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Demethylation Enhances Removal of Pyrimidine Dimers from the Overall Genome and from Specific DNA Sequences in Chinese Hamster Ovary Cells

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We have examined the effects of changes in cytosine methylation on DNA repair in UV-irradiated Chinese hamster ovary (CHO) cells. A hypomethylated derivative of the CHO K_1B_{11} line, B_{11}aza, was established by passaging B_{11} cells over several months in increasing concentrations of 5-azacytidine; greater than 60% demethylation was consistently demonstrated in these conditioned cells. Following a UV dose of 10 J/m^2, the amount of repair replication performed within 24 h was approximately twofold higher in B_{11}aza cells than in control B_{11} cells. Removal of T4 endonuclease V-sensitive sites (ESS) from specific restriction fragments within and around the dihydrofolate reductase (DHFR) gene was then examined in B_{11}aza cells and compared with that in B_{11} cells. Although demethylation had little or no effect on repair in the 5′ half of the DHFR gene, within a nontranscribed sequence immediately downstream from the gene, or within an extragenic region further downstream from the DHFR gene, significant increases in repair were observed at the 3′ end of the DHFR gene and within an extragenic region upstream of the DHFR gene. However, the increases in DNA repair were not accompanied by any changes in overall cellular resistance to UV when colony-forming ability was assayed. We suggest that the level of DNA methylation may play an indirect role in the regulation of DNA repair, perhaps through an effect on chromatin structure or transcriptional activity.

Rodent cells in culture typically exhibit low levels of excision repair following UV irradiation (15, 53), in stark contrast to the high levels characteristic of human cell lines (53). However, the colony-forming abilities of UV-irradiated rodent and human cells are comparable (15, 61). Recent studies demonstrating heterogeneity of DNA repair among specific genomic sequences in these cells may provide an understanding of this paradox. In Chinese hamster ovary (CHO) cells, for example, the 5′ end of the dihydrofolate reductase (DHFR) gene is efficiently repaired after 24 h (60 to 70%), whereas a nontranscribed, 3′-flanking region is poorly repaired (15%), reflecting overall genomic repair levels (6). In Swiss 3T3 mouse cells, DNA repair levels within restriction fragments containing sequences from the transcriptionally active c-*abl* and the transcriptionally quiescent c-*mos* genes appear to correlate with transcriptional activity (33). Increased repair has also been observed in a restriction fragment containing the CHO metallothionein gene following transcriptional activation (43). Finally, in human 6A3 cells, Mellon et al. (36) have observed that two regions within and immediately upstream of the DHFR locus are repaired at a considerably more rapid rate than are the repetitive, transcriptionally silent alpha sequences or the genome overall; however, nearly complete removal of damage is ultimately accomplished in all sequences, as one would expect for these repair-proficient human cells.

On the basis of these results, we have suggested that "essential" DNA sequences may be preferentially repaired. What determines the repairability of a particular sequence? The evidence presented above, along with the recent discovery that transcribed DNA strands are selectively repaired in human and CHO cells (37), suggests that regulation of transcription plays a major role in this determination. However, a definitive test of this hypothesis has yet to be performed, and any one of several parameters which have been correlated with transcriptional activity may play a role in regulating DNA repair; examples include DNase I sensitivity, methylation at cytosine residues, the presence of certain high-mobility-group proteins, and the presence of histone H1 (17). Of these parameters, methylation is one of the more extensively studied, yet its function in eucaryotic cells remains enigmatic (1, 7, 46). Furthermore, no studies to date have addressed the relationship between levels of methylation and DNA repair.

5-Methylcytosine (5-mC) is the only normal methylated base which has been detected in higher eucaryotes. It is derived from cytosine through the activity of a DNA methyltransferase which, in the maintenance mode, appends methyl groups onto cytosine residues in nascent DNA at their C-5 positions following replication. 5-mC constitutes a small percentage of all cytosine residues (typically 1 to 5%) and appears predominantly in very short palindromic sequences, particularly in the statistically underrepresented dinucleotide sequence CpG (35). Quantitatively, greater than 90% of 5-mC residues can be found in CpG sequences, and 50 to 80% of these dinucleotide sequences are methylated in various mouse tissues (44). Interestingly, the distribution of 5-mC within eucaryotic genomes is nonrandom. For example, repetitive DNA sequences are enriched severalfold for 5-mC relative to single-copy sequences (11), and DNA associated with nucleosome cores is methylated to a significantly greater extent than is linker DNA (45, 50), especially in nucleosomes which contain histone H1 (2). Particular regions, most notably those corresponding to active transcription units, tend to be hypomethylated despite the fact that CpG sequences are frequently clustered at the 5′ ends of many genes (35) (exemplary in this respect are the DHFR genes of human [48] and CHO [5, 39, 51] cells). Together

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with data from developmentally regulated gene induction systems and plasmid transfection studies, a large body of evidence has accumulated in support of the hypothesis that changes in methylation affect gene expression. This led us to examine the levels of methylation at *Hpa*II and *Msp*I sites within the DHFR amplicon in CHO B_{11} cells and to correlate these with repair of UV-induced pyrimidine dimers by using our previously developed techniques for fine-structure repair analysis (5). We found that the region of maximal DNA repair efficiency encompasses the solitary region of hypomethylation within the CHO DHFR gene region. This raises the possibility that methylation may play a role in regulating the repair of particular sequences. In the present study, we sought to extend this finding and to test directly the hypothesis that hypomethylation enhances DNA repair. To accomplish this, we established a derivative B_{11} cell line, B_{11}aza, by passage of B_{11} cells in increasing concentrations of 5-azacytidine (5-aCR), a cytidine analog which inhibits methylation in vivo, primarily by covalent modification of the DNA methyltransferase and to a lesser extent by direct misincorporation into DNA, where it cannot be methylated. DNA methylation and repair levels in the total genome and in specific sequences could then be directly compared in the two cell lines. This report represents the first detailed, systematic study of the role of DNA methylation in excision repair in eucaryotic cells.

MATERIALS AND METHODS

Genomic DNA probes. The cosmids c26-1, cA-25, and cS14 (40) were obtained through the courtesy of J. Hamlin (University of Virginia, Charlottesville). The plasmids pMB-5, pB6-7H1A, and pB13-7 (8) were obtained from L. Chasin (Columbia University, New York).

