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Repair of *N*-methylpurines in specific DNA sequences in Chinese hamster ovary cells: Absence of strand specificity in the dihydrofolate reductase gene

(methoxyamine/preferential DNA repair/apurinic sites/methylation damage)

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ABSTRACT **We have developed a quantitative method for examining the removal of *N*-methylpurines from specific genes to investigate their possible differential repair throughout the genome. Chinese hamster ovary cells were exposed to dimethyl sulfate, and the isolated DNA was treated with an appropriate restriction endonuclease. The DNA was heated to convert remaining *N*-methylpurines to apurinic sites to render them alkaline-labile. Duplicate samples heated in the presence of methoxyamine to protect the apurinic sites from alkaline hydrolysis provided controls to assess total DNA. After alkaline hydrolysis, agarose gel electrophoresis, Southern transfer, and probing for the fragment of interest, the ratios of band intensities of the test DNA sample to its methoxyamine-treated control counterpart were calculated to yield the percentage of fragments containing no alkaline-labile sites. The frequency of *N*-methylpurines was measured at different times after dimethyl sulfate treatment to study repair. We found no differences between the rates of repair of *N*-methylpurines in the active dihydrofolate reductase gene and a nontranscribed region located downstream from it in treated cells. Also, similar rates of repair were observed in the transcribed and nontranscribed strands of the gene, in contrast to previous results for the removal of cyclobutane pyrimidine dimers. Thus, there does not appear to be a coupling of *N*-methylpurine repair to transcription in Chinese hamster ovary cells. However, the repair in the dihydrofolate reductase domain appears to be somewhat more efficient than that in the genome overall. Our method permits the quantifying at the defined gene level of abasic sites or of any DNA adduct that can be converted to them.**

The excision-repair of structure-distorting DNA lesions in mammalian chromatin is generally heterogeneous (reviewed in refs. 1–3). Cyclobutane pyrimidine dimers are excised rapidly from the active dihydrofolate reductase (DHFR) gene but persist in the bulk DNA in UV-irradiated Chinese hamster ovary (CHO) cells (4). Furthermore, efficient DNA repair is selective for the transcribed strand of the DHFR gene (5). Some bulky chemical DNA adducts also exhibit differential repair in mammalian cells, including aflatoxin B_1 and psoralen photoadducts, which are removed less efficiently from the unexpressed α-satellite DNA sequences than from the bulk DNA in African green monkey cells (6–8). Within the DHFR gene in human cells, the interstrand cross-linking of DNA by psoralen diadducts is repaired at a faster rate than are psoralen monoadducts in the same region (9). Thus, efficiency of DNA repair may be affected by a variety of factors, including the nature of the damage, its location within the genome, the state of expression of the affected DNA sequences, and the chromatin configuration of the region under study.

Simple methylating agents, such as dimethyl sulfate (DMS), produce a variety of damaged bases in DNA of which 7-methylguanine and 3-methyladenine constitute approximately 90% of the alterations (10). These aberrant bases are removed rapidly from mammalian cellular DNA by 3-methyladenine-DNA glycosylase (11–13) and more slowly by spontaneous depurination (14). Potentially mutagenic apurinic (AP) sites are generated as intermediates that can be repaired by the sequential actions of an AP endonuclease, DNA polymerase, and ligase (15).

Little information is available on the possible sequence-specific repair of DNA damaged by methylating agents, although the selective formation and removal of O^6-methylguanine in isolated fractions of chromatin have been studied. While Galbraith *et al.* (16) observed no differences in the methylation or repair at the O^6 position of guanine in various fractions, Ryan *et al.* (17) reported a significantly higher rate of removal of O^6-methylguanine in active regions of chromatin. They also reported that removal of *N*-methylpurines, unlike O^6-methylguanine, was the same in the different fractions of chromatin they studied.

