# 3-Methyladenine and 7-Methylguanine Exhibit No Preferential Removal from the Transcribed Strand of the Dihydrofolate Reductase Gene in Chinese Hamster Ovary B11 Cells†

Wei Wang, Anuradha Sitaram, and David A. Scicchitano*

*Department of Biology, New York University, New York, New York 10003*

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ABSTRACT: The removal of cylclobutane pyrimidine dimers from cellular DNA occurs preferentially in actively transcribed genes of cells subjected to ultraviolet radiation. In contrast, reports concerning the transcription-dependent repair of *N*-methylpurines formed in cellular DNA following exposure to methylating agents are quite conflicting, with some studies suggesting that no biased clearance of these lesions occurs and others indicating that preferential removal of these adducts transpires in active genetic loci. Even in the cases where no preferential clearance was demonstrated, a slight but statistically insignificant biased removal of *N*-methylpurines from the transcribed strand of active genes was often evident. We proposed that these results might be due to the preferential clearance of only one of the two principal *N*-methylpurines formed, 3-methyladenine, or to the source of the methylating species to which the cells were exposed. Therefore, we investigated the clearance of 3-methyladenine and 7-methylguanine as individual lesions from the amplified dihydrofolate reductase gene of Chinese hamster ovary cells, and we examined the gene-specific removal of *N*-methylpurines formed by several different methylating agents as well. We observed no biased clearance of 3-methyladenine toward the transcribed strand of the locus being examined. This result indicates that any minor gene-specific preferential repair that has been observed previously for *N*-methylpurines *in toto*—which actually reflects the removal of the predominant methylated purine 7-methylguanine—is not due to biased clearance of the transcription-inhibiting 3-methyladenine lesion. Likewise, we found no preferential clearance of *N*-methylpurines from the transcribed strand of the dihydrofolate reductase gene, regardless of the source of the methylating species employed to damage the DNA. These collected data demonstrate that neither 3-methyladenine nor 7-methylguanine is cleared in a transcription-dependent manner from the active dihydrofolate reductase gene of Chinese hamster ovary cells.

Methylating agents are a group of chemical compounds that produce a broad spectrum of mutagenic and toxic damage in the genome. *N*-Methylpurines, of which 7-methylguanine (7mG)[1] and 3-methyladenine (3mA) are the primary constituents, comprise the majority of the lesions induced by these chemicals (Singer & Grunberger, 1983). 7mG is considered to be an innocuous lesion (Pegg, 1984); in contrast, 3mA has been implicated in the toxicity associated with methylating agents, possibly due to its ability to inhibit elongation during DNA and RNA synthesis (Larson et al., 1985; Lindahl, 1986; Strauss et al., 1986; Schwartz, 1989; Racine & Mamet-Bratley, 1993). Both 3mA and 7mG are positively charged when present in DNA and have N-glycosylic bonds that are more unstable than those for unmodified purines, resulting in the formation of alkali-labile sites (ALSs) in DNA following depurination (Singer & Grunberger, 1983).

Removal of damage from cellular DNA is not necessarily a homogeneous process that occurs to the same extent or at the same rate in all portions of the genome (Hanawalt, 1991; Scicchitano & Hanawalt, 1992; Hanawalt & Mellon, 1993). Biased clearance of damage from discrete, transcriptionally active domains of DNA has been documented for a variety of lesions, including those derived from ultraviolet light (Bohr et al., 1985; Madhani et al., 1986; Mellon et al., 1986, 1987), benzo[*a*]pyrene (Chen et al., 1992), benzo[*c*]phenanthrene (Carothers et al., 1992), hydrogen peroxide (Leadon & Lawrence, 1992), and CC-1065 (Tang et al., 1994b), among others. In contrast to these examples, data concerning the transcription-dependent repair of ALSs resulting from the presence of *N*-methylpurines formed in cellular DNA following exposure to methylating agents have been less consistent. ALSs were not removed at a faster rate from the transcribed strand of the Chinese hamster ovary (CHO) B11 dihydrofolate reductase (*dhfr*) gene when compared to either the nontranscribed strand or nearby unexpressed loci following exposure to dimethyl sulfate (DMS). During the course of these studies, however, a statistically insignificant but reproducible bias in the repair of these lesions, with approximately a 5−7% enhancement toward the transcribed strand, was observed (Scicchitano & Hanawalt, 1989, 1990). Independent investigations from other laboratories produced

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* Corresponding author.

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[1] Abbreviations: 7mG, 7-methylguanine; 3mA, 3-methyladenine; ALSs, alkali-labile sites; CHO, Chinese hamster ovary; *dhfr*, dihydrofolate reductase; DMS, dimethyl sulfate; *hprt*, hypoxanthine phosphoribosyltransferase; MMS, methyl methanesulfonate; MNU, *N*-methyl-*N*-nitrosourea; kb, kilobase; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; HBSS, Hanks' balanced salt solution; DMSO, dimethyl sulfoxide; 6mG, $O^6$-methylguanine; mPs, methylphosphotriesters.