Subcloning. The plasmids pZH-20 and pZH-26 were constructed as follows. The parental cosmids (c26-1 and cS14, respectively) were digested with a panel of restriction endonucleases, and the resulting fragments were separated by agarose gel electrophoresis. Following Southern transfer, the restriction fragments were screened for lambda phage and cellular repetitive sequences by sequential hybridization with [^{32}P]dCTP-labeled, nick-translated lambda phage DNA and total B_{11} cellular DNA, respectively. Several fragments lacking these sequences were selected and isolated from low-melting-point agarose gels (34), radiolabeled by nick translation, and hybridized to restricted genomic DNA on nylon support membranes to determine the specificity of each cosmid fragment. Two appropriate fragments were selected and subcloned into compatible restriction sites within the multiple-cloning sequence of pUC18 (42); pZH-20 contains a 3.5- to 3.6-kilobase (kb) *Msp*I fragment of c26-1 subcloned into the *Acc*I site, and pZH-26 contains a 1.7-kb fragment of cS14 subcloned into the *Eco*RI site. On the other hand, the plasmid pZH-27 was constructed by shotgun subcloning the *Pst*I fragments of cA25 into pGEM-3Z (Promega) and selecting DH5α (Bethesda Research Laboratories) transformants by colony hybridization for the absence of lambda phage and cellular repetitive sequences. One subclone was selected and verified to contain a 5.0-kb *Pst*I fragment, which, after nick translation and hybridization to test filters, detects a *Kpn*I fragment of appropriate size.

Cells and media. $K_1B_{11}[0.5]$ CHO cells (referred to elsewhere simply as B_{11} cells) were grown as described by Bohr et al. (6); these cells contain a 50-fold amplification of the DHFR gene achieved through stepwise selection in the presence of methotrexate (22). The B_{11}aza cell line was derived from the B_{11} line by repeated subculture over several months in the presence of incrementally increasing concentrations of 5-aCR (Sigma), which was included in all B_{11}aza cultures except during repair experiments, when 5-aCR was omitted from the medium beginning at least 24 h prior to irradiation. Routine subculturing was performed every 3 to 4 days by seeding approximately 10^6 cells per 10-cm dish. For experiments involving radiochemical labeling, cells were typically seeded at 1×10^6 to 2×10^6 cells per 10-cm dish and incubated for 24 to 48 h prior to introduction of the radiolabel to obtain exponentially growing cells.

Isolation and purification of DNA. Cellular DNA was isolated and purified as described before (3).

Analysis of methylation by HPLC. Much of the high-pressure liquid chromatography (HPLC) method was derived from Flatau et al. (13), with helpful suggestions made by P. A. Jones. Cells were grown in 10 μCi of [6-^{3}H]uridine (New England Nuclear) per ml for 24 h; B_{11}aza cells were grown in the absence of 5-aCR during this labeling period. Cellular DNA was isolated and purified as described above. Following ethanol precipitation, the DNA was resuspended in 1 N NaOH and incubated for 1 h at 50°C to remove RNA. Equal volumes of 1 M Tris (pH 7.4) and 1 N HCl were then added to quench the hydrolysis reaction. The DNA samples were again precipitated with ethanol, lyophilized to dryness, dissolved in 88 to 90% formic acid, and incubated at 175°C for 30 min in sealed ignition tubes (10 by 70 mm; Corning). The samples were then lyophilized to dryness, suspended in 4 mM HCl, and stored at −20°C. Reference base standards (adenine, cytosine, guanine, 5-mC, thymine, and uracil; Sigma) were also dissolved in 4 mM HCl and stored at −20°C. HPLC was performed on a Beckman model 100A equipped with a 25-cm Whatman Partisil 10SCX column and a Whatman type WC5 precolumn at a column temperature of 35°C maintained by a Bio-Rad column heater. The bases were eluted isocratically with 45 mM ammonium phosphate (pH 2.4) at a constant flow rate of 2 ml/min and a concomitant pressure of 950 to 970 lb/in^2 and detected by absorbance at 280 nm (the wavelength at maximum absorption for 5-mC) with a Hitachi model 100-10 spectrophotometer. Fractions were collected at 30-s intervals and assayed for radioactivity in a liquid scintillation spectrophotometer with 9 parts Optifluor (United Technologies, Packard) to 1 part eluate. Genomic methylation levels were quantitated by the equation: % methylation at cytosine residues = [(^{3}H cpm in 5-mC peak)/(^{3}H cpm in 5-mC peak + ^{3}H cpm in cytosine peak)] × 100.

Analysis of methylation by sucrose gradient centrifugation. Cells were grown for 48 h in 0.1 μCi of [*methyl*-^{3}H]thymidine per ml and 10 μM thymidine. DNA prepared as described above was digested with the appropriate restriction endonuclease (Bethesda Research Laboratories) at 5 U of enzyme per μg of DNA for 8 to 12 h at 37°C, followed by precipitation with ethanol and suspension in TE buffer. Samples containing 25,000 cpm of restricted DNA and 2,000 cpm of lambda phage [^{14}C]DNA were layered atop 2.3-ml linear sucrose gradients (5 to 20% [wt/vol] in TE buffer). *Hha*I and *Hpa*II digests of B_{11} DNA were centrifuged at 40,000 rpm for 95 min, and all other samples were centrifuged at 45,000 rpm for 145 min, all at 20°C in an SW50.1 rotor. The gradients were fractionated into approximately 30 fractions by dripping onto Whatman filter paper strips, and the DNA was precipitated in cold 5% trichloroacetic acid and assayed for radioactivity by liquid scintillation spectrophotometry with CrystalFluor (WestChem) in toluene. Number-average mo-

lecular weights were calculated as described by Lehmann (28), from which the relative frequency of recognized sites for each restriction enzyme was calculated. The percentage of *Hpa*II and *Msp*I sites which were modified by methylation was calculated by the equation: % methylation at *Hpa*II and *Msp*I sites = [(number of total CCGG sequences − number of unmethylated CCGG sequences)/(number of total CCGG sequences)] × 100.

