASES OTHER THAN 5-METHYLCYTOSINE NOT DETECTED

By P. D. LAWLEY, A. R. CRATHORN, S. A. SHAH and B. A. SMITH
*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital,
Fulham Road, London SW3 6JB, U.K.*

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1. Incorporation of methyl groups from [methyl-¹⁴C]methionine into DNA of dividing HeLa cells was investigated, essentially by the procedures of Culp, *et al.* (1970).
2. Contrary to the report of the latter, but in agreement with other work on biomethylation of mammalian DNA, 5-methylcytosine was the sole methylated base detected.
3. Chromatographic separations of 3-methylcytosine from 5-methylcytosine and purines are discussed.

Methylation of DNA in mammalian cells by chemical mutagens and carcinogens has been studied extensively (for reviews see Lawley, 1966; Magee & Barnes, 1967), and the known sites of chemical attack by methylating agents are: in adenine, N-1, N-3 and N-7; in cytosine, N-3; in guanine, O-6 (Loveless, 1969; Lawley & Thatcher, 1970) and N-7 atoms. The sites of biomethylation of ribonucleic acids, in which process the proximate agent is *S*-adenosylmethionine, include some of the chemically reactive sites, but in DNA it has generally been considered that these sites are not involved and that C-5 of cytosine residues is the sole site of biomethylation (for a recent compilation of relevant references see Craddock, 1971).

Among biological test objects, the human tumour-cell line HeLa has often been used in alkylation studies (Roberts *et al.*, 1971). It had been assumed generally that the methylated bases detected in DNA after treatment of these cells with carcinogens derive from chemical methylation, not biomethylation. However, since the report by Culp *et al.* (1970), some doubt has been thrown on this assumption; these authors reported 3-methylcytosine and 7-methylguanine as products of biomethylation. These bases were detected by chromatography of acid hydrolysates of DNA isolated from HeLa cells grown in the presence of [methyl-¹⁴C]methionine.

Clearly these findings, if confirmed, would raise several questions about the significance of DNA methylations *in vivo*. For example, the known instability of 7-methyldeoxyguanosine residues to hydrolysis at neutral pH values (Lawley & Brookes, 1963) was taken to indicate that depurination of DNA through this mechanism would lead to cytotoxicity by inactivation of the template activity of DNA. Further, from work on the template pro-

perties of methylated poly(C), Ludlum & Wilhelm (1968) suggested that 3-methylcytosine residues in nucleic acids might cause mutations. If chemically induced methylation of these types in DNA were additional to biomethylation *in vivo*, the interpretation of their possible effects would thus be subject to some complication.

Examination of the results presented by Culp *et al.* (1970) revealed some discrepancies with respect to their identification of 3-methylcytosine. This was reported to be eluted together with thymine, and before cytosine, from a column of Dowex 50 (H⁺ form) eluted with HCl. On the other hand, previous work had shown that 3-methylcytosine was eluted immediately after cytosine, but not separated from it (Brookes & Lawley, 1962; Lawley & Thatcher, 1970).

It was therefore thought desirable to re-examine the problem of biomethylation of DNA in HeLa cells, with a view to resolving this apparent contradiction. Several alternative procedures for separation of methylation products in nucleic acids have been described in the preceding paper (Lawley & Shah, 1972) and it was therefore decided to apply some of these to the present problem.

Materials and Methods

Materials

[methyl-¹⁴C]Methionine (59 μ Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. 3-Methylcytosine was prepared by the method of Brookes & Lawley (1962) as the hydrochloride, and 3-methyldeoxycytidine by the method of Lawley & Brookes (1963) as the methosulphate. 5-Methylcytosine, 5-methyldeoxycytidine and *N*⁶-methyladenine were obtained from Sigma (London)

Chemical Co., London S.W.6, U.K. 7-Methylguanine was prepared from 7-methylguanosine, by the method of Jones & Robins (1963). *N*²-Methylguanine was prepared from *N*²-methylguanosine (Cyclo Chemicals, Los Angeles, Calif., U.S.A.).