To investigate the repair of *N*-methylpurines in specific DNA sequences in CHO cells treated with DMS, we developed a method analogous to that devised for pyrimidine dimers (4). By converting the *N*-methylpurines in DNA to AP sites and hydrolyzing these sites with alkali, their frequency in specific segments of DNA can be determined. Here, we describe the method and the results obtained using it to measure repair of *N*-methylpurines in the DHFR gene in CHO cells exposed to DMS.

MATERIALS AND METHODS

Cell Culture. CHO-B11 cells carrying the amplified DHFR gene (obtained from R. T. Schimke of this Department) were grown in minimal essential medium (MEM) containing 0.2% $NaHCO_3$ and supplemented with glutamine, nonessential amino acids, penicillin and streptomycin, 500 nM methotrexate, and 10% dialyzed fetal bovine serum in humidified 5% CO_2/95% air at 37°C.

Treatment of Cells with Dimethyl Sulfate. After growth in [^{3}H]thymidine (New England Nuclear), cells were incubated in serum-free MEM containing 0.04% $NaHCO_3$ and 10 mM Hepes (pH 7.4) in a humidified 100% air incubator for 1 hr to become equilibrated (18). A freshly prepared solution of DMS (Aldrich) in dimethyl sulfoxide (DMSO) was then added

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Abbreviations: AP, apurinic; DHFR, dihydrofolate reductase; DMS, dimethyl sulfate; DMSO, dimethyl sulfoxide.

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to the medium to give a final concentration of 150 μM DMS. (The concentration of DMSO in the medium did not exceed 0.5%.) After 30 min the medium was replaced by complete MEM also containing 40 μM BrdUrd and 4 μM fluorodeoxyuridine. After the desired recovery time, the cells were lysed with 10 mM Tris, pH 8.0/1 mM EDTA/0.5% $NaDodSO_4$/100 μg of proteinase K (Bethesda Research Laboratories) per ml. These standard conditions were used for treating the cells with DMS unless stated otherwise.

Overall Repair of Genomic DNA Measured by Alkaline Sucrose Gradient Sedimentation. Repair of *N*-methylpurines in total cellular DNA was determined by using a modification of published procedures (19). After treatment with DMS, the cells were lysed and heated at 50°C for 6 hr to convert remaining *N*-methylpurines to AP sites. After this the samples were made 0.1 M in NaOH and heated for an additional 30 min to cleave the DNA at AP sites. Aliquots of each sample were layered on 5–20% alkaline sucrose gradients along with [^{14}C]thymidine-labeled phage λ to provide a standard and then were centrifuged for 110 min at 30,000 rpm in a SW 50.1 rotor (Beckman). Number-average molecular weights were calculated by the method of Lehmann (20).

To demonstrate that 6 hr of heating at 50°C and pH 7.4 was adequate to remove all heat-labile *N*-methylpurines from the DNA, the following control was performed. CHO cells were grown in 10 μM [^{3}H]thymidine (100 Ci/mol; 1 Ci = 37 GBq) for 16 hr and treated with 120 μM DMS in serum-free medium for 30 min at 37°C. The cells were lysed, the lysate was heated at 50°C, and samples were removed at various times and incubated at 37°C for 30 min in 0.1 M NaOH. The alkaline-labile sites were quantified by alkaline sucrose sedimentation. The maximum removal of *N*-methylpurines to produce alkaline-labile sites occurred within 6 hr at 50°C (data not shown), in good agreement with reported values (21, 22).