Analysis of methylation by gel electrophoresis. Analysis of genomic methylation by gel electrophoresis of restriction enzyme digests of cellular DNA was performed as reported previously (5). Quantitation of methylation at *Hha*I or *Hpa*II sites within specific *Bam*HI or *Kpn*I fragments was performed by comparing the intensities of the fragment on autoradiographs between single digests (*Bam*HI or *Kpn*I alone) and double digests with *Hha*I, *Hpa*II, or *Msp*I. If the number of GCGC or CCGG sequences is known and if one assumes that like sequences are demethylated similarly within the limited confines of the particular restriction fragment, then one can estimate the degree to which each site is demethylated as follows. If the relative intensity of two such bands were 0.16, for example, and there were two assayable *Hpa*II sites within the fragment, then each site must be demethylated approximately 60%, since the probability that neither site is demethylated (the prerequisite for retention of the full-length fragment in the double digests) is given by $(1.0 - 0.6)^2 = 0.16$.

Repair replication. The procedure described previously by Smith et al. (49) was used for repair replication.

Repair analysis by gel electrophoresis. Procedures outlined in reference 3 were followed with minor modifications. Most notably, only one hybridization with C_0t_{100} DNA (6) was performed per filter, after which the membrane was washed twice with 5× SSPE (20× SSPE is 3.6 M NaCl, 0.2 M NaH_2PO_4, 22 mM EDTA, pH 7.4) at room temperature, baked for 2 h in vacuo at 80°C, washed for 2 h at 65°C in 0.1× SSPE–0.1% sodium dodecyl sulfate (SDS), and prehybridized as before. This single step has been found to reproduce the suppressive effect of repeated C_0t_{100} hybridizations on repetitive sequences in all subsequent hybridizations with radiolabeled probes.

UV sensitivity. Colony-forming ability was assayed as described in reference 6; 400 cells were seeded per 10-cm dish, and all points were determined in triplicate. Survival was assayed at the following UV doses: 0, 2.5, 5, 7.5, 10, 15, 20, and 30 J/m^2.

RESULTS

Experimental design. The primary objective of this study was to evaluate the role of DNA methylation in DNA repair in UV-irradiated CHO cells. A cell line with dramatically decreased levels of genomic methylation, B_{11}aza, was generated by passaging CHO B_{11} cells in increasing concentrations of 5-aCR. We then examined methylation and DNA repair in bulk DNA and within specific defined sequences in the DHFR gene region. The common genetic background shared by the two cell lines was an important consideration in selecting this approach in that it minimized the potential contribution of variables unrelated to specific changes in methylation.

Generation of the B_{11}aza cell line. The derivation and characterization of the parental B_{11} cell line have been reported previously (22, 25). B_{11}aza cells were generated by growing B_{11} cells in the presence of increasing concentrations of 5-aCR, beginning at 2.2 μM and gradually increasing during routine passage over several months until a final concentration of 100 μM had been reached.

TABLE 1. Quantitation of genomic methylation by methylation-sensitive restriction endonucleases

Cell line	Restriction enzyme	Sequence(s) recognized for cleavage	Sequence not recognized for cleavage	M_n[a] (kb)
B_{11}	*Hha*I	GCGC	G^{5}mCGC	49
	*Hpa*II	CCGG	C^{5}mCGG	53
	*Msp*I	CCGG, C^{5}mCGG		5.7
B_{11}aza	*Hha*I	GCGC	G^{5}mCGC	19
	*Hpa*II	CCGG	C^{5}mCGG	8.2
	*Msp*I	CGGG, C^{5}mCGG		5.5

[a] Number-average molecular weights (M_n) were calculated from the profiles of alkaline sucrose gradients as described in the text.

Overall genomic methylation levels in B_{11} and B_{11}aza cells. Total genomic methylation in B_{11} and B_{11}aza cells was quantitated by two different methods. As a first approximation, we examined the effects of methylation-sensitive restriction endonucleases on the number-average molecular weight of cellular DNA by neutral sucrose gradient sedimentation. We used *Hha*I, which will cleave at the sequence GCGC only when the internal cytosine is unmethylated, and the isoschizomers *Hpa*II and *Msp*I, which cleave at the sequence CCGG only when the internal cytosine is unmethylated (*Hpa*II) or regardless of methylation (*Msp*I). From the data shown in Table 1, we calculated the frequency of unmethylated GCGC (*Hha*I) and CCGG (*Hpa*II) sequences, the frequency of all CCGG sequences (*Msp*I), and thereby the fraction of CCGG sequences which are methylated at internal cytosine residues. This analysis revealed that 89% of *Hpa*II and *Msp*I sites were methylated in B_{11} cells, compared with only 33% in B_{11}aza cells. Thus, a 63% decrease in methylation was induced in B_{11}aza cells as a result of conditioning in 5-aCR. Similar quantitation is not possible for GCGC sequences because of the lack of an appropriate methylation-insensitive restriction enzyme.

Since the above method samples only a subset of methylatable sites, we developed an HPLC assay to quantitate methylation of all cytosine residues. Specifically, we analyzed formic acid hydrolysates of labeled DNA from CHO cells on a cation-exchange HPLC column by collecting fractions of the eluate and assaying for radioactivity. By determining the relative amounts of cytosine and 5-mC (data not shown), we calculated that 3.7% of cytosine residues were methylated in B_{11} cells, while only 1.4% were so modified in B_{11}aza cells. Thus, a 62% decrease in genomic methylation was achieved by passaging B_{11} cells in 5-aCR, a value in close agreement with that obtained by restriction analysis.

Repair replication as a measure of genomic DNA repair. The extent of repair replication following UV irradiation was determined at three different times during the development of the B_{11}aza cell line (Table 2). Cells exposed to 5 and 10 μM 5-aCR exhibited insignificant changes in the levels of repair replication. However, in cells passaged in 100 μM 5-aCR, a twofold (93%) increase in repair replication was measured.