Chromatographic methods

Paper chromatography was carried out on Whatman 3MM paper, with (1) methanol–conc. HCl–water (7:2:1, by vol.) or (2) butan-1-ol–water (17:3, v/v) as solvents. For t.l.c., Polygram Cel 300 UV (Camlab, Cambridge, U.K.) was used with propan-2-ol–aq. NH₃ (sp.gr. 0.88)–water (7:1:2, by vol.) as solvent. Methods for column chromatography are described in detail by Lawley & Shah (1972).

Growth and radioactive labelling of cells

The HeLa cells were obtained by cloning from a line provided by Professor H. Harris in 1964, and frozen stocks were kept in liquid N₂. Cells were grown in suspension culture at a doubling time of about 23 h in a 3 litre fermentor under conditions of controlled temperature (37.5 ± 0.5°C) and pH (7.0 ± 0.2). The culture medium was the minimum essential medium of Eagle (1959), modified for suspension culture by omitting CaCl₂ and increasing concentration of phosphates tenfold, obtained from Grand Island Biological Co., New York, N.Y., U.S.A. (cat. no. F14), plus NaHCO₃ (10.7 g/l), penicillin (2 × 10⁵ units/l), streptomycin (2 × 10⁵ units/l) and foetal bovine serum (Flow Laboratories, Irvine, Scotland) (7%, v/v).

For radioactive labelling, a culture (1.2 litres) at an initial density of 5 × 10⁴ cells/ml was incubated to give a density of 1.1 × 10⁵ cells/ml; adenosine, guanosine and thymidine were then added to a final concn. of 0.1 mM of each, and incubation was continued. After 2 h [*methyl*-¹⁴C]methionine (250 μCi) was added and growth was allowed to continue to a final concn. of 4.2 × 10⁵ cells/ml. Cells were examined microscopically and counted in an electronic counter (Coulter Electronics, Dunstable, U.K.; model F). Cells were then harvested by centrifugation (600g), washed twice with cold phosphate-buffered saline (Grand Island Biological Co.), and stored frozen.

Isolation of DNA

A modification of the method of Kirby & Cook (1967) was used. To the frozen pellet of cells was added a solution (5 ml) containing tri-isopropyl-naphthalene sulphonate (1%, w/v), sodium *p*-aminosalicylate (6%, w/v) in 6% (v/v) butan-2-ol. The mixture was shaken at room temperature until the cells had lysed, and then extracted with 1 vol. of phenol

reagent (phenol, 500 g; 8-hydroxyquinoline, 600 mg; water, 55 ml) by shaking and centrifuging. The aqueous layer was removed, re-extracted with phenol reagent and then added to 2-ethoxyethanol (1 vol.). The fibrous precipitate of DNA was washed with aq. 75% (v/v) ethanol (containing sodium acetate, 2%, w/v), and immediately redissolved in aq. sodium acetate (0.5%)–NaCl (0.37%) (5 ml). Ribonuclease (about 1 mg) was added, and after incubation of the mixture for 15 min at 37°C the DNA was reprecipitated with 2-ethoxyethanol, washed with ethanol and redissolved in sodium acetate (0.5%; 5.5 ml). The solution was assayed for radioactivity and the concentration of DNA estimated by using the value *E*₂₆₀^{1%} 193; the yield was 4 mg and the specific radioactivity 0.04 μCi/mg.

Measurement of radioactivity

Aqueous solutions, e.g. column fractions containing HCl (3.8 ml) or ammonium formate, (6.4 ml) were assayed by scintillation counting (Packard Tri-Carb 375 counter) by using a phosphor (10 ml) containing Triton X-100 (Lennig Chemicals Ltd., Jarrow, U.K.) (4 litres), toluene (6 litres), 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (Koch–Light Laboratories, Colnbrook, Bucks., U.K.) (3.5 g) and 2,5-diphenyloxazole (70 g); the efficiency was about 65% for ¹⁴C, and was not significantly lowered by HCl (3.8 ml) up to a concentration of 2.5 M.