Repair in Specific Regions of the Genome. Cells labeled with [^{3}H]thymidine were treated with DMS, incubated to permit repair, and lysed; their purified DNA was treated with 5 units of *Kpn* I restriction endonuclease per μg of DNA (4, 5). The replicated DNA, density-labeled with BrdUrd, was then removed by density gradient centrifugation, and the fractions containing parental DNA were combined. Two equal portions of these samples, each containing approximately 2 μg of DNA, were heated at 50°C for 6 hr to convert all remaining heat-labile methylated bases to AP sites. During this period, one portion from each set was heated in the presence of 5 mM methoxyamine (Sigma) at pH 7.2 to reduce the AP sites and render them refractory to subsequent alkaline hydrolysis (23). This was necessary to provide control samples containing intact DNA. Then the samples were treated with 0.1 M NaOH for 30 min to cleave the DNA at unprotected AP sites. The DNA was subjected to electrophoresis in 0.5% alkaline agarose gels, transferred to nitrocellulose membranes, and hybridized with ^{32}P-labeled DNA or RNA probes for the DNA fragment of interest (4, 5). After exposing the membranes to Kodak X-Omat AR film (Eastman Kodak) and determining band intensities with a Quick Scan R&D densitometer (Helena Laboratories), we calculated the ratio of the band intensity in the non-methoxyamine-treated lane to its methoxyamine-treated counterpart to determine the fraction of DNA fragments containing no heat-labile bases, P_0. The Poisson distribution was then used to calculate the lesion frequency, $-\ln(P_0)$. To correct for depurination of normal bases during the heating of the DNA, a control experiment was included in which the cells were not treated with DMS. An apparent value of 0.07 lesions per 14 kilobases (kb) was obtained as a background level for our method in untreated cells, and this value was subtracted from the lesion frequency measured for DNA from cells treated with DMS to account for unprotected AP sites and spontaneous depurination. The extent of repair of a particular DNA sequence could then be calculated from the lesion frequencies determined for each time point.

RESULTS

Instability of DMS in DMSO. We discovered that DMS is not very stable in DMSO, which is at variance with data reported in the literature (24). This was demonstrated in an experiment in which CHO cells in 30 ml of serum-free medium were treated with 150 μl of 30 mM DMS in DMSO as described, but the DMS was added to the cells at various times after the solution was prepared. Three hours after preparation of the DMS solution, the initial lesion frequency was seen to decrease by 1 order of magnitude. To avoid this complication, DMS solutions were prepared freshly for each time point in the following studies, and the cells were treated approximately 1 min after addition of DMS to the DMSO.

Repair of Total Cellular DNA. The removal of 3-methyladenine and 7-methylguanine from total cellular DNA was determined for comparison with results for repair of specific regions of the genome. Cells were labeled with 10 μM [^{3}H]thymidine (100 Ci/mol) for 16 hr and treated with DMS at a final concentration of 150 μM. This exposure was chosen to yield a lesion frequency of approximately one heat-labile site per 10 kb and was also within the linear range of the dose–response curve (data not shown). The repair kinetics for total cellular DNA was determined by alkaline sucrose sedimentation as described. Increases in the average size of the DNA with time indicate removal of heat-labile *N*-methylpurines (Fig. 1). Table 1 summarizes the results. The average value for the initial formation of *N*-methylpurines was 1.1 per 14 kb. Within 24 hr 73% of the *N*-methylpurines had been removed from the cellular DNA. This method does not distinguish between AP sites formed at lesion sites during the heating process and any AP sites which were present when the cells were lysed.

Repair in the DHFR Gene. To examine the removal of 3-methyladenine and 7-methylguanine from the DHFR gene, CHO-B11 cells were labeled for 2 days with 10 μM [^{3}H]thymidine at 10 Ci/mol. After treatment with DMS and lysis as described, the repair level in the region of interest was determined. The methoxyamine control was included for evaluating total DNA. This was necessary for determining

