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Hemimethylated duplex DNAs prepared from 5-azacytidine-treated cells

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ABSTRACT

Duplex heavy-light (HL) DNAs synthesized in the presence of BrdUrd and methylation inhibitors were separated from bulk cellular DNA by CsCl density gradient centrifugation and analysed for 5-methylcytosine (5mC) contents by HPLC. DNAs synthesized in the presence of 5 mM ethionine or 2 mg/ml cycloleucine were not detectably hypomethylated, whereas the DNA synthesized in the presence of 2-10 μM 5-azacytidine was undermethylated with respect to control DNA. The heavy, or H-strand, in which up to 5% of the cytosine residues were replaced by intact 5-azacytosine, was undermethylated and the HL duplex DNA was therefore strand asymmetrically methylated. This duplex DNA served as an efficient substrate for a crude DNA methyltransferase preparation which transferred the methyl group from S-adenosylmethionine specifically into cytosine residues within the hypomethylated H strand. Increasing levels of incorporated 5-azacytosine inhibited the action of the methyltransferase suggesting that incorporation of 5-azacytosine into DNA may be responsible for the inhibitory effect of 5-azacytidine on DNA methylation.

INTRODUCTION

There is increasing evidence that DNA methylation may play an important role in controlling eucaryotic gene expression (1,2). Several studies have demonstrated that specific sequences are hypomethylated in cells actively expressing certain genes, whereas the same sequences are methylated in cells inactive in the expression of those genes. This relationship has been found for chicken, rabbit and human globin genes (3-6), the ovalbumin and conalbumin genes (7) and also for viral genes (8,9). Agents which induce a hypomethylation of cytosine residues in DNA also induce changes in gene expression, suggesting that there may be a cause and effect relationship between DNA modification and cellular

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differentiation. Thus 5-azacytidine (5-aza-CR) and other analogs of cytidine modified in the 5-position, induce the formation of muscle cells from non-muscle precursors (10-12), and inhibit the methylation of newly-synthesized DNA (13). 5-Aza-CR has recently been used to reactivate an inactive human X-chromosome (14), and Christman *et al*. (15,16) have proposed that the abilities of dimethylsulfoxide and ethionine to induce Friend cell differentiation may be linked to induction of DNA hypomethylation.

The models of Riggs (17) and Holliday and Pugh (18), which proposed that DNA methylation might play a role in gene expression, also suggested how the modification pattern present in one cell might be semi-conservatively copied in the daughter cells after division. Central to these hypotheses was the proposal that the "maintenance methylases" present in cells would only be capable of the modification of hemimethylated sites. The enzymes would transfer the activated methyl group from S-adenosylmethionine (SAM) to the cytosine residue opposite to, and only opposite to, a methylated CG doublet to ensure the heritability of the pattern. The substrate for the enzyme would therefore be newly-synthesized hemimethylated DNA containing the sequence $\begin{smallmatrix}\text{-mC--G-}\\ \text{--G--C-}\end{smallmatrix}$ and the product would be symmetrically methylated, i.e., $\begin{smallmatrix}\text{-mC--G-}\\ \text{--G-mC-}\end{smallmatrix}$.

Considerable data indicates that such a mechanism operates within cells, since hemimethylated sites do not occur in DNA (19) and the occurrence of tissue-specific methylation patterns implies heritability. Progress in understanding the action of these methylases, which play fundamental roles in the control of gene expression, has thus far been hampered by the lack of adequately defined hemimethylated DNA substrates. DNA extracted from eucaryotic sources would be expected to contain symmetrically methylated modification sites (19) whereas that obtained from procaryotes would either contain no 5mC (and therefore no hemimethylated sites) or symmetrically modified sequences. To overcome these problems, other investigators have either used eucaryotic DNA which was repaired *in vitro* in the absence of methylase (20) or have extracted DNA from ethionine-treated cultures (15) or from cells dividing in the absence of methionine (21) as substrates for an *in vitro* methylation assay.

In this study we have utilized the ability of 5-aza-CR to induce the hypomethylation of newly synthesized DNA (13) to prepare such substrates. DNAs synthesized in the presence of BrdUrd and differing

concentrations of 5-aza-CR were isolated from bulk DNA by CsCl buoyant density gradient centrifugation. These DNAs were characterized with respect to the 5-azacytosine content, the condition of the 5-azacytosine ring and 5mC content. The hemimethylated DNAs accepted methyl groups in an *in vitro* assay specifically into the hypomethylated strand suggesting that the test tube reaction reflected the specificity of the methyltransferase within the cell. The results also provided direct evidence that the presence of the 5-azacytosine ring in DNA inhibits DNA methylation.