Hydrolysis and analysis of DNA

A portion of the *methyl*-¹⁴C-labelled DNA (containing about 100 000 c.p.m.) was isolated from the solution (4 ml) by precipitation with 2-ethoxyethanol as described above and heated with HClO₄ (72%, w/v; 30 μl) for 1 h at 100°C; the hydrolysate was diluted to about 1 ml with water and clarified by centrifugation. Marker bases (200 μg) were added as follows: 3- and 5-methylcytosine, 7-methylguanine, *N*²-methylguanine, *N*⁶-methyladenine. One-half of the hydrolysate was applied to a column (10 cm × 1.5 cm) of Dowex 50 AG 50W (X4; H⁺ form; 400 mesh; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) eluted with a linear gradient of 0.75–2.5 M-HCl (300 ml of each), 3.8 ml fractions being collected. The residue was partially neutralized with 7 M-KOH to about pH 3, and the precipitate of KClO₄ removed by centrifugation; the solution was then applied to a column (80 cm × 1.5 cm) of Dowex 50 (NH₄⁺ form), eluted with 0.3 M-ammonium formate, pH 8.9. In another experiment, a hydrolysate containing 27 000 c.p.m. was neutralized with NH₃ and then applied to a column (85 cm × 1.5 cm) of Sephadex G-10, eluted with 0.05 M-ammonium formate, pH 6.8.

The residual DNA solution was treated with deoxyribonuclease (100 μg) for 1 h at 37°C, and then

adjusted to pH 8.0 with 2M-tris-HCl buffer, venom phosphodiesterase (Worthington Biochemical Corp. Freehold, N.J., U.S.A.) (100 μ g) and bacterial alkaline phosphatase (3 units) being added; incubation was continued for 16h, when t.l.c. of a portion (about 10 μ l) of the solution showed that conversion into deoxyribonucleosides was complete. Markers of 3- and 5-methyldeoxycytidine were added, and the digest was then applied to a column (60cm $\times$ 1.5cm) of Dowex 50 (NH_4^+ form) eluted with (i) 0.3M-ammonium formate (120 6.4ml fractions) and (ii) 1M-ammonium formate (60 fractions), at pH 8.9.

Results

Growth of HeLa cells for about two generations in the presence of [methyl- ^{14}C]methionine was accompanied by incorporation of labelled methyl groups into cellular DNA, as reported by Brown & Attardi (1965) and Culp *et al.* (1970). The concentration of methionine in the medium used in the present work is nominally 0.1 mM, so that the specific radioactivity of this amino acid in the medium was about 2.1 mCi/mmol. Assuming that the specific radioactivity of methyl groups in DNA was of this order, and that all of the methylation was in fact in DNA,

Fig. 1. Chromatogram of an enzymic digest of HeLa-cell DNA from cells grown in the presence of [methyl- ^{14}C]methionine

A column (80cm $\times$ 1.5cm) of Dowex 50 (NH_4^+ form) was used, eluted first with 0.3M-ammonium formate, pH 8.9, for 120 6.4ml fractions, and then with 1M-ammonium formate. (a) U.v. absorption in the fractions at 254nm; (b) radioactivity in the fractions. The deoxynucleosides derived from the DNA are: Thd, thymidine; dCyd, deoxycytidine; dGuo, deoxyguanosine; dAdo, deoxyadenosine. 5-Me-dCyd and 3-Me-dCyd represent methyldeoxycytidines added as markers.

the observed specific radioactivity of $13.5 \mu\text{Ci}/\text{mmol}$ of DNA P would correspond to an extent of methylation of about 6.8 mmol of methyl groups/mol of DNA P. This may be compared with the value 8.7 mmol/mol reported by Brown & Attardi (1965), derived from similar experiments.