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corroborative data using the same damaging agent, cell line, and gene (May et al., 1993). Likewise, no differences in the removal of ALSs from the active *dhfr* and hypoxanthine phosphoribosyltransferase (*hprt*) genes and the silent Duchenne muscular dystrophy gene of cultured T-lymphocytes were observed following exposure to methyl methanesulfonate (MMS) (Bartlett et al., 1991). In contrast, when *N*-methyl-*N*-nitrosourea (MNU) was used as the source of damage, the *dhfr* gene in CHO cells was cleared of approximately 60% of the ALSs in 24 h, whereas the c-*fos* gene was not repaired; the total genomic clearance of ALSs in the same time frame was 70% (LeDoux et al., 1990b). The clearance rates reported in these studies were slower than those for repair following exposure to DMS (Scicchitano & Hanawalt, 1989, 1990). Likewise, removal of ALSs was significantly more efficient from the actively expressed insulin gene in rat RINr 38 cells when compared to the unexpressed insulin gene from rat RINr B2 cells following exposure to MNU; importantly, the initial lesion frequencies in both cell lines were the same (LeDoux et al., 1990a). Individual strand repair was not ascertained in the loci examined in the last two studies mentioned.

These results suggest that preferential removal of *N*-methylpurines from transcribed regions of DNA might exist under certain circumstances. We reasoned that the incongruities in the reported data might be attributable to the biased clearance of one of the two principal *N*-methylpurines formed, 3mA, or to the source of the methylating species to which the cells were exposed. In order to address these issues, we investigated two distinct aspects of *N*-methylpurine repair in an active gene: (i) the specific nature of transcription-dependent repair as it relates to 3mA; and (ii) the role of the methylating agent used in the studies. With regard to item (i), if 3mA were shown to exhibit strand-biased repair kinetics due to its ability to impede elongation during RNA synthesis, this might offer an explanation for the slight but statistically insignificant repair bias toward the transcribed strand of the *dhfr* gene in CHO B11 cells (Scicchitano & Hanawalt, 1989). When *N*-methylpurine repair was analyzed as a whole by assessing the clearance of ALSs, the results primarily reflected the removal of the most abundant lesion, 7mG, which accounts for approximately 80% of the *N*-methylpurines. Repair of 3mA would have been masked in such experiments, and any potentially biased removal of it would not have been seen easily unless its clearance were assessed independently. In terms of item (ii), the other consistent feature of reports where biases in *N*-methylpurine-induced ALSs were observed was that the source of methylation damage was MNU which damages DNA via $S_N1$ kinetics (Ledoux et al., 1990a,b). In cases where $S_N2$ methylating agents such as DMS were used as sources of genomic damage, no relationship between transcription and DNA repair was observed (Scicchitano & Hanawalt, 1989, 1990; Bartlett et al., 1991). Therefore, an examination of the effect of the source of the methylating species on transcription-dependent *N*-methylpurine repair was undertaken.

In this paper, transcription-dependent DNA repair will refer to the differential removal of damage from the transcribed strand of an active genetic domain as opposed to the nontranscribed strand; the process can result in a biased, preferential clearance of lesions from the transcribed strand of the expressed locus. Additional important factors that contribute to heterogeneous repair of the genome, including the accessibility of the lesion to repair proteins and the effects of chromatin structure, should be distinguished from this phenomenon (Hanawalt, 1993).

For these studies, we concentrated our efforts on comparing repair rates between the transcribed and nontranscribed strands of the *dhfr* gene, with the immediate goal being to determine if transcription-dependent repair was at work on the adducts in question. The specific target genetic locus was the 14 kilobase (kb) *Kpn*I restriction fragment of the amplified *dhfr* gene of CHO B11 cells; this particular region encompasses approximately half of the gene and spans the first four exons (Mellon et al., 1987; Scicchitano & Hanawalt, 1989). This was chosen for ease of detection during Southern analysis, and because it allowed us to make comparisons among numerous published studies concerning preferential DNA repair where adduct clearance in this region was examined.

Our results demonstrate that preferential removal of transcription-impeding 3mA from the transcribed strand of the *dhfr* gene of CHO B11 cells does not occur. Also, the source of the methylating species has no effect on the clearance of *N*-methylpurine-induced ALSs: They are cleared equally well from both strands of the *dhfr* gene. These data are discussed in light of current observations concerning the removal of other types of DNA damage, methylation-induced mutagenesis, and their implications for the mechanism and global nature of transcription-dependent DNA repair.

## MATERIALS AND METHODS

*Materials.* CHO B11 cells harboring the amplified *dhfr* gene were from the laboratory of Robert Schimke (Department of Biological Sciences, Stanford University, Stanford, CA) (Kaufman & Schimke, 1981). The restriction endonuclease *Kpn*I was obtained from GibcoBRL Life Technologies (Gaithersburg, MD); all other enzymes were obtained from either Promega (Madison, WI), New England Biolabs (Beverly, MA), or United States Biochemical (Cleveland, OH). Methylating agents were procured from a variety of sources: DMS, MMS, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI), and MNU was obtained from Sigma Chemical Co. (St. Louis, MO). *Escherichia coli* strain MS23 and the plasmid pCY5 harboring the *E. coli tag* gene were the generous gifts of Dr. Mutsuo Sekiguchi (Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan). Purified T4 endonuclease V was kindly provided by Dr. R. Stephen Lloyd (Center for Molecular Science, University of Texas Medical Branch, Galveston, TX). Plasmids used as a source of probes in this work were made available by Dr. Isabel Mellon (Department of Pathology, University of Kentucky, Lexington, KY) and Drs. Graciela Spivak and Ann Ganesan (Department of Biological Sciences, Stanford University, Stanford, CA).

