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Short Communication

Quantitation of 5-methylcytosine by one-dimensional high-performance thin-layer chromatography

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ABSTRACT

A method for the quantitative analysis of DNA 5-methylcytosine by one-dimensional high-performance thin-layer chromatography using alkylamino modified silica (HPTLC-NH_2) plates is described. The preparative method is simple, involving enzymatic digestion of DNA with micrococcal nuclease and phosphodiesterase II to 3′-monophosphate nucleosides, conversion by T_4 polynucleotide kinase to ^{32}P-labeled 3′,5′-bisphosphate nucleosides, and chromatographic separation of nuclease P_1-cleaved 5′-monophosphate nucleosides. The weak, basic anion exchanger property of the HPTLC-NH_2 plate enables separation of multiple samples in one dimension, whereas traditional polyethyleneimine cellulose plates require development of individual samples in two dimensions for analysis of 5-methylcytosine.

INTRODUCTION

The presence of 5-methylcytosine (5mC)[a] in nucleic acids was first described in *Mycobacterium tuberculosis* by Johnson and Coghill [17] in 1925. Since then, many studies have sought to determine its biological role, resulting in a variety of methods for the analysis of this minor base. Past techniques for measuring 5mC required sophisticated instrumentation such as high-performance liquid chromatography [1,2], gas chromatography-mass spectrometry [3,4], or antibodies specific for 5mC [5,6]. Other procedures utilizing thin-layer chromatography coupled with a postlabeling technique [7,8] required development in two dimensions such that only a single sample could be analyzed on one plate. Using HPTLC-NH_2 plates [9], we have developed a one-dimensional technique for 5mC determination that permits the simultaneous analysis of ten samples. In contrast to HPLC, this method allows the analysis of large batches of samples in a shorter period of time. The results obtained through this method are comparable with several HPLC methods and are within the range of previously published results for V79 cells [10]. Since changes in DNA methylation represent an early marker for neoplastic transformation [11] and exposure to cytotoxic drugs [12,13], this procedure could facilitate the screening of large

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[a] Abbreviations: 5mC = 5-methylcytosine; HPTLC = high-performance thin-layer chromatography; 5mdCMP = 5-methyl-2′-deoxycytidine 5′-monophosphate; dCMP = 2′-deoxycytidine 5′-monophosphate; dAMP = 2′-deoxyadenosine 5′-monophosphate; dGMP = 2′-deoxyguanosine 5′-monophosphate; dTMP = thymidine 5′-monophosphate; dH_2O = deionized distilled water.

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numbers of patients or monitoring the effects of drug treatment upon DNA methylation.

EXPERIMENTAL

Materials

Isobutyric acid and 5′-monophosphate nucleosides 5mdCMP, dCMP, dAMP, dGMP, dTMP, ATP and TLC chambers (inside dimensions 121 mm × 108 mm × 83 mm) were obtained from Sigma (St. Louis, MO, USA) as were the enzymes micrococcal nuclease and phosphodiesterase II. T_4 polynucleotide kinase and nuclease P_1 were purchased from Boehringer Mannheim (Indianapolis, IN, USA). Glass-based HPTLC plates, 10 × 10 cm, coated with silica gel NH_2 F254s were obtained from EM Separations (Gibstown, NJ, USA). Radiolabeled [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from DuPont (Boston, MA, USA) and [2-^{14}C]deoxycytidine (45-55 mCi/mmol) was from Moravek Biochemicals (Brea, CA, USA) Kodak Diagnostic Film X-Omat RP was purchased from Eastman Kodak (Rochester, NY, USA).

Cell line

V79 Chinese hamster cells were a kind gift of Morgan Harris, University of California, Berkeley, CA, USA. Cells were grown in Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% dialyzed fetal bovine serum (JRH Biosciences, Lenexa, KS, USA).

DNA preparation

Total cellular DNA was extracted using a modified Marmur procedure as previously described [14]. Only DNA samples with an A_{260}/A_{280} ratio of 1.8 were used since protein contamination was found to result in poor chromatographic separation. For each sample, 6 μg of DNA was precipitated using 1/50th volume of 5 *M* sodium chloride and two volumes of ethanol at −20°C.

