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Repair methylation of parental DNA in synchronized cultures of Novikoff hepatoma cells

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ABSTRACT

Parental and filial DNA strands were isolated from a Novikoff rat hepatoma cell line, synchronized by S-phase arrest with excess thymidine, that had completed up to one round of DNA replication in the presence of (^{14}C-methyl)methionine and (6-^{3}H)bromodeoxyuridine. Both strands were methylated, the proportion of total methyl label in parental DNA increasing slightly with time in S-phase. The studies were repeated with (^{14}C-methyl)methionine and (^{3}H)deoxycytidine to determine if parental methylation occurred on extant or repair-inserted cytosine residues. Both (^{14}C) and (^{3}H) were found in parental DNA. The (^{14}C)/(^{3}H) ratio of parental DNA-5-methylcytosine was about twice that in filial DNA while the (^{3}H) data showed twice the concentration of 5-methylcytosine in parental compared to filial DNA. Thus parental methylation occurred on repair-inserted cytosine residues and resulted in overmethylation. That the DNA damage and repair was due to S-phase arrest was shown by repeating the studies using a sequential mitotic-G_1 arrest method. With this method little (^{14}C) or (^{3}H) was found in parental DNA. We conclude that S-phase arrest leads to DNA damage and repair with subsequent overmethylation of repair-inserted cytosines; that sequential mitotic-G_1 arrest minimizes DNA damage; and, that the latter technique, suitable for synchronization of large quantities of cells, may prove useful in relatively artifact-free studies of eukaryotic DNA replication.

INTRODUCTION

Early studies of eukaryotic DNA methylation demonstrated that the process occurred at the polymeric level of DNA but gave no information on whether methylation serves in some capacity to direct DNA replication or transcription[1,2,3]. Studies of the temporal relationship of methylation to the cell cycle, which includes examination of parental versus progeny strand methylation, could reveal explicit functional roles for the process. Although prokaryotic systems seem to restrict methylation to nascent strands of DNA at or near the replication point[4], eukaryotic systems may not necessarily be comparable. Three hours after release from a-

methopterin blockade of L929 cells, 17% of DNA methylation occurred on parental DNA and 83% on progeny strands[5]. Methylation of parental DNA has been noted in synchronous cultures of Physarum polycephalum[6] and may have occurred in mouse adrenal cells synchronized with hydroxyurea[7]. Methylation of parental strands of DNA was not detected in Chinese hamster ovary cells synchronized by mitotic selection[8] or in mouse mammary gland explants induced to synthesize DNA by hormonal stimuli[9].

Although preliminary studies from this laboratory[10] indicated that methylation of parental DNA in synchronized Novikoff hepatoma cells occurred, the fact that parental strand methylation did not increase with time in S-phase was somewhat enigmatic. This report shows that methylation of parental DNA in the Novikoff system is due almost entirely to methylation of cytosine residues inserted into parental DNA by DNA repair processes, that such "repair methylation" may well result in overmethylation of DNA, and that a sequential mitotic-G_1 arrest synchronization procedure may circumvent damage to DNA apparently induced by synchronization techniques that depend upon S-phase arrest.

MATERIALS AND METHODS

(G-^{3}H)-deoxycytidine (8.15 Ci/mM) and (6-^{3}H)-5-bromodeoxyuridine were purchased from New England Nuclear; (^{14}C-methyl)-methionine (56 mCi/mM) from Amersham-Searle; amethopterin from Lederle Labs.; unlabeled deoxyribonucleosides and Colcemid from Cal-Biochem; optical grade Cs_2SO_4 from Harshaw Chemicals; and hydroxyurea from Sigma.

All studies used a Novikoff rat hepatoma cell line (N1-S1) isolated by Morse and Potter[11] cultured in suspension as previously described[3]. Cells for synchrony were grown in 300 ml of Swim's number 69 medium to a density of 0.5 to 0.7 x 10^6 cells per ml. In the S-phase arrest technique, thymidine was added to a concentration of 10^{-3}M; 10 hours later deoxyadenosine, deoxycytidine and deoxyguanosine (all 10^{-5}M) were added to circumvent the thymidine block. Colcemid (5 x 10^{-7}M) was added at the same time. After reincubation for 8 hours the cells were harvested and resuspended in Colcemid- and methionine-free medium containing adenosine (10^{-4}M), amethopterin (1.8 x 10^{-5}M) and deoxyadenosine, deoxyguanosine and deoxycytidine (10^{-5}M each). (6-^{3}H)-5-bromode-

oxyuridine and (^{14}C-methyl)methionine at levels of 0.417 uCi and 0.33 uCi per ml, respectively, were initially used to monitor DNA replication and methylation. In other studies, (G-^{3}H)-deoxycytidine and (^{14}C-methyl)methionine were used, both at levels of 0.625 uCi/ml. At resuspension, 90-93% of the cells were in Colcemid arrest. All cells divided about 4 hours after resuspension then entered S-phase which, in this perturbed system, lasts about 10 hours.