Analysis of methyl- ^{14}C -labelled DNA by hydrolysis with HClO_4 and chromatography on Dowex 50 (H^+ form) eluted with HCl gave results closely similar to those reported by Culp *et al.* (1970). The principal feature was that the major part of the radioactivity (56%) was obtained in a single peak, eluted from the column coincident with the u.v. absorption due to added marker 5-methylcytosine; the peak occurred at a value R_{Ad} 0.48 (where R_{Ad} denotes fraction no. of elution of product/fraction no. of elution of adenine). But a considerable amount (35%) was eluted in earlier fractions not associated with any base, and a relatively small amount with thymine (3%). The u.v.-absorption peaks were not

symmetrical in this system, despite the use of a gradient of increasing concentration of HCl ; the possibility that some labelled purines were present in the 'tail' of the eluted peak of radioactivity could not be eliminated, but no defined peak of radioactivity was eluted after 5-methylcytosine. The presence of labelled 6-methylaminopurine, which in this system is the last base to be eluted (R_{Ad} 1.26), was definitely eliminated, within limits of about 0.2% of the total radioactivity (56000 c.p.m.).

Another unsatisfactory feature of this system is that 3-methylcytosine (R_{Ad} 0.44) is not separated from 5-methylcytosine (R_{Ad} 0.48) and methylguanines are not well separated (7-methylguanine, R_{Ad} 0.69; N^2 -methylguanine, R_{Ad} 0.6).

A satisfactory separation of these methylcytosines is achieved by degrading nucleic acids to the level of nucleosides, followed by chromatography on Dowex 50 (NH_4^+ form) (Lawley & Shah, 1972). For the present case the results of this separation are shown in

Fig. 2. Rechromatography of fractions containing methylcytosines derived from a chromatogram of a perchloric acid hydrolysate of methyl- ^{14}C -labelled HeLa-cell DNA

Portions of the fractions (a, 21–23; b, 24–26) from a chromatogram on Dowex 50 (H^+ form) eluted with HCl were evaporated, and the residues were rechromatographed on a column ($85 \text{ cm} \times 1.5 \text{ cm}$) of Sephadex G-10 eluted with 0.05 M -ammonium formate, $\text{pH} 6.8$. —, Radioactivity; --- (b only), u.v. absorption due to added marker bases, 3- and 5-methylcytosine (3-MeCyt and 5-MeCyt respectively).

Fig. 1. The major product was 5-methyldeoxycytidine (56%) and no radioactivity was found associated with the marker 3-methyldeoxycytidine, which was clearly separated from its isomer. Some radioactivity appeared with thymidine (4%) and in the region of elution of deoxyguanosine (7%). The remainder of the radioactivity was eluted before thymidine.

To separate the bases 3- and 5-methylcytosine, Sephadex G-10 eluted with 0.05 M-ammonium formate was investigated as a possibly suitable system. Fig. 2 shows the results obtained when the peak fractions containing the methylcytosines were evaporated and the residues applied to this type of column. No radioactivity was found to be associated with 3-methylcytosine.

When a whole acid hydrolysate of DNA was examined in this system (Fig. 3), two peaks of radioactivity were obtained. Although the 'tail' of the first peak (fractions 13–14) was near to the position of elution of marker 3-methylcytosine (fractions 15–16)

Fig. 3. Chromatogram of a perchloric acid hydrolysate of methyl- ^{14}C -labelled HeLa-cell DNA

The hydrolysate was neutralized with ammonia and applied to Sephadex G-10 eluted with 0.05 M-ammonium formate. The positions of elution of u.v. absorption due to normal bases guanine and adenine, and added marker methylated bases, are shown by arrows; the normal pyrimidines eluted near 5-methylcytosine and could not be distinguished in this system. 3-MeCyt and 5-MeCyt, 3- and 5-methylcytosine respectively; Gua, guanine; Ade, adenine, N^6 -MeAde, N^6 -methyladenine; 7-MeGua, 7-methylguanine; N^2 -MeGua, N^2 -methylguanine.

it was not coincident with it. The major peak (53%) of radioactivity was coincident with the u.v. absorption of marker 5-methylcytosine. Added markers of 7-methylguanine, N^2 -methylguanine and N^6 -methyladenine were eluted in the later fractions, but contained no radioactivity.