Nucleic acid digestion and kinase reactions

DNA (6 μg) from each sample was digested into 3′-monophosphate nucleosides with micrococcal nuclease (5 units) and phosphodiesterase II (0.25 units) for 3 h at 37°C in 30 μl of 2.5 m*M* Tris pH 8.8 containing 2.5 m*M* $CaCl_2$. The 3′-monophosphate nucleosides were converted to ^{32}P-labeled 3′,5′-bisphosphate nucleosides by reacting 5 μl of the above digest with T_4 polynucleotide kinase (5 units) and 25 μCi of [γ-^{32}P]ATP in 50 m*M* Tris pH 7.6, 10 m*M* $MgCl_2$, 5 m*M* dithiothreitol, 10 μM ATP, and 1 m*M* spermidine in a total volume of 25 μl for 3 h at 37°C. The labeled 3′,5′-bisphosphate nucleosides were then converted to 5′-[^{32}P]monophosphate nucleosides by adding nuclease P_1 (5 units) in a volume of 5μl of 0.6 m*M* $ZnCl_2$ (for a final concentration of 0.1 m*M* $ZnCl_2$) for 1 h at 70°C. This enzymatic hydrolysis is essentially identical to that published by Wilson *et al.* [8]. The digests were then frozen at −20°C until HPTLC analysis.

HPTLC separation and quantitation of the 5′-monophosphate nucleosides[a]

Using a 10 × 10 cm glass HPTLC-NH_2 plate with fluorescent indicator, unlabeled dCMP and 5mdCMP were first applied (1 and 2 μl of 1 mg/ml stocks, respectively) to facilitate the detection of the nucleotides of interest. After centrifuging the digest at 10 000 *g* for 10 min, 0.5 μl of the labeled sample was applied over the standards. The plate was developed in isobutyric acid:dH_2O:NH_4OH (17.7:8.0:0.3, v/v) until the solvent front was within 2 mm of the top of the plate. The plate was then dried to completeness (under vacuum at 80°C for 30 min). The plate was again developed in the same dimension in fresh solvents, dried and exposed to X-ray film for ten minutes for sample evaluation. The dCMP and 5mdCMP spots were identified (Fig. 1A) by UV-induced fluorescence (254 nm). Spots corresponding to the standards were marked on the glass side of the plate, excised with a diamond scribe and subjected to scintillation counting with aqueous cocktail. The 5mC content of the DNA was determined from the radioactivity found in the dCMP and 5mdCMP

[a] While we found this HPTLC technique useful for the analysis of 5′-monophosphate nucleosides, the 3′-monophosphate nucleosides did not produce sufficient resolution for analysis.