The sequential mitotic-G_1 arrest method involved addition of Colcemid (5×10^{-7}M) for 10 hours (about one population doubling time), centrifugation and wash of the cells in Colcemid-free medium, and resuspension into medium containing 5×10^{-3}M hydroxyurea. Ten hours later the cells were harvested, washed once, and resuspended in medium containing labeled precursors. Under these conditions, DNA synthesis reproducibly begins ca. 45 minutes after resuspension, continues for a six hour period, then ceases. Figure 1 shows the cumulative incorporation of ^{3}H-deoxythymidine into DNA in cells synchronized by this technique.

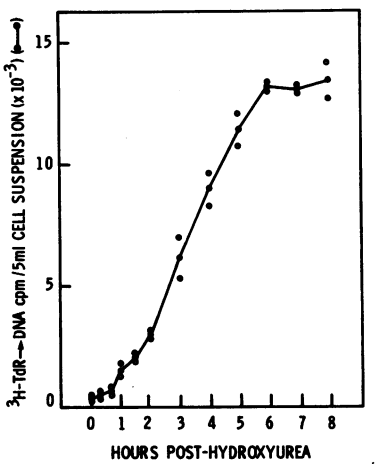

Fig.1. Incorporation of ^{3}H-deoxythymidine into DNA in Novikoff hepatoma cells synchronized by the mitotic arrest-G_1 arrest technique. Filled circle: average incorporation in paired daughter cultures; open circle: the average of a duplicate analysis of each daughter culture.

At varying times after final resuspension with either technique, cells were harvested, washed in saline, suspended in 10 ml of 0.1M Tris (pH 7.5)-0.01M NaCl-0.0015M $MgCl_2$ (TSM), stirred on

ice for 30 minutes, homogenized with a Potter-Elvehjem homogenizer, 1.0 ml Triton X-100 added, and the tube mixed; nuclei were recovered by centrifugation, washed once with TSM and frozen at -30°C. DNA was extracted from frozen-thawed nuclei using the methods described previously[3,12]. The purified deproteinized RNA-free DNA was unspooled in 2 to 3 ml 0.015M NaCl-0.0015M sodium citrate (pH 11.6), diluted to a final concentration of 50 to 100 ug of DNA per 0.5ml of the above buffer, heat-denatured for 15 minutes, quick-cooled in ice, and layered onto 4.5 ml of a cesium sulfate solution prepared by adding 3.200g Cs_2SO_4 to 4.5ml of 0.015M NaCl-0.0015M sodium citrate (pH 11.6)(η = 1.3725); final volume was 5.0 ml. After centrifugation (SW 50.1 rotor) at 35000 rpm for 48 hours at 25°, gradients were fractionated with an ISCO gradient fractionator and a model UA-4 absorbance monitor. Refractive indices of the 0.1ml fractions were determined and the corresponding Cs_2SO_4 densities calculated. Fractions were counted in 10 ml of InstaGel (Packard) using double label settings with efficiencies of 43.7% for (^{14}C) and 20.3% for (^{3}H); (^{14}C) spill into the (^{3}H) channel was 10.4%. All steps from the initial resuspension during synchrony to the gradient preparation were carried out in nearly complete darkness to minimize light-induced strand breaks in the bromodeoxyuridine-substituted DNA.

Parental and progeny DNA fractions were separately pooled, dialyzed, lyophilized, and hydrolyzed to free bases with 90% HCOOH at 175°C for 35 minutes. The digest was chromatographed on Whatman No.1 paper in two dimensions using n-butanol:0.2N NH_4OH (6:1 v/v) in the first dimension and isopropanol:con.HCl:H_2O (65:17.2:17.8 v/v) in the second dimension. Determination of (^{14}C)/(^{3}H) ratios in 5-methylcytosine was accomplished by elution of the 5-methylcytosine spot with 0.1N HCl, lyophilizing the eluate, redissolving the eluate in 0.5ml H_2O, adding 15ml of Bray's counting fluid, and counting at absolute efficiencies of 44% for (^{14}C) and 21.5% for (^{3}H); (^{14}C) spill into the (^{3}H) channel in this system was 14% with no tritium counts recorded in the (^{14}C) channel.