*Cell Culture.* CHO B11 cells were maintained as follows. The cells were cultured in minimal essential medium containing 0.2% $NaHCO_3$ that was supplemented with nonessential amino acids, penicillin, streptomycin, 500 nM methotrexate, and 10% dialyzed fetal bovine serum. The cells were grown at 37 °C in a humidified 5% $CO_2$/95% air atmosphere.

*Purification of 3mA−DNA Glycosylase I.* 3mA−DNA glycosylase I was purified from *E. coli* MS23 harboring the pCY5 plasmid carrying the *tag* gene encoding the glycosylase. The procedure for purification was as described in the literature (Sakumi et al., 1986).

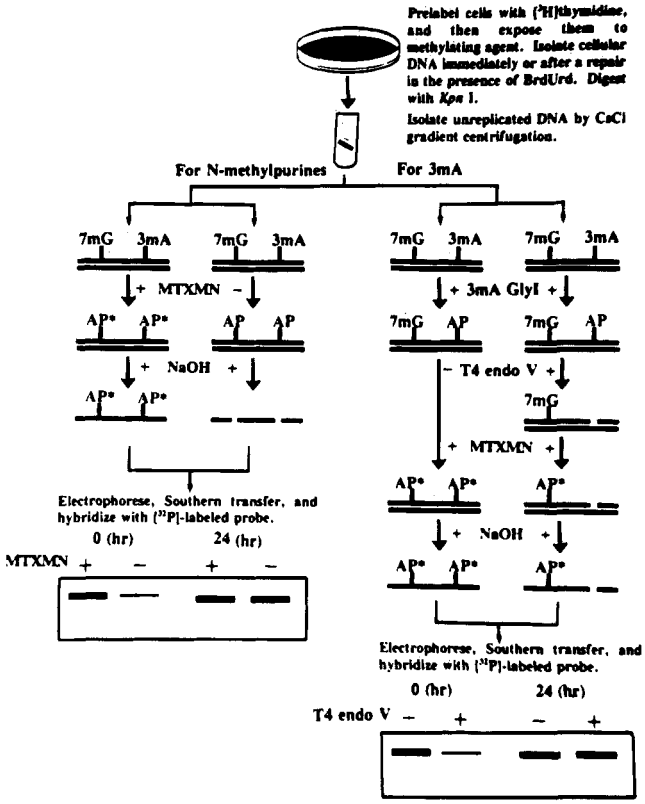


FIGURE 1: Methods for assessing repair of ALSs derived from *N*-methylpurines or 3mA are shown schematically. Methoxyamine (MTXMN) is used in both protocols as a means of protecting ALSs from degradation by NaOH.

*Exposure of Cells to Methylating Agents.* These procedures were adapted from previous studies (Scicchitano & Hanawalt, 1989; Bartlett et al., 1991). Prior to exposure to methylating agents, the CHO B11 cellular DNA was labeled by growing the cells in medium supplemented with 10 $\mu$M [*methyl*-$^3$H]thymidine (0.1 $\mu$Ci/mL) for approximately 16 h. The medium was then removed, the cells were washed twice with Hanks' balanced salt solution (HBSS), and 10 mL of fresh serum-free medium was added to each flask. The desired methylating agent was dissolved in dimethyl sulfoxide (DMSO), and 50 $\mu$L was added to the cells to obtain the desired concentration of agent. DMSO alone was added to several flasks to obtain undamaged control DNA. The cells were incubated at 37 °C for 30 min and then washed twice with HBSS to remove any residual methylating compound. Cells were immediately lysed or permitted to recover in complete medium supplemented with 40 $\mu$M 5-bromo-2′-deoxyuridine and 4 $\mu$M 5-fluoro-2′-deoxyuridine to density label cellular DNA.