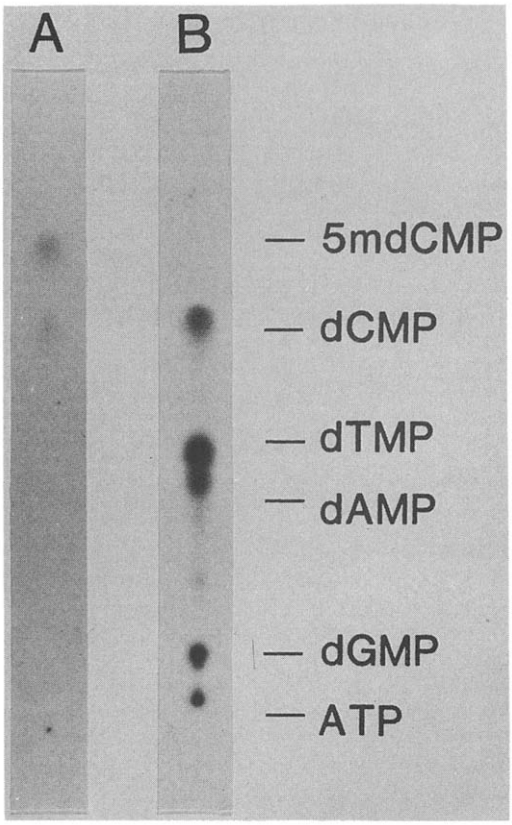

Fig. 1. (A) Postlabeled V79 cell DNA digest on HPTLC-NH_2 after one-dimensional development in isobutyric acid–dH_2O–NH_4OH (17.7:8.0:0.3, v/v/v). The dCMP and 5mdCMP standards were detected under UV light (254 nm). (B) Autoradiogram of the above lane. R_F values are: ATP, <0.01; dGMP, 0.05; dAMP, 0.26; dTMP, 0.29; dCMP, 0.45; 5mdCMP, 0.54.

using the following equation: % 5mC = [5mdCMP/(5mdCMP + dCMP)] × 100.

HPLC analysis

For the purpose of comparison, 5mC content of V79 cells was also determined by treating log phase cells with [2-^{14}C]deoxycytidine and the DNA was analyzed as described previously [16]. Briefly, V79 cells were exposed to [2-^{14}C]deoxycytidine for 24 h, and DNA was purified and subsequently hydrolyzed to free bases in 88% formic acid for 30 min at 180°C. Formic acid was removed by evaporation under a stream of nitrogen gas and each sample was taken up in a small volume of 0.1 *M* HCl. Using a Bio-Rad HPLC system, the labeled bases were separated on an Aminex A-9 column (Bio-Rad) at 60°C using 0.2 *M* ammonium acetate pH 5.5 at a flow of 1.0 ml/min. Fractions were collected at 30-s intervals and assessed for radioactivity by scintillation counting as above.

RESULTS AND DISCUSSION

The HPTLC-NH_2 plate offers an alternative procedure for the separation of 5′-monophosphate nucleosides. This one-dimensional method, which allows up to ten samples to be analyzed per plate, permits reproducible determination of the percent of 5mC in total DNA. The postlabeling procedure and HPTLC conditions resulted in high incorporation of label and adequate separation of product so that a 0.5 μl application gave average counts of 155 000 and 2300 dpm against a background of <100 dpm for dCMP and 5mdCMP, respectively. The percent of 5mC in total V79 cell DNA as determined by postlabeling and HPTLC was 1.50 ± 0.05. As seen in Table I, this compares favorably with HPLC values reported earlier by Citti *et al.* [10], and with values obtained in our laboratory. As can be seen by the autoradiogram in Fig. 1B, the four major nucleotides dCMP, dTMP, dAMP and dGMP are present in sufficient amounts for quantitation by standard densitometric methods. However, since 5mC normally represents only a small percentage of the total cytosines in mammalian DNA, it was not possible to produce a sufficient exposure of the 5mdCMP spot without

TABLE I

5mC CONTENT OF V79 CELL DNA

Method	%5mC ± standard error
HPTLC	1.50 ± 0.05[a]
HPLC	1.42 ± 0.20[b]
HPLC	1.52 ± 0.04[c]

[a] $n = 4$, mean of means.
[b] Results from Citti *et al.* [10], number of determinations unknown.
[c] Results from V79 cell DNA prelabeled with [2-^{14}C]deoxycytidine, $n = 4$.

saturating the film over the other nucleotide spots.

The limits of this one-dimensional HPTLC technique have yet to be fully defined. We have measured 5mC levels of clinical tumor specimens from patients receiving various chemotherapy agents which tend to alter methylation levels over a wide range. Concentrations of 5mC measured in these samples ranged between 1.0 and 8.5% of total cytosines (data to be published elsewhere). The method reported here thus produces reliable quantitation over at least this range of 5mC concentrations, which encompasses values which could be reasonably expected in mammalian DNA. With the emergence of storage phosphor technology, the overall sensitivity and accuracy could likely be increased [15].

CONCLUSIONS

HPTLC using alkylamino-modified silica plates offers a simple, practical method for the determination of 5mC content in microgram quantities of postlabeled DNA. This technique is different from earlier two-dimensional TLC procedures since the HPTLC-NH_2 plate offers the advantage of multiple sample analysis on a single plate.

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