The observed increase in repair replication could reflect differences in nucleotide pool sizes between the B_{11} and B_{11}aza cell lines. To examine this possibility, we determined the incorporation of [^{3}H]thymidine ([^{3}H]dThd) per microgram of replicated DNA in each trial. Should nucleotide pool sizes differ significantly between the two cell lines, one

TABLE 2. Determination of genomic DNA repair by quantitation of repair replication

Exp[a]	Repair period (h)	Repair replication ([^{3}H]dThd incorporation per μg of parental-density DNA [cpm])			Semiconservative replication ([^{3}H]dThd incorporation per μg of hybrid-density DNA [cpm])		
		B_{11}	B_{11}aza	% Difference	B_{11}	B_{11}aza	% Difference
1	4	42	45	7.1	1,057	1,060	0.3
	8	59	71	20	902	887	1.7
	12	66	66	0	NA[b]	917	
	24	91	107	18	783	1,074	37
2	4	43	33	−23	NA	NA	
	12	66	57	−14	NA	NA	
	24	89	79	−11	21,674	18,044	−17
3	24	99	191	93	40,811	45,024	10

[a] The three experiments listed here differed principally in the concentration of 5-CR in which the B_{11}aza cells were maintained: 5 μM 5-aCR in experiment 1, 10 μM 5-aCR in experiment 2, and 100 μM 5-aCR in experiment 3.

[b] NA, Not available.

would expect a corresponding disparity in the incorporation of tritium label during replication. As shown in Table 2, these differences were sufficient to account for the ambiguous results obtained in the first two experiments. However, in the third experiment, the small 9.4% difference in [^{3}H]dThd incorporation did not indicate sufficient nucleotide pool size variation to account for the 93% increase in repair replication detected. We conclude that the increase in repair replication noted for B_{11}aza cells is significant.

Methylation changes within the DHFR amplification unit. Having examined DNA methylation and DNA repair at the genomic level, we next turned to address these questions at the level of specific sequences within the DHFR amplification unit by using gel electrophoresis to size-fractionate DNA digested by the same methylation-sensitive restriction enzymes used in our studies of genomic methylation, followed by Southern transfer to nylon membranes and hybridization to nick-translated probes to detect defined sequences (Fig. 1). We used *Bam*HI and *Kpn*I in single digests in order to generate restriction fragments in common with our repair assays and in combination with *Hha*I, *Hpa*II, and *Msp*I in double digests. Appropriate calculations (see Materials and Methods for details and an example) for the 7-kb *Bam*HI fragment at the 3′ end of the DHFR gene (fragment D in Fig. 2) revealed that the single *Hha*I site within the fragment was 46% demethylated in B_{11}aza cells, whereas the single *Hpa*II site was 89% demethylated. These results support earlier observations in two ways: heterogeneity is present in the extent of demethylation for different sequences, and *Hpa*II sites appear to be more easily demethylated than *Hha*I sites. Note also that an average of the two figures yields a value of 68% demethylation, in close agreement with the results obtained by two methods for the overall genome.

Removal of ESS from defined sequences within the DHFR amplification unit. To quantitate DNA repair within specific sequences, we used a sensitive assay for pyrimidine dimers described previously (6) which uses the pyrimidine dimer-specific enzyme T4 endonuclease V (13, 36) in combination with alkaline gel electrophoresis and standard Southern blotting techniques. In a previous report (5), we carefully delineated a repair domain centered about the DHFR gene,

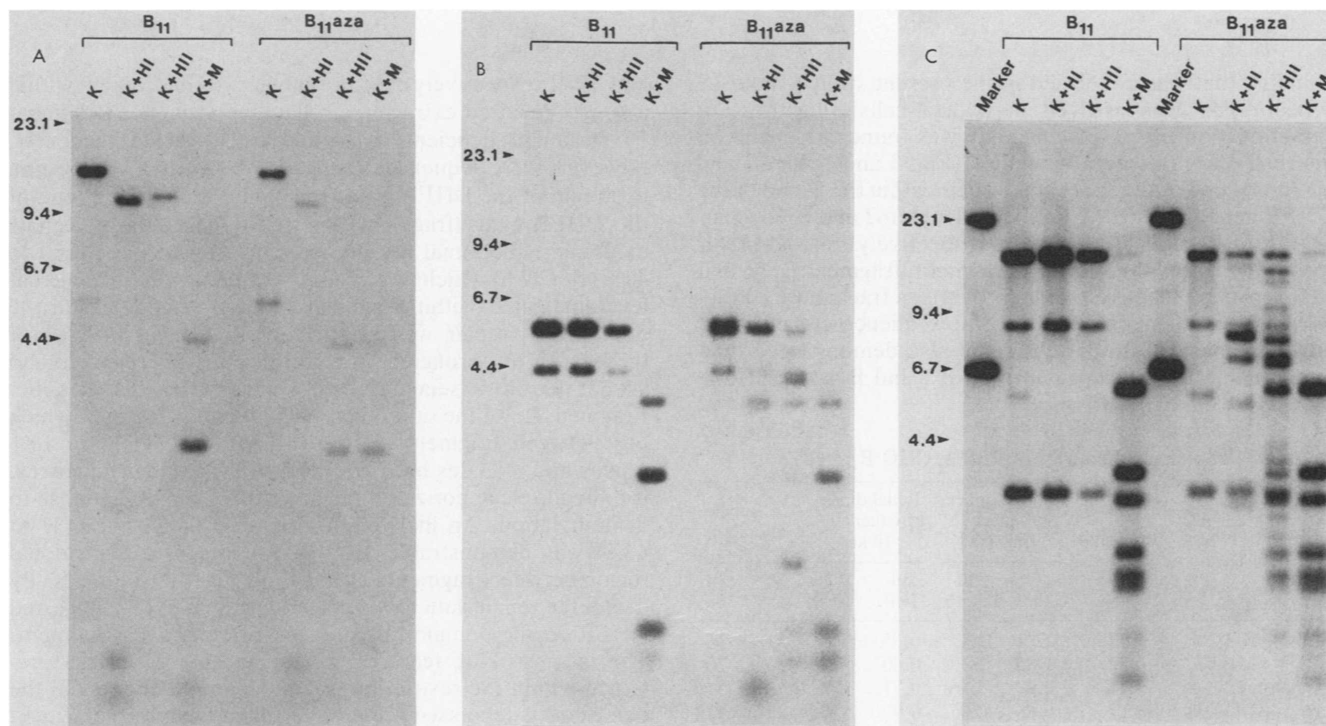

FIG. 1. Southern blot analysis of demethylation induced by 5-aCR treatment within specific regions of the DHFR locus. Total cellular DNA from B_{11} and B_{11}aza cells was digested with *Kpn*I (K) alone or in combination with *Hha*I (HI), *Hpa*II (HII), or *Msp*I (M), electrophoresed in parallel through 0.6% agarose gels, Southern transferred to Genatran nylon membranes, and hybridized with the following nick-translated probes: (A) pMB5, (B) pB6-7H1, and (C) cS14. Sizes are indicated in kilobases.