*Assessment of Repair of 3mA in Discrete Domains of Genomic DNA.* The clearance of 3mA-induced ALSs from discrete domains of the genome was determined by adapting methods that were previously published (Bohr et al., 1985; Scicchitano & Hanawalt, 1990); the procedure is summarized in Figure 1. The removal of 3mA was assessed by using purified 3mA−DNA glycosylase I from *E. coli* in conjunction with T4 endonuclease V. The source of methylation damage was DMS; this agent was chosen because it produces primarily 3mA and 7mG. The cells were exposed to the agent as described above. Following the desired repair time, the medium was removed, and the cells were washed with HBSS and lysed with a solution of 10 mM Tris·HCl (pH 8.0)/1 mM EDTA/0.5% SDS containing 100 $\mu$g/mL proteinase K. The DNA was isolated by phenol extraction and cut with the restriction endonuclease *Kpn*I at a concentration of 5 units/$\mu$g of DNA. Replicated DNA was removed by centrifugation through CsCl. Fractions containing parental DNA were combined, dialyzed to remove CsCl, and concentrated with a Model SC110 Speed Vac (Savant Instruments, Inc., Farmingdale, NY). The samples for each recovery time were divided into two aliquots, and 3mA was removed specifically by the addition of 3mA−DNA glycosylase I to a final concentration of 2.5 ng/$\mu$g of DNA in 20 mM Tris·HCl (pH 7.5)/100 mM NaCl/8.5 mM 2-mercaptoethanol/10 mM EDTA. The resulting apurinic sites were incised in one aliquot of each pair by the addition of T4 endonuclease V at a concentration of 0.6 ng/$\mu$g of DNA; the other aliquot was not incised and acted as the source of full-length DNA. All samples were then heated at 60 °C for 6 h in the presence of 5 mM methoxyamine at pH 7.5 to remove heat-labile 7mG and reduce the apurinic sites to protect them from alkaline hydrolysis. This final step was done to ensure that no additional ALSs were formed after the removal of 3mA. The resulting fragments of DNA were resolved in 0.5% alkaline agarose gels, transferred to MSI Magna NT nylon membranes (Fisher Scientific, Pittsburgh, PA), and hybridized with $^{32}$P-labeled DNA to visualize repair in both strands of the locus or with $^{32}$P-labeled RNA probes to analyze repair in the individual strands. Radiolabeled probes were prepared as described elsewhere (Mellon et al., 1987; Scicchitano & Hanawalt, 1989). All hybridizations were performed with QuikHyb (Stratagene, La Jolla, CA) (Deeley et al., 1991). Bands corresponding to the full-length *Kpn* I restriction fragments located within the *dhfr* gene were quantified using a Model GS-250 Molecular Imager (Bio-Rad Laboratories, Hercules, CA). For each sample, the ratios of the band intensities for the DNA hydrolyzed with T4 endonuclease V to that which was not incised were calculated to yield a value of $P_0$. By using the Poisson distribution, the actual lesion frequency was determined as $-\ln(P_0)$.

*Assessment of Repair of N-Methylpurines in Discrete Domains of Genomic DNA.* The clearance of *N*-methylpurine-induced ALSs from discrete domains of the genome was determined by methods previously published (Bohr et al., 1985; Scicchitano & Hanawalt, 1989); the procedure is described in Figure 1. Following the desired repair time, the medium was removed, the cells were washed and lysed, the DNA was isolated and cut, and the replicated DNA was removed as described above. Equal portions of each DNA sample were separated into two aliquots and heated at 50 °C for 6 h. During this period, one sample was incubated in the presence of 5 mM methoxyamine at pH 7.5; this provided essential, intact control samples of DNA at each time point. Unprotected ALSs were hydrolyzed with 30 mM NaOH for 30 min. Denaturing agarose gel electrophoresis, Southern analysis, and quantitation of bands were performed as described above for the repair of 3mA. For each sample, the ratios of the band intensities for the unprotected DNA to the methoxyamine-protected control DNA were calculated to yield $P_0$, and the actual lesion frequency was determined as $-\ln(P_0)$.

## RESULTS

*Purification of 3mA−DNA Glycosylase I.* The notion that 3mA might be subject to biased DNA repair due to its ability to impede transcription could only be tested if a method for

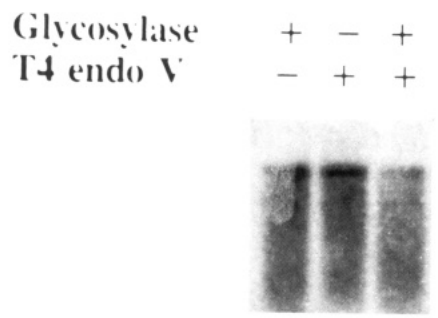


FIGURE 2: Autoradiogram demonstrating enzymatic incision of DNA containing 3mA. DNA was rapidly isolated from CHO B11 cells exposed to 1 mM DMS for 30 min. It was cut with *Kpn*I, and the fragments were treated in the presence (+) or absence (−) of 3mA−DNA glycosylase I and T4 endonuclease V (T4 endo V) as indicated. The DNA was then heated for 6 h at 50 °C in the presence of methoxyamine. The samples were resolved by denaturing agarose electrophoresis using a 0.5% gel in 30 mM NaOH/1 mM EDTA. The DNA was then transferred to a membrane and hybridized with $^{32}$P-labeled DNA probes for the *dhfr* gene. The resulting bands were quantified with a GS-250 Molecular Imager.