MATERIALS AND METHODS

General. Transformed mouse embryo cells (MCA Cl-15-C), which are a derivative of the C3H/10T½CL8 line (22), were used for DNA preparations because they grew to higher cell densities than the parental line. These cells can be induced to form myotubes by 5-aza-CR (12) and were propagated in 850 cm^2 roller bottles in Eagle's basal medium (GIBCO) containing 10% heat-inactivated fetal calf serum and penicillin and streptomycin. Roller bottles (Falcon Plastics, Oxnard, CA), containing actively-growing MCA Cl-15-C cells, were treated with potential methyltransferase inhibitors in medium containing 5 µg/ml of 5-bromodeoxyuridine (BrdUrd) in a darkened 37°C room. Four roller bottles were used per treatment and the cells were harvested by trypsinization after a 20 hr exposure time. The cell pellets (approximately $1\text{-}2 \times 10^8$ cells) were then suspended in SSC (0.15 M NaCl, 0.015 M sodium citrate) for DNA extraction.

Cesium Chloride Density Gradient Centrifugation. DNA was extracted from cell pellets by a modification of the Marmur (23) method as previously described (13). The DNA was sheared by 5 passages through a 25 gauge hypodermic needle, mixed with saturated CsCl and the refractive index adjusted to 1.4030. The final DNA concentration was approximately 75 µg/ml and the solution was centrifuged for 48 hr at 36,000 rpm in an SW 50.1 rotor (Beckman Instruments, Fullerton, CA) at 20°C. The centrifuge tubes were pierced with hypodermic needles (20 G) and 20 consecutive fractions of 6 drops each collected. DNA was located by its absorbance at 260 nm and the heavy-light (HL) fraction with the highest absorbance and 2-3 fractions more dense than it, were pooled as potentially hemimethylated DNA. Similarly, the light-light (LL) peak fraction and the 2 fractions less dense than it, were pooled and represented DNA which had

not replicated during exposure to BrdUrd. The pooled fractions were dialysed against 10% SSC (0.015 M NaCl, 0.0015 M sodium citrate) and stored at -80°C.

5-Methylcytosine Determinations. DNA samples were dialysed extensively against 0.01 M ammonium acetate, lyophilized and hydrolysed in 88% formic acid for 25 min at 180°C. Hydrolysates were dissolved in 0.1 M HCl and the bases separated on a Whatman Partisil SCX column fitted to a Spectra-Physics SP-8000 high performance liquid chromatograph. The mobile phase was 0.1 M KH_2PO_4 pH 2.5 and the bases were detected by their absorbance at 280 nm. The areas underneath the absorbance curves were integrated electronically and the 5mC/5mC+C ratios determined from a calibration curve constructed as described by Singer et al. (24).

Preparation of DNA Methyltransferase. Nuclei were prepared from the spleens of female BDF_1 mice essentially as described by Simon et al. (25) except that 1 mm dithiothreitol (DTT) and 10 mM Tris-HCl pH 7.8 were included in all buffers and nuclei were centrifuged through a 2.4 M sucrose cushion in a Beckman SW 27 rotor at 18,000 rpm for 90 min. Purified nuclei were extracted with 0.8 M KCl, 1 mM DTT, 50 mM Tris-HCl pH 7.8 for 30 min at 4°C with stirring. The KCl concentration was lowered to 0.3 M by the addition of 10 mM Tris-HCl, 1 mM DTT and extraction was continued for a further 30 min. The insoluble chromatin was sedimented at 20,000 g for 15 min, enzyme activity remained in the supernatant.

Enzyme Assays. Assays were carried out in a final volume of 80 µl containing 50 mM Tris-HCl pH 7.8, 5 mM EDTA, 0.5 mM DTT, 5 µM S-[methyl-^{3}H] adenosyl-methionine (2 µCi/assay, New England Nuclear, 15 Ci/mmol), 1.0 µg DNA and crude enzyme (approximately 10 µg protein/assay). Tubes were incubated for 30 min at 37°C and the reaction terminated by incubating for 20 min at 60°C in the presence of 60 µg pronase (self digested for 2 hr at 37°C). Carrier salmon sperm DNA was added (20 µg/assay) and the DNA precipitated in 10% trichloroacetic acid (TCA) for 10 min at 4°C. The precipitate was washed twice with cold 5% TCA, redissolved in 0.5 ml 0.5 M NaOH and heated at 65°C for 20 min. DNA was reprecipitated as above, washed extensively with cold 5% TCA and hydrolysed in 5% TCA at 100°C for 30 min. Remaining insoluble material was removed by centrifugation and the radioactivity in the supernatant determined after the addition of 5 ml Biofluor (New England Nuclear, Boston, Mass.).