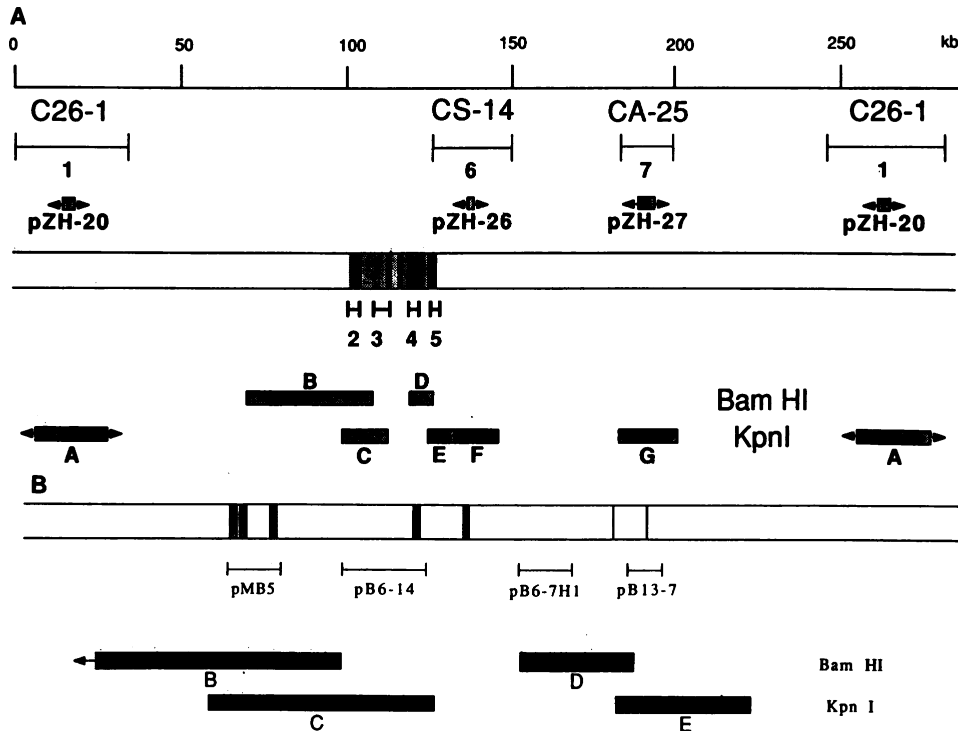

FIG. 2. Schematic diagram of the B_{11} DHFR locus, indicating the location of relevant probes and restriction fragments discussed in the text. (A) Overall view of the DHFR amplification unit in B_{11} cells. In unamplified CHO cells, c26-1 lies downstream of the DHFR gene, but the precise location of the breakpoint in B_{11} cells has yet to be determined. The DHFR transcription unit is represented by the central shaded rectangle, the more darkly shaded regions indicating exon sequences. Letters represent restriction fragments in which DNA repair was assayed, and numbers represent the genomic probes used to detect these specific restriction fragments. The presence of arrows at either end of some boxes representing probes and restriction fragments signifies some uncertainty in the precise location of these sequences. (B) Enlarged, more detailed diagram of the DHFR transcription unit. The shaded regions along the genomic map indicate exon sequences, with the more lightly shaded box at the 3′ end representing the 3′ untranslated region.

a finding that was confirmed in the present study (Table 3). When repair was assayed in B_{11}aza cells, the effect of demethylation on repair efficiency was found to depend on the restriction fragment examined (Fig. 3 and Tables 3 and 4). At one end of the spectrum, repair within the 5′ end of the DHFR gene (fragment C) remained high (62 and 65% repair in 24 h in B_{11} and B_{11}aza cells, respectively), as did repair within the large 30-kb *Bam*HI fragment (fragment B, 56 and 68% repair, respectively) which overlaps fragment C. Fragment G, representing an extragenic sequence considerably downstream from the DHFR gene, also demonstrated relatively high levels of repair in both B_{11} and B_{11}aza cells (48 and 55%, respectively). At the other extreme, repair within a poorly repaired extragenic 3′-flanking sequence (fragment F) remained deficient (0 to 20%). Of greatest interest, however, were sequences representing an extragenic region upstream of the DHFR gene (fragment A) and the 3′ end of the DHFR gene (fragment D). In B_{11} cells, these regions exhibited suboptimal levels of repair (approximately 30 to 40% after 24 h) which were clearly distinct from the deficient levels detected within fragment F. In B_{11}aza cells, on the other hand, repair was significantly increased in each of these cases to "proficient" levels (68 and 66%, respectively) similar to those seen at the 5′ end of the DHFR gene. Fragment E, on the other hand, a 9-kb *Kpn*I fragment which lies between fragments D (66% repair) and F (0 to 10% repair) and includes both DHFR and 3′-flanking sequences, is difficult to categorize on the basis of its repair response to demethylation. An increase of arguable significance (37 to 49%) was demonstrable and may represent a transitional region between fragments D and F. In Fig. 4, we graphically depict the repair data to illustrate the status of the suggested DHFR repair domains before and after 5-aCR treatment. The most striking features include the increased ESS removal within the restriction fragments noted above and the persistently depressed repair levels observed in fragment F.

TABLE 3. Removal of ESS in CHO B_{11} cells

Fragment	Probe used	Fragment size (kb)	Initial dimer frequency/fragment		Initial dimer frequency/10 kb		% Repair after 24 h	
			10 J/m²	20 J/m²	10 J/m²	20 J/m²	10 J/m²	20 J/m²
A	pZH-20	22	1.21	ND[a]	0.55	ND	33	36
B	pMB-5	30	1.80	ND	0.60	ND	56	70
C	pMB-5	14	0.99	1.86	0.71	1.33	62	54
D	pB6-7H1	7	0.68	0.97	0.97	1.38	25	42
E	pB13-7	9	ND	1.39	ND	1.54	ND	37
F	pZH-26	14	0.91	1.78	0.65	1.27	0	0
G	pZH-27	25	1.45	ND	0.58	ND	48	43

[a] ND, Not determined.