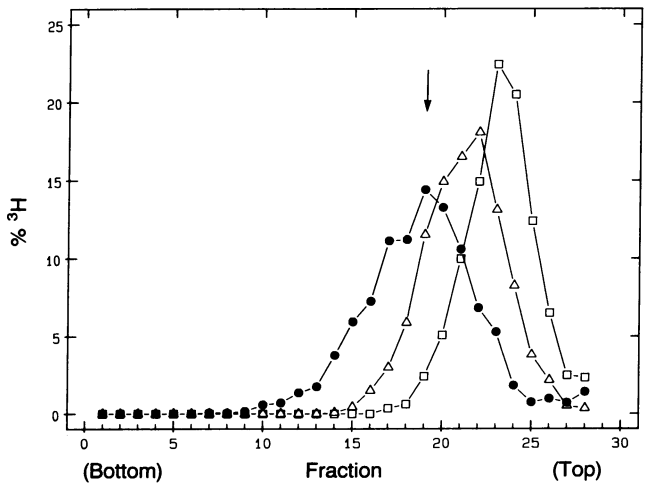

FIG. 1. Radioactivity profiles of alkaline sucrose gradients. The profiles demonstrate repair in total cellular DNA after CHO cells were treated with 150 μM DMS for 30 min at 37°C in serum-free medium. Sedimentation is toward the left. Cells were harvested at 0 hr (□) and 24 hr (△) after treatment with DMS. Control cells (●) were not treated with DMS. The arrow indicates the position of the ^{14}C-labeled phage λ DNA marker, taken to be 48 kb.

Table 1. Alkaline-labile lesion frequency and % repair for different regions of the CHO genome

Region	Lesion frequencies per 14 kb per repair time in hours					
	0 hr	3 hr	6 hr	10 hr	12 hr	24 hr
TCD	1.11 ± 0.07	0.85 ± 0.07 (23)	0.71 ± 0.03 (35)	0.71 ± 0.03 (35)	—	0.31 ± 0.03 (72)
DHFR gene	1.54 ± 0.09	0.94 ± 0.02 (39)	0.82 ± 0.02 (47)	—	0.31 ± 0.07 (80)	−0.04 ± 0.04 (103)
3′ flanking	1.50 ± 0.10	1.07 ± 0.01 (29)	0.97 ± 0.02 (35)	—	0.46 ± 0.10 (70)	0.05 ± 0.05 (97)
T strand	1.44 ± 0.18	0.84 ± 0.04 (42)	0.68 ± 0.05 (53)	—	0.32 ± 0.10 (78)	0.05 ± 0.11 (97)
NT strand	1.45 ± 0.05	0.95 ± 0.05 (34)	0.79 ± 0.01 (46)	—	0.39 ± 0.22 (73)	0.01 ± 0.02 (99)

Values are means ± SEM followed by the percent repair in parentheses. TCD, total cellular DNA; T, transcribed; NT, nontranscribed.

the fraction of DNA fragments containing no heat-labile bases by calculating the ratio of the non-methoxyamine-treated DNA to its protected counterpart. To demonstrate the efficiency of the method, a plasmid DNA labeled with [^{3}H]thymidine was severely damaged with DMS, heated for 6 hr at 50°C in the presence or absence of methoxyamine, treated with alkali, and analyzed by electrophoresis. At least 84% of the AP sites generated became refractory to basic hydrolysis after the methoxyamine reduction of the deoxyribose moiety present at the AP site (Fig. 2 and Table 2).

In the case of the 14-kb *Kpn* I fragment of the DHFR gene, which encompasses the first four exons of the transcription unit (Fig. 3), complete repair occurred within 24 hr after cellular exposure to DMS as determined by probing with nick-translated pZH4 (4, 5) (Fig. 4 and Table 1). The removal of *N*-methylpurines and repair of AP sites in a region flanking the DHFR gene on the 3′ side (Fig. 3) was also complete within 24 hr (Fig. 4 and Table 1). This region was probed with the nick-translated pZH22 (4, 5). The initial lesion frequency in this fragment, which is located entirely outside of the transcription unit of the DHFR gene, was the same as that for the *Kpn* I restriction fragment within the gene.

Repair of the Transcribed and Nontranscribed Strands in the DHFR Gene. In view of the recent discovery of the selective repair of pyrimidine dimers in transcribed DNA strands (5), it was of interest to determine whether differences exist in the removal of *N*-methylpurines from the transcribed and nontranscribed strands of the DHFR gene. This was done by probing each strand of the *Kpn* I restriction fragment located within the gene with ^{32}P-labeled RNA probes prepared as described by Mellon *et al.* (5). No differences were observed for repair of the respective strands. The initial lesion frequencies in the two strands were also the same, being approximately 1.5 heat-labile *N*-methylpurines per 14-kb fragment (Fig. 4 and Table 1). This value corresponded to that found when using the nick-translated probes described above. The data for all of the gene fragments examined and for total cellular DNA are summarized in Table 1. While no significant differences exist among these specific regions in terms of repair rates, there is clearly a difference between the rates of removal of *N*-methylpurines from the DHFR domain and the rate for the repair of total cellular DNA. The time required to remove 50% of the damage from the DHFR gene and the nearby region was approximately 6 hr, while that required for a similar fraction of the damage to be removed from the overall genome was 14 hr.