Product Analysis. DNA which had been methylated in vitro, was mixed with a small quantity of untreated DNA containing ^{14}C-cytosine to act as a marker, dissolved in 10% SSC and made 0.1 M with respect to NaOH. An equal volume of saturated Cs_2SO_4 dissolved in 0.1 M NaOH was added, the refractive index adjusted to 1.3750 and the solution centrifuged at 36,000 rpm for 48 hr at 20°C in the SW50.1 rotor. The radioactivity in consecutive 6 drop fractions was determined following precipitation with TCA and filtration on Whatman GF/C filters.

The DNA product was also hydrolysed in 70% $HClO_4$ at 60°C, neutralized with KOH at 4°C, centrifuged and the bases separated by 2-dimensional chromatography on cellulose plates (E. Merck, Darmstadt, Germany) in the presence of unlabelled carrier 5mC and thymine. The solvent systems used were isopropanol: HCl:water (65:16.7:18.3) in the first dimension and n-butanol:methanol:water:NH_3(60:20:20:1) in the second dimension (26). Bases were located under UV light, extracted with 0.1 N HCl at 37°C for 2 hr and radioactivity determined after the addition of 5 ml Biofluor. The same method was used to recover ^{14}C-5-azacytosine from DNA. Intact ^{14}C-5-azacytosine was located by comigration with excess unlabelled 5-azacytosine (Sigma) and other radioactive products detected by autoradiography on Kodak X-ray film.

Extent of Substitution of 5-azacytosine for cytosine. Cultures were treated for 20 hr with various concentrations of 5-azacytidine-4-^{14}C (53mCi/mmol, Stanford Research Institute). Incorporation of labelled analog into DNA was determined following hydrolysis in 0.3 N KOH for 24 hr at 37°C. Replicate dishes (5 per treatment) were pooled for DNA determinations by the method of Abraham et al (27).

RESULTS

Isolation and Characterization of Hemimethylated DNA.

Duplex DNA replicated in the presence of 5-aza-CR or other potential methyltransferase inhibitors and BrdUrd was separated from unreplicated DNA by CsCl buoyant density gradient centrifugation (Fig 1). DNA-containing fractions with a density greater than 1.715 gm/cc were pooled and contained heavy-light (HL) or potentially hemimethylated duplex DNA. Those fractions which contained DNA with a density of less than 1.6780 gm/cc were also pooled and represented light-light (LL) parental DNA which had not replicated during the 20

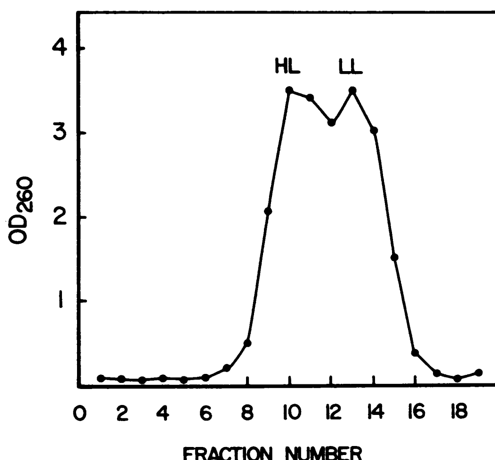

Figure 1. Neutral CsCl equilibrium centrifugation of DNA extracted from MCA Cl-15-C cells treated for 20 hr with BrdUrd (5 µg/ml) and 5-aza-CR (3 µM). The bottom of the gradient is to the left.

hr treatment with BrdUrd. This duplex DNA would be expected to be symmetrically methylated and served as a useful control for 5mC determinations and methyltransferase assays.