Effects of demethylation on UV survival. We also wished to determine whether the global demethylation induced by 5-aCR treatment, the twofold increase in repair synthesis, and the regional increases in ESS removal contribute in any

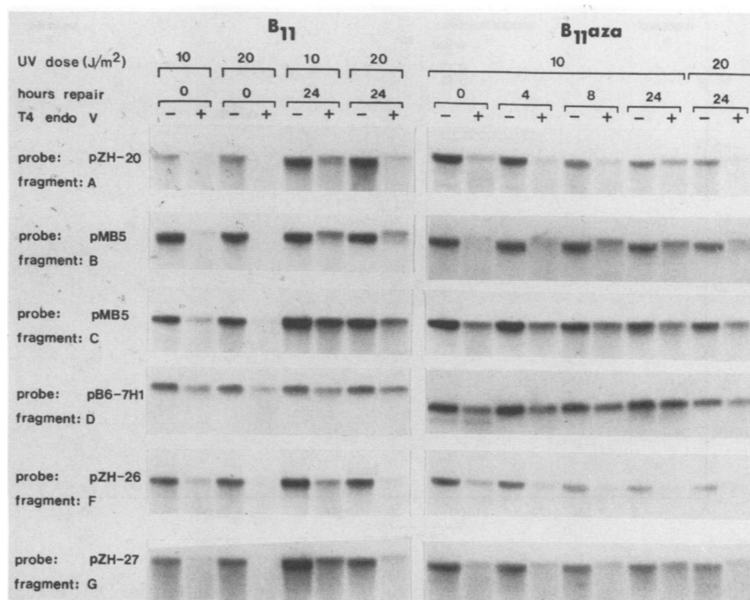

FIG. 3. Southern blot analysis of ESS removal from specific regions of the DHFR gene locus. CHO B_{11} or B_{11}aza cells were irradiated and allowed to repair damage for the periods indicated, and parental-density DNA was isolated by equilibrium centrifugation in neutral CsCl gradients. Two aliquots of each sample were apportioned and treated with T4 endonuclease (endo) V or mock buffer, electrophoresed in parallel through 0.5% alkaline agarose gels, and transferred to Genatran nylon membranes. These were subsequently hybridized to the nick-translated probes shown and exposed to X-ray film following appropriately stringent washes. Only that small portion of each autoradiogram which includes the band of interest is shown. The probe used and the restriction fragment visualized are indicated to the left in each case (see Fig. 1 for the key to the restriction fragments). Note: the autoradiographs depicted are not necessarily those which were quantitated to yield the data given in Tables 3 and 4.

way to enhanced cellular survival. Therefore, we assayed colony-forming ability after UV irradiation in B_{11} and B_{11}aza cells. In two separate trials, we consistently found no significant change in the survival of UV-irradiated cells resulting from passage in 5-aCR (data not shown).

DISCUSSION

This report represents the first detailed, systematic study of the relationship between DNA methylation and DNA repair of UV-induced damage in mammalian cells. A few studies have previously examined methylation levels in newly synthesized DNA following DNA damage in mammalian cells and detected significant hypomethylation in repair patches (24) and in activated genes (29). However, no study has yet dealt with the converse problem of how DNA repair is affected by changes in methylation. To approach this problem, we continuously passaged CHO B_{11} cells in increasing concentrations of 5-aCR to generate a derivative cell line, B_{11}aza, that exhibits 60 to 70% demethylation both within defined sequences and in the genome overall. In the context of these dramatic local and global alterations in methylation levels, significant changes in DNA repair were detected. The amount of repair replication in the overall genome was increased twofold after 24 h in B_{11}aza cells, a difference which cannot readily be accounted for by altered nucleotide pool sizes (as judged by incorporation into replicated DNA). At higher resolution, we found that ESS removal within different regions of the DHFR amplification unit in these cells was affected uniquely within each region despite similar levels of local demethylation.

The differential effects of demethylation on excision repair within different regions of the genome are quite provocative and may provide some insight into possible regulatory factors if one were to systematically correlate these effects with known characteristics of each fragment. Progressing 5′ to 3′ (Fig. 1A), we begin with fragment A, which is presented upstream of the DHFR gene in Fig. 1A but is actually far removed downstream of the gene in the unamplified case (52). Recent work by Looney et al. (30) has localized a

TABLE 4. Removal of ESS in CHO B_{11}aza cells

Fragment	Probe used	Fragment size (kb)	Initial dimer frequency/fragment (10-J/m² dose)	Initial dimer frequency/10 kb (10-J/m² dose)	% repair after:			
					4 h, 10 J/m²	8 h, 10 J/m²	24 h, 10 J/m²	24 h, 20 J/m²
A	pZH-20	22	1.42	0.64	0	25	68	57
B	pMB-5	30	1.99	0.67	37	59	68	75
C	pMB-5	14	1.06	0.76	19	45	65	66
D	pB6-7H1	7	0.71	1.02	0	48	75	66
E	pB13-7	9	0.84	0.93	15	36	61	49
F	pZH-26	14	1.18	0.84	0	0	4	19
G	pZH-27	25	1.72	0.69	3	25	55	64

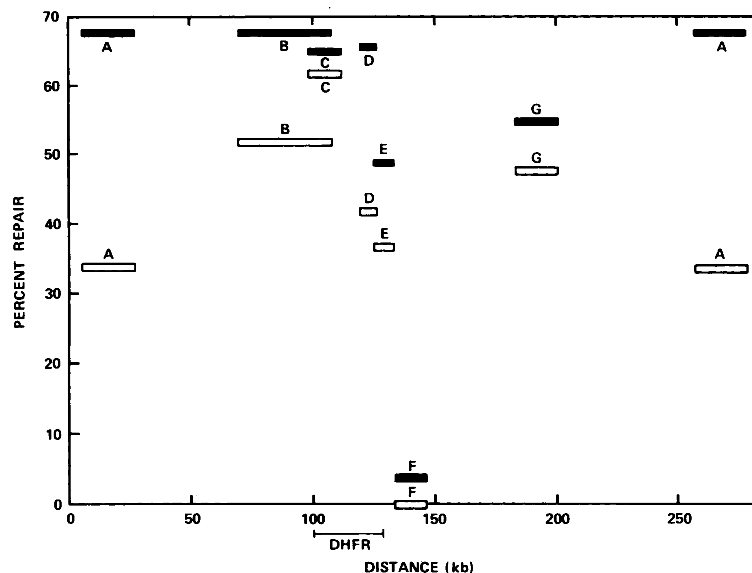

FIG. 4. Schematic diagram illustrating the effects of demethylation on DNA repair within the DHFR repair domain in CHO cells. The location and size of each fragment are indicated on the abscissa, and repair efficiencies are indicated on the ordinate. Open bars represent data for untreated B_{11} cells, and solid bars represent data for 5-aCR-conditioned B_{11}aza cells. The data shown for fragments A, B, C, F, and G are 24-h repair time points after a 10-J/m^2 dose, and the data for fragments D and E are 24-h repair time points after a 20-J/m^2 dose. Data at different doses were chosen as being representative for different fragments in order to demonstrate results obtained with initial dimer frequencies optimal for analysis.