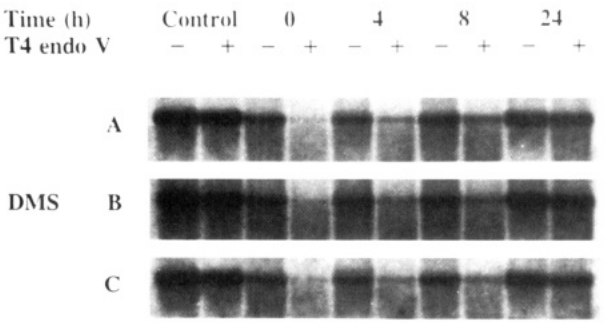


FIGURE 3: Autoradiogram demonstrating the repair of ALSs induced by the removal of 3mA in the *dhfr* gene of CHO B11 cells. CHO B11 cells were exposed to 1 mM DMS for 30 min, and the DNA was rapidly isolated. It was cut with *Kpn*I, and the samples were divided into two equal aliquots containing 2 $\mu$g of DNA. The samples were treated with 3mA−DNA glycosylase I. Following this, one sample from each pair was digested with T4 endonuclease V (T4 endo V) as indicated (+); no T4 endo (−) was added to the other sample in each pair in order to obtain full-length control DNA. The DNA was then heated for 6 h at 50 °C in the presence of methoxyamine, and the samples were resolved by denaturing agarose electrophoresis using a 0.5% gel in 30 mM NaOH/1 mM EDTA. The DNA was then transferred to a membrane and hybridized with $^{32}$P-labeled probes to reveal repair in (A) the *dhfr* gene, (B) the transcribed strand of the *dhfr* gene, or (C) the nontranscribed strand of the *dhfr* gene. The same filter was used throughout for each of these analyses. $^{32}$P-Labeled DNA or RNA probe was removed each time by washing the filter in 0.4 M NaOH at 42 °C for 30 min and neutralizing it in 0.2 M Tris·HCl (pH 7.5)/0.5% SDS. The resulting bands were quantified with a GS-250 Molecular Imager.

specifically removing it from damaged DNA could be devised. Such a protocol is described under Materials and Methods and in Figure 1. It is dependent upon obtaining purified 3mA−DNA glycosylase I, an enzyme that specifically removes 3mA from DNA, leaving an apurinic site in its place. It must be emphasized that this particular enzyme has no other associated activities: It is quite specific for 3mA and does not remove other damaged bases from DNA (Sakumi & Sekiguchi, 1990). The method for purifying it was based on that described by Sakumi et al. (1986). By following their procedure, we obtained a fraction of protein containing three bands as assessed by SDS−polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue R. The most intense band had an $M_r$ of 21 000, corresponding to the size of 3mA−DNA glycosylase I. In functional assays, this fraction contained enzyme activity that was diminished in the presence of free 3mA, an inhibitor of this particular glycosylase (data not shown).

*Quantitation of 3mA-Induced ALSs Formed in Cellular DNA following Exposure to DMS.* Prior to establishing the actual repair rates for 3mA in a transcribed region of DNA, a dose of DMS suitable for inducing approximately one 3mA lesion per 14 kb of DNA needed to be established—recall that this is the size of the *Kpn*I restriction fragment located in the CHO B11 *dhfr* gene being examined. DMS generates 7mG and 3mA as approximately 74% and 18% of the DNA damage, respectively (Singer & Grunberger, 1983). ALSs due to 3mA were measured using 3mA−DNA glycosylase I and T4 endonuclease V as shown in Figure 1, with the exception that the DNA was not subjected to CsCl buoyant density gradient centrifugation. This step was not required because the cells were lysed immediately after the desired exposure time, and no DNA repair recovery period was allowed. Prior to using this technique, it was shown that both 3mA−DNA glycosylase I and T4 endonuclease V were necessary for cutting the DNA at sites having a 3mA lesion: Either enzyme alone did not incise the DNA as shown in Figure 2. By using these two enzymes and measuring the ALSs at various doses of DMS, it was determined that exposing CHO B11 cells to 1 mM DMS for 30 min at 37 °C resulted in one 3mA per 14 kb *Kpn*I restriction fragment (data not shown).

*Repair of 3mA-Induced ALSs in the dhfr Gene of CHO B11 Cells.* In order to determine if 3mA exhibits biased repair in the transcribed strand of the *dhfr* gene, CHO B11 cells were exposed to 1 mM DMS for 30 min. Figure 3 shows the autoradiograms for the repair of 3mA at different time points in both strands of the *dhfr* gene as well as the individual transcribed and nontranscribed strands of this same locus. Repair of 3mA did occur, and the results are presented in Table 1. The clearance of 3mA from both strands of the *dhfr* gene reached a level of 81% in 24 h; repair in the transcribed and nontranscribed strands reached 77% and 84%, respectively, in the same time frame. No significant differences in the repair rates for this adduct could be observed between the DNA strands in the *dhfr* gene at this level of damage. In order to be certain that strand-biased repair did not occur at earlier time points, we examined the removal of 3mA at 2 h; both strands were cleared of 3mA-induced ALSs to a level of 20%, indicating that no preferential repair of this lesion occurs at early times following exposure to a methylating agent (data not shown).

*Quantitation of ALSs in Cellular DNA following Exposure to $S_N1$ or $S_N2$ Methylating Agents.* The notion that biased DNA repair exists for ALSs generated by $S_N1$ methylating agents but not for those sites formed following exposure to $S_N2$ methylating chemicals needed to be explored systematically. In order to begin these investigations, it was necessary to establish effective doses for the methylating agents chosen for these studies such that each would yield approximately one *N*-methylpurine-induced ALS per 14 kb of DNA. CHO B11 cells were exposed to several concentrations of MMS, MNU, or MNNG, and the number of ALSs formed by each agent was established, again with the exception that the DNA was not subjected to CsCl buoyant density gradient centrifugation. The results of these studies indicated that the following doses of each methylating agent would yield the desired level of damage of one ALS per 14 kb fragment of

Table 1: Removal of 3mA from the *dhfr* Gene of CHO B11 Cells following Exposure to DMS[a]

| | ALSs/14 kb *Kpn*I fragment at time (h) | | | | % repair at time (h) | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 4 | 8 | 24 | 0 | 4 | 8 | 24 |
| *dhfr* gene[b] | 1.32 ± 0.17 | 0.73 ± 0.22 | 0.63 ± 0.19 | 0.25 ± 0.03 | 0 | 45 ± 8 | 53 ± 8 | 81 ± 3 |
| TS[c] | 1.23 ± 0.21 | 0.76 ± 0.15 | 0.71 ± 0.20 | 0.27 ± 0.04 | 0 | 40 ± 8 | 43 ± 8 | 77 ± 7 |
| NTS[d] | 1.18 ± 0.14 | 0.75 ± 0.14 | 0.62 ± 0.16 | 0.20 ± 0.03 | 0 | 40 ± 8 | 48 ± 8 | 84 ± 4 |

[a] "±" is standard error of the mean. [b] *dhfr* gene, both strands of the *Kpn* I fragment. [c] TS, transcribed strand. [d] NTS, nontranscribed strand.