The 5mC contents of these DNAs were estimated by high performance liquid chromatography on Whatman Partisil SCX columns after acid hydrolysis (Fig 2). 5-Methylcytosine (retention time 612 sec.) was well separated from cytosine (retention time 371sec.) and the areas under the curves were integrated electronically. The contents of 5mC in LL or HL DNAs prepared from control cultures or cultures exposed to ethionine or cycloleucine did not differ significantly from each other (Table 1). These general inhibitors of SAM metabolism were therefore not efficient in the production of hemimethylated DNAs.

The HL DNAs synthesized in the presence of 5-aza-CR, on the other hand, showed dose-dependent decreases in the levels of 5mC. Since the parental light strands in these HL DNAs would be expected to contain the normal levels of 5mC, we also calculated the percentage decrease in 5mC contents of the H strands (i.e., the strands synthesized in the presence of 5-aza-CR). These values, shown in Table 1, were in fairly close agreement with our earlier determinations for the percentage inhibition of DNA methylation using radioactive precursors (13). The HL DNAs were therefore hemimethylated and were characterized further for levels of 5-

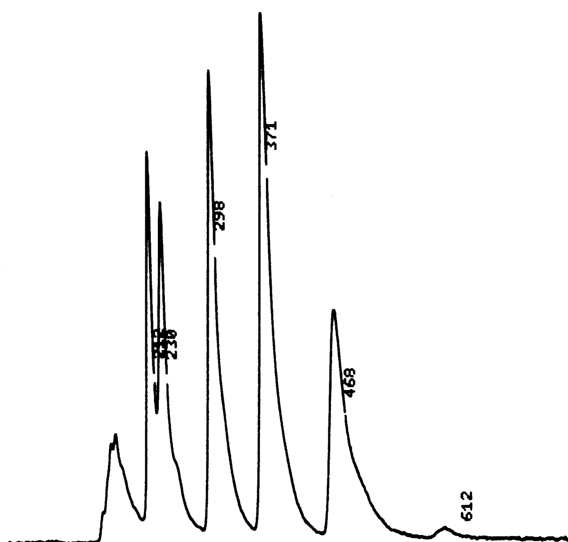

Figure 2. High performance liquid chromatogram on Partisil SCX of formic acid hydrolysate of HL DNA extracted from cells treated with 5-aza-CR (3 μM) in the presence of BrdUrd. Retention times are printed in seconds and correspond to bromouracil (212), thymine (230), guanine (298), cytosine (371), adenine (468) and 5-methylcytosine (612). The bases were detected by their absorbance at 280 nm and the areas under the curves integrated electronically.

azacytosine and methyl-accepting abilities. Table 1 also shows that with the exception of the LL DNA extracted from cells treated with 10 μM 5-aza-CR, the LL DNAs were not measurably demethylated with respect to control DNAs.

Methyl-Accepting Abilities of DNA Substrates

The LL and HL DNAs extracted from cells treated with ethionine, cycloleucine or 5-aza-CR were tested for their abilities to accept ^{3}H-methyl groups from SAM in the presence of a crude mouse spleen extract (Table 2). The LL DNAs extracted from control cultures or cultures treated with the methylase inhibitors, were less efficient than the corresponding HL DNAs in accepting methyl groups *in vitro*. DNA extracted from E. Coli B, which does not contain 5 mC (28), accepted 44.8 pmoles of CH_3/mg DNA/hr under the same assay conditions (results not shown) suggesting that there was little *de novo* methylation activity in the spleen extract.

Table 1. 5-Methylcytosine Contents of DNA Substrates.

Treatment	$\frac{5mC}{5mC+C}$ x 100 LL DNA	HL DNA	5mC in H Strand % Control
None	3.25	3.36	100%
Ethionine (5mM)	3.51	3.30	97%
Cycloleucine (2mg/ml)	3.50	3.50	108%
5-Aza-CR (2μM)	3.24	2.36	38%
5-Aza-CR (3μM)	3.35	2.08	21%
5-Aza-CR (10μM)	2.83	1.70	0%

DNAs prepared from cultures treated with the indicated compounds in the presence of BrdUrd were separated into heavy-light (HL) and light-light (LL) fractions by CsCl density gradient centrifugation. The ratio of 5mC/5mC+C in these DNAs was determined by HPLC and the percentage decrease in the 5mC content of the H strand calculated. Results given are for the means of 2-4 separate HPLC determinations.

The HL DNAs prepared from cells treated with ethionine or cycloleucine demonstrated higher methyl-accepting abilities with the crude methyltransferase than the HL DNA extracted from control cells. Thus, although we had not been able to measure a decrease in the 5mC content of these substrates (Table 1) a low number of hemimethylated sites must have been generated in the newly synthesized DNA by exposure to ethionine or cycloleucine.