matrix attachment region (MAR) within the 5′ half of c26-1, and a possible transcription unit whose 5′ end overlaps the 3′ end of c26-1 has also been detected (P. Foreman and J. Hamlin, personal communication). From preliminary mapping data (data not shown), we believe that fragment A lies towards the 3′ end of c26-1, relating it more closely with the putative transcription unit. It is within this complicated context that we observed a significant increase in DNA repair (from 34 to 68%) with demethylation. Fragment B incorporates a small portion of the 5′ end of the DHFR gene and extends upstream for many kilobases to include most, if not all, of the divergent transcription unit which has been uncovered in CHO cells (39) as well as in mouse (9) cells. Furthermore, a MAR has also been pinpointed just upstream of the DHFR transcription unit, well within the confines of fragment B. Thus, while a MAR and a transcription unit have been physically linked to but not definitively mapped within fragment A, their presence has been firmly established within fragment B. When one then examines ESS removal within fragment B, one finds relatively proficient repair in both B_{11} and B_{11}aza cells, although a slight increase may be discernible following 5-aCR treatment (56 and 68%, respectively). However, comparing fragments A and B, one finds that repair in untreated B_{11} cells is significantly greater within fragment B than within fragment A. Could this difference somehow reflect the disparate relationships between MARs and transcription units in each case? Zehnbauer and Vogelstein, for example, suggest that a strong functional coupling between transcription and replication exists, teleologically because of the optimization achieved if the relevant multienzyme complexes "were structurally positioned within a skeletal structure such as the nuclear matrix" (60). If, as expected, excision repair in mammalian cells is similarly directed by a multienzyme complex, it is an obvious progression to include repair with replication and transcription at common fixed sites such as MARs as an optimal arrangement. A direct association between repair and transcription is strongly suggested by the work of Mellon et al. (37), and such a unified theory would certainly help to explain the aforementioned results in fragments A and B.

Fragments C, D, and E are either entirely (fragments C and D) or partially (fragment E) encompassed by the DHFR transcription unit and, at last count, do not harbor any MARs within their confines. However, DNA repair appears to follow a decreasing gradient in the 5′ to 3′ direction in B_{11} cells, progressing from 54 to 42 to 37% (24-h repair following a UV dose of 20 J/m^2). Following demethylation, repair in fragments C and E is marginally increased to 66 and 49%, respectively, whereas repair within fragment D is more substantially increased (to 66%). Put another way, demethylation results in an expansion of the region in which ESS removal is maximally efficient to include most of the 3′ half of the DHFR gene, with possibly a slight diminution in repair efficiency at the extreme 3′ end. However, this slight decrease may in fact be attributable to that fraction of fragment E which lies beyond the 3′ border of the DHFR transcription unit (if one believes in a strict quantitative relationship between repair and transcription). Such an argument would conveniently obviate the need to suggest a gradient of repair within the DHFR gene in B_{11}aza cells at all. On the other hand, the gradient observed in 5-aCR-naive B_{11} cells is not so easily dismissed, particularly concerning the data from fragment D. One possible explanation is that the putative repair complex follows a processive modus operandi but somehow loses competence or experiences a greater probability of dissociation from its substrate with increasing distance from its point of initiation (hypothetically located in the vicinity of the MAR immediately upstream of the DHFR gene).

Finally, we come to fragments F and G. Fragment F is a 14-kb *Kpn*I fragment immediately downstream from fragment E, yet unlike any of the other fragments, it exhibits little or no repair (0 to 20% after 24 h) in either B_{11} or B_{11}aza

cells. In contrast, fragment G exhibits relatively proficient repair in both cases (48 and 55%, respectively). Details concerning these two fragments remain sketchy, yet it is probable that they differ in their transcriptional activity (fragment F seems to be quiescent, whereas fragment G yields detectable message in nuclear run-on experiments; P. Foreman and J. Hamlin, personal communication) and that fragment F harbors or is very close to a recently mapped replication initiation site.

The discovery of strand-specific repair at the CHO DHFR locus introduces a heterogeneity which requires judicious use of the Poisson expression, which assumes random introduction and removal of lesions from a homogeneous population of restriction fragments (discussed in reference 37). Two implications of this finding are particularly relevant to this study: an overestimate of repair may be expected as the initial dimer frequency per fragment increases, and an asymptotic maximum of 50 to 60% repair exists if one assumes little or no repair of nontranscribed strands. In consideration of the first implication, we used two UV doses (10 and 20 J/m^2) whenever possible in order to ensure an optimal initial dimer frequency (approximately 1.0 dimer per fragment) for subsequent Poisson calculations. Another practical consideration which is closely tied in with this problem of UV dose is densitometric quantitation of bands on autoradiographs. As the initial dimer frequency per fragment increases, the disparity in band intensities between T4 endonuclease V-treated and untreated lanes increases, pushing both the limit of sensitivity of the densitometer at the lower end and the linear response range of the film at the upper end. This explains, for example, why we have presented data at different UV doses in Fig. 4 and why data representing identical repair periods but different UV doses may differ more than one would otherwise hope or expect. The particular relevance to this study of the second point raised is that one loses the sensitivity to detect increases in repair at 24-h time points if repair in the 5-aCR-untreated case is already high (e.g., fragments B and C). Therefore, it would be profitable to examine earlier time points in the hope that kinetic differences would be uncovered. Such data for B_{11} cells have been reported previously by us (5), and we turn to these as a baseline for comparison against B_{11}aza data presented here. Repair in B_{11} cells after a 20-J/m^2 dose and 8 h of repair was reported to be 37 and 43% in fragments B and C, respectively; for B_{11}aza cells, we now report values of 59 and 45%, respectively, following a UV dose of only 10 J/m^2. Thus, following demethylation, a significant increase in repair is apparent for fragment B but not for fragment C.