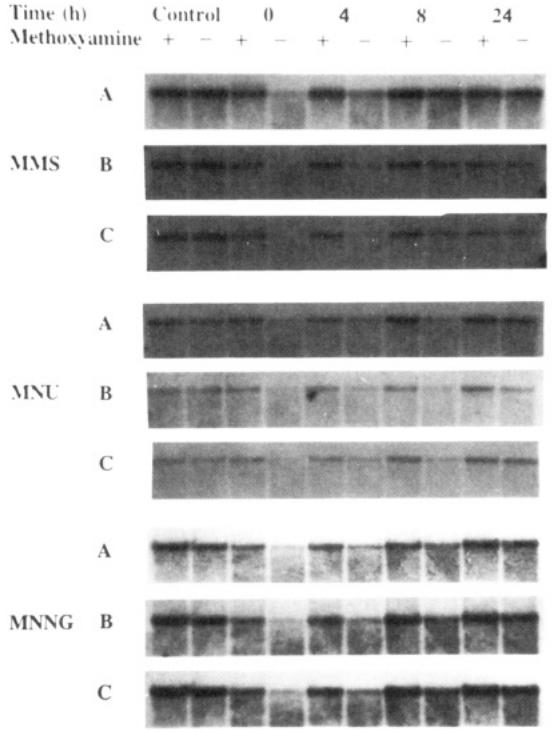


FIGURE 4: Autoradiograms demonstrating the repair of *N*-methylpurines in the *dhfr* gene following exposure of CHO B11 cells to MMS, MNU, or MNNG are shown. The DNA was isolated following immediate lysis of the cells or at a repair time as indicated, and it was then digested with *Kpn*I. The samples were then divided into two aliquots containing 2 $\mu$g of DNA in each and heated for 6 h at 50 °C in the absence (−) or presence (+) of methoxyamine. Following denaturing electrophoresis and Southern analysis, the filters corresponding to each methylating agent were hybridized to reveal repair in (A) the *dhfr* gene, (B) the transcribed strand of the *dhfr* gene, or (C) the nontranscribed strand of the *dhfr* gene. The same filter was used throughout for each of these analyses. $^{32}$P-Labeled DNA or RNA probe was removed each time by washing the filter in 0.4 M NaOH at 42 °C for 30 min and neutralizing it in 0.2 M Tris•HCl (pH 7.5)/0.5% SDS. The resulting bands were quantified with a GS-250 Molecular Imager.

DNA: 1.5 mM MMS, 2.0 mM MNU, and 25 $\mu$M MNNG (data not shown).

*Repair of Methylation-Induced ALSs in the dhfr Gene of CHO B11 Cells.* In order to assess the removal of *N*-methylpurines from the *dhfr* gene of CHO cells, MMS, MNU, or MNNG was used as a source of damage at the doses established above. The removal of ALSs from both strands of DNA and from each of the individual strands of the *Kpn*I restriction fragment was measured. Figure 4 shows the autoradiograms for the experiment for each of the agents. For MMS, MNU, and MNNG, efficient repair was observed in all cases. Furthermore, both the transcribed and nontranscribed strands of the *dhfr* locus were very efficiently cleared of ALSs. Quantitative analyses of the autoradiograms were performed, and the data that were generated are in Table 2, where the actual lesion frequencies and percent repair rates are presented. For all three methylating agents examined, repair reached approximately 80−90% in 24 h when clearance of *N*-methylpurines was measured in both strands of DNA. The differences among the rates of repair associated with each methylating agent were not statistically significant at the time points studied. Repair rates in each individual strand of the locus were very efficient, along the same order as that observed for both strands together. These data demonstrate that no observable biased repair exists for *N*-methylpurines between the transcribed and nontranscribed strands of the *dhfr* locus, both in terms of actual ALSs per fragment and in terms of percent repair, regardless of the source of the methylating species used in the experiment.

## DISCUSSION

As our results indicate, we observed no preferential clearance of 3mA-induced ALSs from the transcribed strand of the *dhfr* locus: Repair of these adducts occurred equally well in both strands of the gene. To the best of our knowledge, this is the first time that the specific clearance of this lesion from a discrete genetic domain has been reported. This result indicates that DNA adducts which impede transcription elongation do not necessarily exhibit biased repair kinetics toward the transcribed strand of active genes; however, as will be discussed shortly, it should not necessarily be inferred from these data that a transcription-dependent mechanism is not, at least in part, responsible for clearing 3mA from this region.