The HL DNAs synthesized in the presence of 5-aza-CR, which contained decreased levels of 5mC (Table 1), were very efficient acceptors of ^{3}H-methyl groups and the accepting abilities of the DNAs were lost on DNA denaturation (Table 2). However, the HL DNA synthesized in the presence of high concentrations (10 μM) of 5-aza-CR was a less efficient substrate for the methyltransferase than the DNA synthesized in cultures treated with lower analog concentrations. This difference between the efficiencies of the substrates was also maintained under conditions where the DNA was in excess (Fig. 3). Under these conditions, the addition of 100 μM 5-aza-CR directly to the *in vitro* assay had no effect on the

Table 2. Methyl Accepting Abilities of DNA Substrates.

Treatment	pMoles CH_3 incorporated/mg DNA substrate/hr		
	LL DNA	HL DNA	Denatured HL DNA
None	109.3	209.5	82.6
Ethionine (5mM)	93.3	492.6	N.D.
Cycloleucine (2mg/ml)	43.8	234.4	N.D.
5-Aza-CR (0.5μM)	148.4	443.5	N.D.
5-Aza-CR (2.0μM)	572.6	1141.4	77.0
5-Aza-CR (3.0μM)	664.4	1480.3	71.1
5-Aza-CR (10μM)	231.1	837.2	50.9

DNAs prepared from cultures treated with the indicated agents in the presence of BrdUrd were separated into heavy-light (HL) and light-light (LL) fractions by CsCl buoyant density gradient centrifugation. The abilities of these DNAs to accept methyl groups from SAM in the presence of a crude spleen methyltransferase were then determined under conditions where DNA was limiting (1 μg DNA/assay). Some DNA samples were heat-denatured prior to assay by heating to 100°C for 10 min followed by rapid cooling. N.D. = not determined.

methyl-accepting ability of hemimethylated DNA (results not shown). Thus, while the analog itself had no direct effect on the *in vitro* reaction, the presence of high levels of 5-azacytosine in DNA appeared to inhibit the action of the enzyme.

Extent of 5-Azacytosine Substitution

The extent of substitution of 5-azacytosine for cytosine in the DNA of MCA Cl-15-C cells was determined in the experiment shown in Fig 4. The amount of 5-azacytosine incorporated was dependent on the 5-aza-CR concentration in the medium, and 1.37% of the cytosine residues in total DNA were replaced by 5-azacytosine at a concentration of 10 μM 5-aza-CR. Since approximately 50% of the DNA replicated during the treatment period (Fig 1), and the 5-azacytosine would be incorporated only into the H strand, the highest level of substitution of the fraudulent base for cytosine in this strand would be 4 x 1.37% or 5.48%. The methyl-

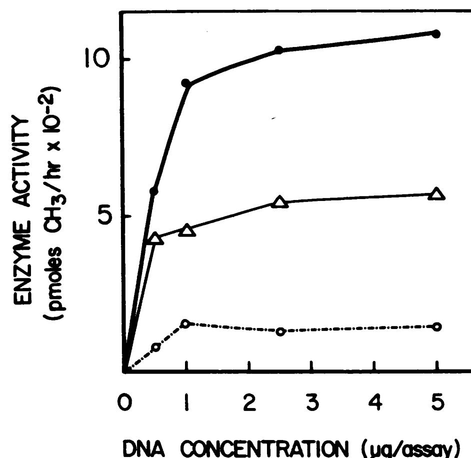

Figure 3. Effect of substrate concentration on methyl-accepting abilities of HL DNAs. HL DNAs were extracted from control cultures (O) or cultures treated with 2 µM (●) or 10 µmM (Δ) 5-aza-CR. The abilities of different concentrations of these DNAs to serve as methyl-acceptors in an *in vitro* methylation assay were then determined.

accepting abilities of limiting concentrations of HL DNAs prepared from cultures treated with these same doses of 5-aza-CR are also shown in Fig 4. Thus, while increasing the level of 5-aza-CR in the culture medium led to an increased incorporation of the analog and a decrease in 5mC content (Table 1), the presence of high levels of 5-azacytosine in DNA inhibited the action of the methyltransferase.