To demonstrate that 5-methylcytosine derived from deoxyribonucleic acid, separately pooled parental and progeny strands were enzymatically digested and chromatographed in a borate buffer

system[13]. All (^{14}C-methyl)-labeled bases could be accounted for as 5-methyldeoxycytidine or 5-methyldeoxycytidine-5'-monophosphate.

RESULTS AND DISCUSSION

Figure 2 illustrates the pattern of DNA methylation and DNA replication in Novikoff cells that completed 77% of replication after synchronization by the excess thymidine-Colcemid method.

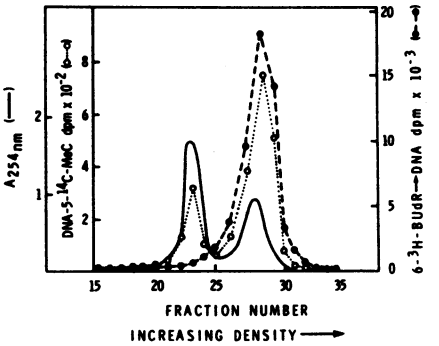

Fig.2. Alkaline Cs_2SO_4 isopycnic gradient separation of 5-bromodeoxyuridine-substituted Novikoff cell DNA from cells that had completed 77% of DNA replication. Details in text. Fraction volume, 0.1ml. (——), A_{254nm} trace; (●----●), incorporation of (6-^{3}H)-5-bromodeoxyuridine into DNA; (o....o), incorporation of (^{14}C-methyl)methionine into DNA.

Table 1 shows the results of cumulative methylation of parental and filial strands of DNA at three stages of S-phase when replication is 21%, 34% and 77% complete.

Since filial DNA is more highly methylated than parental DNA, the label appearing under the parental peak might represent filial DNA that cosediments with light parental DNA or else be due to incomplete denaturation. However, the proportion of (^{3}H)-label under the parental peaks ranges from 2% to 6% whereas the proportion of (^{14}C) activity ranges from 17% to 27%. If the (^{3}H) activity represented cosedimentation or incomplete denaturation, the two percentages would not be very divergent. Secondly, when parental and filial peaks from the 77% replicated sample were separately rerun on alkaline Cs_2SO_4 gradients, the proportion of (^{14}C) label in the parental peak remained nearly the same whereas the (^{3}H) activity dropped from 6.4% to 3.6%. Reisolation from the 34% replication study gave the same result.

Table 1 also shows that, although the incorporation of (6-^{3}H)-5-bromodeoxyuridine into DNA increases with percent replication,

% Replication	(^{3}H) dpm per mg		(^{14}C) dpm per mg	
	Filial DNA	Parental DNA	Filial DNA	Parental DNA
21%	788,743.	18,355.	32,897.	5,732.
34	1,073,705.	34,188.	61,768.	12,098.
77	1,945,671.	123,835.	72,567.	19,499.
77*	2,053,308.	73,136.	69,692.	16,982.

% Replication	$\frac{(^3H)parental}{(^3H)filial}$	$\frac{(^{14}C)parental}{(^{14}C)filial}$	$\frac{(^{14}C)filial}{(^3H)filial}$	$\frac{(^{14}C)parental}{(^3H)parental}$
21%	2.3%	17.4%	0.042	0.312
34	3.2	19.6	0.057	0.354
77	6.4	26.9	0.037	0.157
77*	3.6	24.4	0.034	0.232

Table 1. Comparison of the incorporation of (6-^{3}H)-5-bromodeoxyuridine into isolated parental and progeny DNA strands at three different stages of replication in Novikoff cells synchronized by the excess thymidine-Colcemid technique. The final specific activity of the (^{14}C-methyl)methionine used in these studies was ten-fold less than used in the experiments shown in Tables 2 and 3. (*) denotes the fact that the isolated parental and progeny strands in this experiment were separately reisolated from a second alkaline Cs_2SO_4 density gradient centrifugation.

the cumulative methylation of parental and filial DNA increases to a proportionately lesser extent. Although the methylation of newly synthesized DNA has been noted to decrease in later portions of S-phase [8], the decreased methylation of parental DNA might represent the completion of methylation of cytosine residues inserted into parental DNA by DNA repair processes. By allowing Novikoff cells to complete one round of replication in the presence of unlabeled 5-bromodeoxyuridine and (G-^{3}H)-deoxycytidine, it was determined that less than 3.5% of the cytosine residues incorporated into DNA were found in parental DNA. Since knowledge of intracellular pool sizes of deoxycytidine in this study and S-adenosylmethionine in the experiments in Table 1 was unavailable, it was not possible to determine if the methylation of parental DNA represented the methylation of extant parental cytosine residues and/or the methylation of repair-inserted cytosines.