Separations of methylcytosines by paper chromatography were not satisfactory. The R_F values of 3-methylcytosine were 0.66 in solvent (1) and 0.32 in solvent (2); those of 5-methylcytosine were 0.65 in solvent (1) and 0.31 in solvent (2).

These methylcytosines were, however, separated on Dowex 50 (NH_4^+ form) at pH 8.9, the R_{Ad} values being 0.74 for 5-methylcytosine and 0.90 for 3-methylcytosine. This system also showed that, apart from thymine (3% of total radioactivity), 5-methylcytosine was the sole identified labelled base (56%), the remainder of the radioactivity being eluted before thymine.

Discussion

The present work shows that extensive radioactive labelling of HeLa-cell DNA can be achieved by growing cells through about two generations in the presence of [methyl- ^{14}C]methionine, but that only a part (56%) of this is present in an identified methylated base, i.e. 5-methylcytosine. A small proportion (about 3%) of the radioactivity was recovered in thymine. The residue was found in unidentified fragments eluted from chromatographic columns before any base or nucleoside derived from DNA.

The apparent extent of methylation of the DNA could be calculated from the known amount of radioactivity used and the composition of the medium. After allowing for the proportion of radioactivity in the DNA preparation not associated with the identified methylated base, the value 3.7 mmol of 5-methylcytosine/mol of DNA P is obtained. This is about one-third of the value consistently reported for other mammalian DNA species (cf. Shapiro, 1968). A probable cause of the discrepancy is the uncertainty in the factor of dilution of the radioactive methionine.

This estimate is not, of course, the principal reason for using this method for detection of methylated bases in DNA; the purpose was to identify the nature of these bases. No significant (i.e. greater than about 0.2% of the total) amount of any methylated base other than 5-methylcytosine was found.

Three chromatographic systems used here sufficed to separate the methylcytosines, two as bases and one as deoxynucleosides. The commonly used system of chromatography on Dowex 50 (H^+ form) was not satisfactory for separation of these bases. But they could be separated on Dowex 50 (NH_4^+ form), eluted with 0.3 M-ammonium formate at pH 8.9 (a system devised for nucleoside separation by Junowicz

& Spencer, 1969), and on Sephadex G-10 eluted with 0.05M-ammonium formate, pH 6.8. The deoxynucleosides were well separated on the Dowex 50 (NH_4^+ form) system, because of the considerable difference in their pK'_a values (5-methyldeoxycytidine, pK'_a about 4.3, and 3-methyldeoxycytidine, pK'_a about 8.7). The higher pK'_a of the latter ensured its relatively greater retention on the column at pH 8.9. For its elution, the more concentrated 1M-ammonium formate was required; this was achieved within a comparatively small volume of eluent, and relatively low extents of methylation of nucleic acids at the N-3 position of cytosine can be detected by this method (Lawley & Shah, 1972).

Although the identification of the biomethylated base in DNA was thus conclusive, the occurrence of unidentified labelled material in the preparation was clearly unsatisfactory. Similar results have been found in studies of chemical methylation of DNA in cells, when the extent of methylation is low in comparison with the extents of methylation of non-nucleic acid macromolecular cellular constituents (P. D. Lawley & D. J. Orr, unpublished work). A possible explanation is therefore that adsorption of labelled cellular material to DNA had occurred during the extraction of the nucleic acid. This question requires further investigation.

Some comment should perhaps be made about the discrepancies between the present work and that of Culp *et al.* (1970), whose results from analogous experiments were in fact broadly similar to those obtained here. It seems inescapable that the identification by Culp *et al.* (1970) of 3-methylcytosine as a product of biomethylation was mistaken. This base is not, as reported by these authors, eluted together with thymine in the system they used, but together with and between cytosine and 5-methylcytosine. Their identification of labelled methylguanines in DNA was also not confirmed by the present work, as shown particularly in Fig. 3, although chemical methylation

to yield 7-methylguanine in nucleic acids can be detected readily by this system (Lawley & Shah, 1972).

It seems therefore that the typical methylation products induced in DNA by chemical alkylation are not present in HeLa-cell DNA, where the sole biomethylated base is 5-methylcytosine.

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