The s-triazine ring of 5-azacytosine is known to be unstable in aqueous solutions (10,29) and we therefore determined the integrity of the ring in DNA. DNA extracted from cells labelled with ^{14}C-5-aza-CR was hydrolysed in 70% $HClO_4$ for 18 hr at 60°C and the bases separated by two dimensional thin layer chromatography. Greater than 85% of the ^{14}C radioactivity was found in intact 5-azacytosine even after these harsh hydrolysis procedures, demonstrating that the radiochemical had indeed been incorporated into DNA and also that the intact s-triazine ring structure was present in the DNA substrates.

Analysis of Reaction Product

HL DNAs obtained from control cultures or cultures treated with 10 µM 5-aza-CR were incubated with ^3H-SAM in the presence of the crude methyltransferase. The reaction products were analysed by alkaline Cs_2SO_4

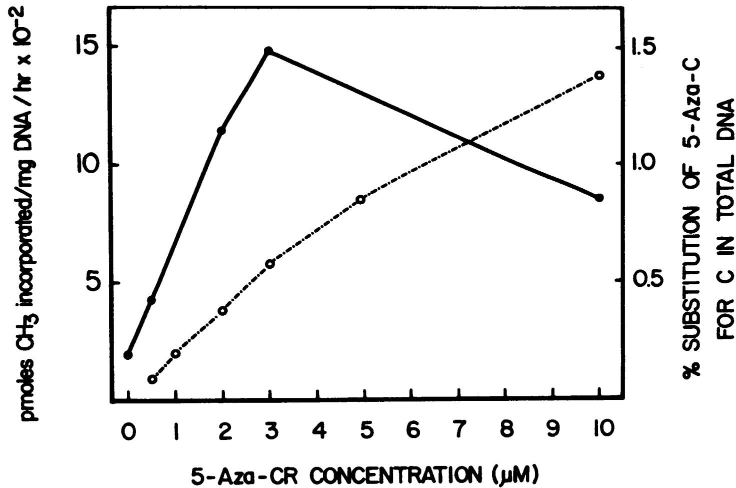

Figure 4. Effect of increasing concentrations of 5-aza-CR on the incorporation of 5-azacytosine into DNA and methyl-accepting abilities of HL DNAs. MCA Cl-15-C cells were treated for 20 hr in the presence of BrdUrd (5 μg/ml) and the indicated concentrations of ^{14}C-5-aza-CR. The extent of substitution of ^{14}C-5-azacytosine for cytosine (O) in total cellular DNA was then determined (see text for determination of extent of substitution in the newly synthesized or H strand). The abilities of HL DNAs extracted from cultures treated under the same conditions, to serve as substrates in the methylation assay are also shown (●, data from Table 2).

density gradient centrifugation to determine whether the ^{3}H-methyl groups were incorporated into the hypomethylated H strand (Fig 5). The low level of incorporation of ^{3}H-methyl groups into control HL DNA was confined almost exclusively to the H strand. Since this BrdUrd-containing strand had been synthesized immediately before extraction from the living cells, it probably contained potential modification sites which had not been methylated before cell lysis. The much-increased incorporation of ^{3}H-methyl groups which occurred with the HL DNA prepared from cultures treated with 5-aza-CR was also confined to the H or 5-azacytosine-containing strand. Thus methyl groups were transferred specifically to the hypomethylated strand.

Two dimensional chromatography of acid hydrolysates of the HL DNA reaction products demonstrated that 83-88% of the incorporated radioactivity was present in 5mC (results not shown). The test-tube reaction therefore satisfied several of the criteria for cellular

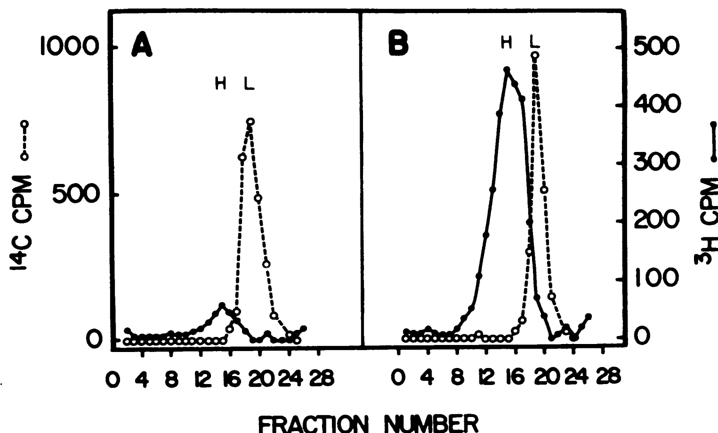

Figure 5. Alkaline Cs_2SO_4 equilibrium density gradients of *in vitro* methylation reaction products. The HL DNA extracted from control (A) or cultures treated with 10 µM 5-aza-CR (B) was methylated *in vitro* in the presence of ^3H-SAM then mixed with marker ^{14}C-labelled light-light DNA and centrifuged to equilibrium on alkaline Cs_2SO_4 gradients. The bottoms of the gradients are to the left, (●) ^3H-cpm; (O) ^{14}C-cpm.