Therefore, both (^{14}C-methyl)methionine and (G-^{3}H)-deoxycytidine and unlabeled 5-bromodeoxyuridine were added to cells at the time of resuspension from Colcemid. After about 50% replication,

DNA was isolated, parental and progeny strands separated and pooled from six replicate gradients of the same sample of DNA, and cytosine and 5-methylcytosine chromatographically isolated from formic acid hydrolysates of each strand. Comparison of the ratio of $(^{14}C)/(^{3}H)$ in 5-methylcytosine in parental to that in progeny DNA is independent of knowledge of pool sizes and provides the following: (1) if the $(^{14}C)/(^{3}H)$ ratios of 5-methylcytosine in parental versus progeny DNA are identical, then (^{14}C)-label in parental strands represents only methylation of repair-inserted cytosine residues; (2) if the ratio in parental DNA is higher than the ratio in progeny DNA-5-methylcytosine, then (^{14}C)-label is due either to the methylation of extant parental cytosine residues, or overmethylation of cytosine residues inserted into parental DNA by DNA repair, or a combination of methylation of extant and repair-inserted cytosine residues. The results of this study are shown in Table 2.

Strand	Base	(^{3}H) dpm/mg DNA*	(^{14}C) dpm/mg DNA*
Progeny	Cyt	8,525,530.	-
	MeCyt	212,410.	570,740.
Parental	Cyt	259,890.	-
	MeCyt	12,930.	54,935.

Strand	$\frac{\text{Total } (^{3}H)\text{parental}}{\text{Total } (^{3}H)\text{progeny}}$	$\frac{(^{3}H)\text{ MeCyt}}{(^{3}H)\text{ MeCyt} + (^{3}H)\text{ Cyt}}$	$\frac{(^{14}C)\text{ MeCyt}}{(^{3}H)\text{ MeCyt}}$
Progeny	-	2.43%	2.69
Parental	3.1%	4.74	4.25

Table 2. Incorporation of (^{14}C-methyl)methionine and (G-^{3}H)-deoxycytidine into parental and progeny strand DNA-5-methylcytosine in cells synchronized by excess thymidine-Colcemid. (*) refers to the amount of DNA in the separated strands as determined by the Ceriotti method[14].

About 3% of the (^{3}H)-labeled cytosine residues were found in parental DNA. The $(^{14}C)/(^{3}H)$ ratio of parental 5-methylcytosine was about 60% greater than that ratio in progeny 5-methylcytosine indicating either the methylation of extant parental cyosine residues or the relative overmethylation of cytosine residues inserted into parental DNA by repair processes. The ratio of (^{3}H)-labeled 5-methylcytosine to the total (^{3}H)-label in cy-

tosine plus 5-methylcytosine (middle column, lower portion of the table) is an indication of the extent of methylation of de novo incorporated cytosine residues. It is clear that the parental cytosine residues are overmethylated vis a vis cytosine residues in progeny DNA.

Since S-phase arrest synchronization techniques are known to induce abberrations and artifacts in studies on DNA replication[15], an alternative synchrony method in which cells are never arrested in S-phase was developed. The essence of the technique is outlined in Methods. The (^{14}C-methyl)methionine and (G-^{3}H)-deoxycytidine study was repeated using the Colcemid-hydroxyurea synchronized cells. In this study, the cells were harvested 8 hours after resuspension into hydroxyurea-free medium. DNA replication was about 84% complete as measured by paper weights of the A_{254} tracings of parental and progeny peaks. In contrast to DNA isolated from cells synchronized by the excess thymidine-Colcemid technique (Fig.2), little or no (^{14}C) or (^{3}H) label was found under the parental DNA peak using the mitotic-G_1 arrest method.