To determine whether the phenomenon of enhanced DNA repair following demethylation has any biological significance, we compared the UV sensitivities of B_{11} and B_{11}aza cells in colony-forming assays following UV irradiation. No significant differences in survival rates were noted at any UV dose examined. However, these results might not be entirely unanticipated. If one assumes that the CHO DNA repair machinery is capable of unambiguously targeting essential sequences at the expense of unessential sequences, then any improvement in the repair of the latter might be phenotypically silent in survival assays. Thus, this negative result could be argued as confirmation of our basic hypothesis.

Mechanistically, how could changes in methylation influence or direct DNA repair processes in the eucaryotic cell? In principle, one may envision both *cis*- and *trans*-acting effects of demethylation, the former being defined by a requirement for linkage in the genetic sense between the critical site(s) of demethylation and the specific locus under examination with respect to repair. In consideration of the sizable literature demonstrating gene activation following 5-aCR treatment, it is possible, for example, that the expression of a significant number of genes has been up-regulated in B_{11}aza cells. If transcription does play a primary role in determining the efficiency of DNA repair, then such gene activation may contribute towards the twofold increase in repair replication by enhancing the repairability of a corresponding fraction of the genome. Alternatively, among the up-regulated genes may be one or more genes coding for proteins involved in DNA repair itself. Indeed, such a mechanism has already been proposed to explain the high-frequency reversion of mutant X-ray-sensitive CHO-K_1 cells to wild-type phenotypes following 5-aCR treatment (21). A second possible effect of 5-aCR is related to its adverse effects on RNA and protein synthesis (27, 31, 32, 47, 56). These metabolic perturbations might induce a DNA repair system, leading to an increased level of DNA repair. Regardless of their nature, such adverse effects of 5-aCR were a major concern to us, and we attempted to minimize them by growing B_{11}aza cells in 5-aCR-free medium for at least 24 h prior to the introduction of DNA damage, as well as during the repair period itself. As a control, we assayed DNA methylation in B_{11}aza cells grown for up to 3 days in the absence of 5-aCR; no significant remethylation was detectable. Since the half-life of 5-aCR is approximately 6 h in phosphate-buffered saline (pH 7.4) at 37°C (P. G. Constantinides, M.Sc. thesis, University of Stellenbosch Medical School, Stellenbosch, South Africa, 1977), it is probable that very little 5-aCR remains at the time that repair is initiated. In addition, RNA turnover would allow clearance of 5-aCR-substituted RNA species from cells, which is important because the primary action of 5-aCR in all instances so far studied is to inhibit methyltransferases, and this activity appears to be dependent on the incorporation of 5-aCR into the appropriate enzyme substrate (i.e., RNA or DNA) (23).

Of more immediate interest are the *cis*-acting effects of demethylation. Seemingly, methylation must act in concert with other factors to determine repair efficiency. Since methylation has frequently been associated with an "inactive" or nuclease-resistant chromatin structure (17, 26), one possible scenario might involve one or more methylated DNA-binding proteins which would assemble an additional level of chromatin complexity capable of excluding DNA repair enzymes. That chromatin structure can indeed interfere with DNA repair has been demonstrated previously (20). Also, a putative methylated DNA-binding protein has been partially purified from human placental nuclei (54), and binding-substrate experiments reveal that the dinucleotide CpG in its methylated state is necessary but not sufficient for its binding (12, 55).

From the data presented here, a persuasive case can be made for transcription as a principal determinant of repair. Methylation, while not absolute in its effect on repair, does seem to play an active role, perhaps within the context of preexistent transcriptional activity. An interesting feature of the enhanced repair observed in demethylated cells is that the asymptotic maximum of roughly 60% repair is maintained. This suggests that the optimal level of repair under any circumstances is already achieved in specific regions in normal cells; demethylation merely serves to improve suboptimal repair elsewhere in the genome. Since optimal repair in 5-aCR-untreated cells is achieved by virtually complete repair of the transcribed strand accompanied by very poor repair of the nontranscribed strand, one might predict that methylation selectively enhances repair of the nontran-

scribed strand. Experiments to test this hypothesis are under way.

The stimulation of DNA repair by demethylation appears to depend on the degree of demethylation induced, since no significant increase in repair replication was noted at early stages of genesis of the B_{11}aza cell line. This idea is supported by data from parallel experiments with Southern blot analysis, which qualitatively demonstrate markedly less demethylation at these preliminary stages. In the context of a methylated DNA-binding protein discussed above, one might suggest that enough protein must be released in order to allow access by repair enzymes. Alternatively, one can imagine the existence of a subset of methylated sites which are relatively refractory to demethylation but crucial in determining the efficiency of DNA repair. This idea is supported by evidence demonstrating various degrees of demethylation at different sites and by the fact that specific critical sites have been proposed in the regulation of gene expression (23, 44).

As a point of interest, we have noted that the efficiency of 5-aCR in inducing demethylation varies from site to site. For instance, in comparing methylation at *Hha*I (GCGC) and *Hpa*II and *Msp*I (CCGG) sites, it was consistently found that less demethylation was induced at *Hha*I sites than at *Hpa*II and *Msp*I sites. This difference was noted whether total genomic DNA was analyzed by velocity sedimentation through neutral sucrose gradients or specific sequences were examined by probing membranes derived from agarose gels. Since methylation at cytosine residues occurs primarily within the central CpG dinucleotides common to both recognition sequences, the possibility of local sequence effects is raised, although alternative mechanisms such as dissimilar local chromatin conformations which might concomitantly define a region's repair characteristics cannot be eliminated.

The function of 5-mC residues in eucaryotic cells is probably multifaceted and may be complicated by different roles at different sites and nonunanimity of function among distinct but analogous sites (for example, witness the varied correlations between gene expression and methylation at the 5′ ends of genes). More recently, evidence has been presented suggesting the existence of methylation-based mismatch repair systems in eucaryotes similar to those found in *Escherichia coli* (16, 18). Now, we propose yet a third possible role for methylation in eucaryotic cells, as a determinant of DNA repair.

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