"maintenance" DNA methylation in that ^3H-methyl groups were transferred from ^3H-SAM into cytosine residues which were specifically located in the undermethylated strand of hemimethylated duplex DNA.

DISCUSSION

The DNA substrates prepared from 5-aza-CR treated cultures were characterized with respect to 5mC and 5-azacytosine contents and represent the first defined hemimethylated DNA substrates to be used in an *in vitro* methylation assay. These substrates were efficient methyl acceptors and the results clearly demonstrated the preference of the crude methylase for hemimethylated duplex DNA. The fact that methyl groups were transferred specifically to cytosine residues in the hypomethylated strand has not previously been shown and indicated that the enzyme had the properties of a "maintenance" methylase (17, 18). It was also significant that duplex hemimethylated DNA was a 20 fold better substrate for the enzyme than single stranded DNA.

We are unable to explain the increased methyl-accepting abilities of LL DNAs prepared from cultures treated with 5-aza-CR. However it is possible that these fractions contained some contaminating newly synthe-

sized hemimethylated DNA which had not incorporated enough BrdUrd to cause a significant density shift on the gradients. The occurrence of GC-rich regions in DNA which would incorporate low levels of BrdUrd but contain many potential methylation sites should also be considered. However, it is unlikely that the results were due to the appearance of symmetrically demethylated sites in the LL DNA, since E.Coli B DNA, which contains no 5mC, was a poor substrate for the spleen enzyme.

Recently, Tanaka et al. (30) showed that bulk DNA isolated from Ehrlich's ascites tumor cells treated with 5-aza-CR was an efficient acceptor of methyl groups in vitro. We have preferred to utilize density-labelled DNA in order to remove unreplicated DNA from the preparation. The occurrence of bromouracil in the hypomethylated strand not only allowed for the isolation of hemimethylated DNA from bulk DNA but also for the characterization of the in vitro product. The presence of the thymine analog would probably not have adversely affected the efficiencies of the substrates, since the detailed study of Singer et al. (24) showed that BrdUrd did not influence methylation levels in rat hepatoma cell DNA and BrdUrd-containing DNA is methylated in living cells (13,19). The presence of the 5-azacytosine ring, on the other hand, inhibited the enzyme activity, but we were unsuccessful in demonstrating by HPLC that DNA substrates prepared from ethionine or cycloleucine treated cultures were hypomethylated. Ethionine and cycloleucine are inhibitors of all cellular methylations and may not therefore be as useful as 5-aza-CR in the preparation of hemimethylated DNAs.

Christman et al. (16) have suggested that DNA prepared from Friend cells induced to differentiate by ethionine or other agents was hypomethylated based on its ability to accept methyl groups in vitro. However, as in the present study, the degree of hypomethylation was too small to be measured by HPLC. Also, although DNAs replicated in the absence of methionine (21) or repaired in vitro in the absence of methyltransferase (20) are efficient acceptors of methyl groups, all of these preparations would contain appreciable quantities of contaminating symmetrically modified DNA. Thus, while the presence of 5-azacytosine in duplex DNA does inhibit the action of the methyltransferase, it nevertheless allows for the preparation of milligram quantities of an efficient, defined substrate.

Our results also provided evidence that the presence of 5-azacytosine in DNA directly inhibited the action of the methyltransferase. Incorporated 5-azacytosine has recently been shown to inhibit irreversibly E.Coli K12

DNA (cytosine-5) methyltransferase (28) so that the ability of the fraudulent base to inhibit DNA methylation appears to be quite general. We do not yet know whether this is due to the arrest of methyltransferase which has been proposed to "walk" down the helix (31) or whether the enzyme binds irreversibly to potentially modifiable sites containing 5-azacytosine residues. The results do however, provide an explanation for our earlier observations that the substitution of less than 5% of cytosine residues by 5-azacytosine led to an 85% inhibition of cytosine methylation in living cells (13).