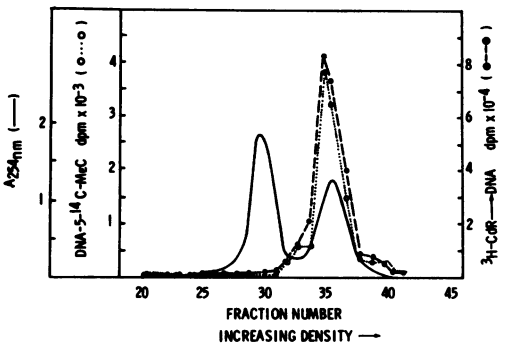

Figure 3. Alkaline Cs_2SO_4 isopycnic gradient separation of 5-bromodeoxyuridine-substituted Novikoff cell DNA from cells that had completed about 84% of DNA replication. Details in text. Fraction volume, 0.1ml. (——), A_{254nm} trace; (•-----•), incorporation of (G-^{3}H)-deoxycytidine into DNA; (o....o), incorporation of (^{14}C-methyl)methionine into DNA.

The parental and progeny DNA strands were isolated from six alkaline Cs_2SO_4 density gradient centrifugations of the same sample of DNA as analyzed in Fig.3, pooled, hydrolyzed, cytosine and 5-methylcytosine chromatographically isolated, and analyzed as in Table 2. The results are shown in Table 3.

Strand	Base	(^{3}H) dpm/mg DNA*	(^{14}C) dpm/mg DNA*
Progeny	Cyt	19,068,420.	-
	MeCyt	209,380.	855,930.
Parental	Cyt	30,495.	-
	MeCyt	3,830.	14,265.

Strand	$\frac{\text{Total } (^3H)\text{parental}}{\text{Total } (^3H)\text{progeny}}$	$\frac{(^3H)\text{ MeCyt}}{(^3H)\text{ MeCyt} + (^3H)\text{ Cyt}}$	$\frac{(^{14}C)\text{ MeCyt}}{(^3H)\text{ MeCyt}}$
Progeny	-	1.09%	4.09
Parental	0.18%	1.12	3.73

Table 3. Incorporation of (^{14}C-methyl)methionine and (G-^{3}H)-deoxycytidine into parental and progeny strand DNA-5-methylcytosine in cells synchronized by the Colcemid-hydroxyurea technique. (*) refers to the amount of DNA in the separated and analyzed strands as determined by the Ceriotti method[14].

Less than 0.2% of the (^{3}H)-labeled cytosine residues were found in parental DNA. The (^{14}C)/(^{3}H) ratio of parental 5-methylcytosine was nearly the same as that ratio in progeny strand 5-methylcytosine indicating that the parental methylation that did occur was due to the methylation of repair-inserted cytosine residues. In this instance, however, the low level of "repair methylation" was not characterized by overmethylation since the ratio of (^{3}H)-labeled 5-methylcytosine to the total (^{3}H)-label in cytosine plus 5-methylcytosine was identical in both parental and progeny DNA strands.

CONCLUSIONS

Methylation of parental strands of DNA has been reported in cell systems synchronized by S-phase arrest procedures[5,7,10]. These results stand in contrast to studies reporting the absence of parental strand methylation in Chinese hamster ovary cells synchronized by mitotic selection[8] and in mouse mammary gland explants induced to replicate DNA by hormonal stimuli[9]. Although these discrepant findings might be ascribed to differences in the cell systems analyzed, the results presented in this paper using the same cell system synchronized by two fundamentally different methods indicate that parental strand methylation probably reflects the methylation of cytosine residues inserted into parental DNA in response to DNA repair processes induced by S-phase arrest. These studies also confirm the observations of Borek and

colleagues studying prokaryotes[16,17] (and, more recently, eukaryotes[18]) that DNA repair processes are accompanied by a relative overmethylation of repair-inserted cytosine residues. The significance of such overmethylation is not presently clear but the present observation of a normal extent of methylation of cytosine residues when DNA repair is not extensive (Table 3) may indicate that overmethylation is not an unfailing accompaniment of DNA repair.

Finally, a method of cell synchronization has been presented in which damage to cellular DNA inducing DNA repair processes is reduced quite substantially compared to a S-phase arrest technique. Selection of mitotic cells from monolayer cell cultures followed by hydroxyurea-induced G_1 arrest has been used in cell synchrony studies[19] but this method is not applicable to suspension cell cultures. The sequential Colcemid-induced mitotic arrest followed by hydroxyurea-induced G_1 arrest method described here easily provides tightly synchronized cells in the large numbers needed for many biochemical studies and may prove useful for relatively artifact-free studies of eukaryotic DNA replication.

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