Although it is difficult to conceive of how the cell would discriminate between an *N*-methylpurine formed by one methylating agent over another, we felt it to be of some importance to discern if the source of methylation damage influences the clearance of *N*-methylpurine-induced ALSs as suggested by reports in the literature (Scicchitano & Hanawalt, 1989, 1990; LeDoux et al., 1990a,b; Bartlett et al., 1991; May et al., 1993). To do this, we investigated their repair in the *dhfr* locus of CHO B11 cells following exposure to the $S_N2$ methylating agent MMS, or the $S_N1$ methylating agents MNU and MNNG; we excluded the $S_N2$ methylating agent DMS from this series because gene-specific repair results for *N*-methylpurine-induced ALSs induced by this compound have been published (Scicchitano & Hanawalt, 1989; May et al., 1993). The essential difference between these two types of compounds is the degree to which oxygen atoms in DNA are methylated, with $S_N1$ agents being much more oxygen-reactive and thus producing significant quantities of $O^6$-methylguanine (6mG) and methylphosphotriesters (mPs). 6mG is removed from DNA by $O^6$-alkylguanine−DNA alkyltransferase, and mPs are not believed to be repaired in mammalian cells (Singer & Grunberger, 1983; Friedberg, 1985). It must be emphasized that 6mG is a stable purine derivative and does not contribute to the formation of ALSs, and mPs are sensitive to extensive hydrolysis at levels of alkali that are much higher than those required to cleave ALSs (Crathorn & Shooter,

Table 2: Removal of *N*-Methylpurines from the *dhfr* Gene of CHO B11 Cells following Exposure to Methylating Agents[a]

| | | ALSs/14 kb KpnI fragment at time (h) | | | | % repair at time (h) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 4 | 8 | 24 | 0 | 4 | 8 | 24 |
| MMS | *dhfr* gene[b] | 1.38 | 0.78 | 0.10 | 0.27 | 0 | 43 | 93 | 80 |
| | TS[c] | 1.07 | 0.58 | 0.27 | 0.23 | 0 | 46 | 75 | 79 |
| | NTS[d] | 1.13 | 0.66 | 0.40 | 0.23 | 0 | 42 | 68 | 80 |
| MNU | *dhfr* gene[b] | 0.86 ± 0.01 | 0.39 ± 0.01 | 0.27 ± 0.09 | 0.10 ± 0.04 | 0 | 56 ± 4 | 69 ± 9 | 89 ± 4 |
| | TS[c] | 0.82 ± 0.05 | 0.38 ± 0.08 | 0.22 ± 0.01 | 0.16 ± 0.04 | 0 | 54 ± 8 | 73 ± 3 | 81 ± 6 |
| | NTS[d] | 0.82 | 0.41 | 0.19 | 0.09 | 0 | 50 | 77 | 89 |
| MNNG | *dhfr* gene[b] | 1.22 ± 0.42 | 0.57 ± 0.11 | 0.54 ± 0.20 | 0.25 ± 0.05 | 0 | 53 ± 8 | 57 ± 5 | 79 ± 3 |
| | TS[c] | 0.99 ± 0.06 | 0.52 ± 0.12 | 0.33 ± 0.06 | 0.14 ± 0.11 | 0 | 48 ± 14 | 67 ± 5 | 86 ± 12 |
| | NTS[d] | 1.16 ± 0.11 | 0.70 ± 0.02 | 0.48 ± 0.13 | 0.20 ± 0.08 | 0 | 40 ± 4 | 60 ± 7 | 83 ± 8 |

[a] "±" is standard error of the mean. [b] *dhfr* gene, both strands of the *Kpn* I fragment. [c] TS, transcribed strand. [d] NTS, nontranscribed strand.

1982). Our results indicate that no significant bias in the repair of ALSs exists between the transcribed and nontranscribed strands of the *dhfr* gene, regardless of the source of the methylating agent employed in the experiment; in all cases, removal of ALSs from either strand of this locus was very efficient in 24 h. The repair seen in these experiments primarily reflects that associated with the clearance of 7mG, the predominant *N*-methylpurine formed. These results are in agreement with previous studies demonstrating no significant bias in the removal of ALSs from the individual strands of the *dhfr* locus following exposure to the $S_N2$ methylating agent DMS (Scicchitano & Hanawalt, 1989, 1990). In conjunction with the data obtained for 3mA clearance, our results indicate that no preferential repair of either 3mA or 7mG is observed in the *dhfr* locus of CHO B11 cells.

An important consideration in these studies is how the clearance rates for 3mA and 7mG compare to those for other adducts in the identical gene and cell line. The rate of 3mA clearance is only slightly less than that reported for the rate of cyclobutane dimer clearance from the transcribed strand of the *dhfr* gene in CHO cells as assessed by sensitivity to T4 endonuclease V, an enzyme that incises DNA at these lesions (Mellon et al., 1987). Note that the initial lesion frequencies for dimers and 3mA were about the same. Very limited repair of dimers is observed in the nontranscribed strand of the gene, a result that is in sharp contrast to the data for 3mA presented here, where the clearance of this methylated base is so efficient in both strands that no preferential removal of this moiety is observed. Even if a portion of the 3mA lesions were subject to a transcription-dependent repair mechanism, the overall efficiency of the base excision repair pathway would mask any biases. In the case of dimers, preferential removal of the lesions from the transcribed strand is extraordinarily dramatic precisely because the nontranscribed strand is not cleared of these adducts to any great extent.