It was also interesting that although the hypomethylated strand containing the fraudulent base could be methylated in the test tube, it remained hypomethylated in living cells following removal of 5-aza-CR, and this led to the formation of symmetrically demethylated sites sensitive to Hpall cleavage (13). This difference may be due to an increased enzyme-substrate ratio in the test tube, or alternatively may reflect cellular mechanisms which allow for the maintenance of a hemimethylated state during one cell division, so that new symmetrically-demethylated sites may be generated during differentiation. The use of cytosine-analogs, modified in the 5-position, should therefore allow us to probe more completely the cellular mechanisms responsible for the maintenance and evolution of methylation patterns.

REFERENCES

1. Razin, A., and Riggs, A.D. (1980) Science 210, 604-610.
2. Burdon, R.H., and Adams, R.L.P. (1980) Trends Biochem. Sci., 5, 294-297.
3. McGhee, J.D. and Ginder, G.D. (1979) Nature 280, 419-420.
4. Waalwijk, C. and Flavell, R.A. (1978) Nucleic Acids Res., 5, 4631-4641.
5. Shen, C-K.J. and Maniatis, T. (1980) Proc. Natl. Acad. Sci. USA 77, 6634-6638.
6. Van der Ploeg, L.H.T., and Flavell, R.A. (1980) Cell 19, 947-958.
7. Mandel, J.L., and Chambon, P. (1979) Nucleic Acids Res., 7, 2081-2103.
8. Desrosiers, R.C., Mulder, C., and Fleckenstein, B. (1979) Proc. Natl. Acad. Sci., USA, 76, 3839-3843.
9. Sutter, D., and Doerfler, W. (1980) Proc. Natl. Acad. Sci. USA 77, 253-256.
10. Constantinides, P.G., Jones, P.A., and Gevers, W. (1977) Nature 267, 364-366.
11. Constantinides, P.G., Taylor, S.M., and Jones, P.A. (1978) Devel. Biol. 66, 57-71.
12. Taylor, S.M. and Jones, P.A. (1979) Cell 17, 771-779.
13. Jones, P.A. and Taylor, S.M. (1980) Cell 20, 85-93.
14. Mohandas, T., Sparkes, R.S. and Shapiro, L.J. (1981) Science 211, 393-396.
15. Christman, J.K., Price, P., Pedrinan, L., and Acs, G. (1977) Eur. J. Biochem 81, 53-61.

16. Christman, J.K., Weich, N., Schoenbrun, B., Schneiderman, N., and Acs, G. (1980) J. Cell Biol 86, 366-370.
17. Riggs, A.D. (1975) Cytogenet. Cell Genet. 14, 9-25.
18. Holliday, R. and Pugh, J.E. (1975) Science 187, 226-232.
19. Bird, A.P. (1978) J. Mol. Biol. 118, 49-60.
20. Adams, R.L.P., McKay, E.L., Craig, L.M. and Burdon, R.H. (1979) Biochim. Biophys. Acta. 561, 345-357.
21. Turnbull, J.F., and Adams, R.L.P. (1976) Nucleic Acids Res. 3, 677-695.
22. Reznikoff, C.A., Bertram, J.S., Brankow, D.W. and Heidelberger, C. (1973) Cancer Res. 33, 3239-3249.
23. Marmur, J. (1961) J. Mol. Biol. 3, 208-218.
24. Singer, J., Stellwagen, R.H., Roberts-Ems, J. and Riggs, A.D. (1977) J. Biol. Chem. 252, 5509-5513.
25. Simon, D., Grunert, F., v. Acken, U., Döring, H.P. and Kröger, H. (1978) Nucleic Acids Res. 5, 2153-2167.
26. Randerath, K. and Randerath, E. (1967) Methods Enzym. 12, 323-347.
27. Abraham, G.N., Scaletta, C. and Vaughn, J.H. (1972) Anal. Biochem. 49, 547-549.
28. Friedman, S. (1981) Mol. Pharmacol. 19, 314-320.
29. Notari, R.E. and de Young, J.L. (1975) J. Pharm. Sci. 64, 1148-1157.
30. Tanaka, M., Hibasami, H., Nagai, J., and Ikeda, T. (1980) Aust. J. Exp. Biol. Med. Sci. 58, 391-396.
31. Drahovsky, D. and Morris, N.R. (1971) J. Mol. Biol. 57, 475-489.