*N*-Methylpurines are not the only adducts that have been identified that do not exhibit a bias in repair in the *dhfr* locus of CHO B11 cells. Transcription-dependent repair of lesions derived from the potent carcinogen benzo[*a*]pyrene has been studied using the *E. coli* UvrABC excinuclease to incise DNA at the sites of adducts. No significant biased clearance of these lesions was observed (Tang et al., 1994a). [Interestingly, a biased clearance of benzo[*a*]pyrene lesions was seen in the *hprt* gene of human cells (Chen et al., 1992).] No transcription-dependent preferential removal of *N*-acetoxy-2-acetylaminofluorene adducts was seen in the *dhfr* gene either (Tang et al., 1989). Clearly, preferential repair of genomic DNA damage is highly dependent on the type of damage, its location in the genome, the activity of the locus, and the cells being examined.

Biased removal of DNA adducts from the transcribed strands of active genes implies that mutations resulting from such damage should also be biased. Furthermore, they should stem primarily from changes initiated by DNA adducts that remain unrepaired in the nontranscribed strand. Such biased mutational spectra have been reported for several agents. Ultraviolet light induced a strand bias in mutations in the *hprt* gene of hamster cells (Menichini et al., 1991), benzo[*a*]pyrenediol epoxide elicited biased mutations in the *hprt* gene of human cells (Chen et al., 1990), and benzo[*c*]-phenanthrenediol epoxide yielded mutations in the *dhfr* gene of CHO cells (Carothers et al., 1991). In all these cases, strand-biased clearance of the adducts in question was also observed (Mellon et al., 1987; Carothers et al., 1992; Chen et al., 1992). For $S_N1$ methylating agents, a bias in GC → AT transitions stemming from guanine located on the nontranscribed strand has been reported in V79 Chinese hamster cells (Zhang & Jenssen, 1991), rodent skin fibroblasts (Jansen et al., 1994), and human cells (Yang et al., 1991). The lesion most likely responsible for such mutations is 6mG, and the strand bias in mutation frequency has been attributed to preferential clearance of this adduct from the transcribed strand of the particular locus being examined. Both AT → TA and GC → TA transversion mutations have also been observed; these base changes are probably due to the formation of apurinic sites caused by spontaneous or enzymatic removal of *N*-methylpurines (Loeb, 1985; Yang et al., 1991; Zhang & Jenssen, 1991; Jansen et al., 1994). The transversion mutations are rarer than transition mutations, and, as far as we could ascertain, no strand bias for their formation has been seen. These data are consistent with our results demonstrating extremely efficient repair of 3mA- and 7mG-induced ALSs from both strands of an actively transcribed genetic locus.

How, then, do these collected data fit into a system whereby the transcribed strands of active genes are repaired differentially from their nontranscribed counterparts and other quiescent genetic domains? A general model is that the transcribed strand can be cleared of damage by two mechanisms: transcription-dependent and transcription-independent repair pathways. Transcription-dependent removal of damage would involve stalled elongation of the transcription complex due to the presence of an adduct that blocks its progression. A transcription−DNA repair coupling factor would then target repair machinery to that region (Hanawalt, 1992, 1993). The transcription-independent repair would be mediated by the action of a glycosylase or nucleotide excision repair, depending upon the adduct, in a

fashion identical to that for the nontranscribed strand. Essentially, if the transcription-independent repair pathway were extremely efficient, as in the case of 3mA, the cell would be less reliant upon a transcription-dependent pathway to clear the adducts. No preferential repair of the transcribed strand would be noticed in this case solely because the glycosylase responsible for clearance of *N*-methylpurines is extremely efficient, thus masking any transcription-dependent repair that might be occurring. In cases where differences in the repair of *N*-methylpurines have been observed between active and quiescent domains, repair might be faster in the expressed loci due to enhanced accessibility of these open regions to 3mA−DNA glycosylase. This model could potentially account for much of the observed data concerning methyl-induced ALS clearance.

An important issue which has not yet been clarified concerns the actual nature of the transcription-dependent repair pathway. Is it linked to the nucleotide excision machinery, base excision machinery, or both? In mammalian cells and *E. coli*, transcription-dependent repair of cyclobutane pyrimidine dimers requires the presence of nucleotide excision repair; these results were obtained by using mutants deficient in this pathway, where no transcription-dependent removal of these lesions was seen (Mellon & Hanawalt, 1989; Lommel & Hanawalt, 1993; Isabel Mellon, personal communication). For transcription-blocking lesions normally cleared by base excision, such as 3mA, it is conceivable that a stalled transcription complex could target the nucleotide excision pathway to the region to remove the damage merely because it blocks elongation, without regard to the actual adduct. In the case of DNA adducts where repair is normally mediated by base excision, studies in mutants lacking the essential glycosylase should help distinguish among these possibilities, but such experimental data have yet to be reported. For *N*-methylpurine clearance, no mutant mammalian cells lacking the 3mA−DNA glycosylase have been identified, making it difficult to ascertain whether or not nucleotide excision could be responsible for removing a lesion like 3mA from the transcribed strand. Such experiments might be more amenable to other model systems in which transcription-dependent DNA repair has been demonstrated and for which 3mA−DNA glycosylase mutants are available—*E. coli*, for example.

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