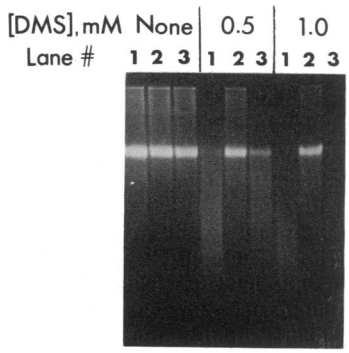

FIG. 2. Protection of AP sites from alkaline degradation by treatment with methoxyamine. A plasmid labeled with [^{3}H]thymidine was linearized by treatment with *Bam*HI and damaged with various millimolar levels of DMS (indicated above the lane numbers) for 2.5 hr at 37°C. The samples for each dose of DMS were heated at 50°C as follows: 6 hr without methoxyamine (lanes 1), 6 hr with 5 mM methoxyamine (lanes 2), and 6 hr without methoxyamine followed by treatment with 5 mM methoxyamine at 37°C for 30 min (lanes 3). All of the samples were then heated at 37°C for 30 min in 0.1 M NaOH and subjected to electrophoresis on a 0.7% agarose gel. After the ethidium bromide staining procedure, the bands were cut from the gel and assayed for radioactivity to quantitate the level of protection (see Table 2).

DISCUSSION

We have demonstrated that the *N*-methylpurines formed within the transcribed unit of the DHFR gene in CHO cells are repaired at the same rate as that found in a nontranscribed region downstream from it. This result is very different from that seen for the removal of pyrimidine dimers from these regions in CHO cells irradiated with UV light (4). Removal rates for dimers in this 3′ flanking region were similar to that for total cellular DNA, being approximately 20% in 24 hr, while removal of dimers within the gene reached a level of 70% in that period.

Mellon *et al.* (5) reported differences in the removal of pyrimidine dimers between the nontranscribed and transcribed strands of the DHFR gene. They found that in CHO cells, the transcribed strand was cleared of dimers within 4 hr after exposure of the cells to UV radiation, while dimers persisted in the nontranscribed strand up to 24 hr. This is clearly not the case for the removal of *N*-methylpurines. Removal of these aberrant bases and the resulting AP sites after treatment with DMS occurred at the same rate in both strands of the DHFR gene.

Other investigators have also reported no differences in repair rates of *N*-methylpurines among different regions of chromatin. Ryan *et al.* (17) treated rats with dimethylnitrosamine, isolated the liver nuclei, and then fractionated the DNA into an active chromatin fraction, two fractions con-

Table 2. Protection of AP sites from alkaline degradation with methoxyamine

DMS, mm	Lane no. in Fig. 2	cpm	% protection from alkaline degradation
None	1	2262	100
	2	2306	100
	3	1936	100
0.5	1	292	13
	2	1954	85
	3	823	43
1.0	1	91	4
	2	1943	84
	3	361	19

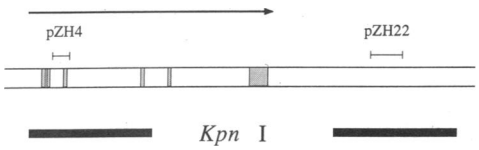

FIG. 3. DHFR gene restriction fragments and probes. The gray bands indicate the positions of the exons. The arrow shows the length of the DHFR gene as well as the direction of transcription. The black bands below the gene represent the positions of the two 14-kb *Kpn* I restriction fragments. The restriction fragment located within the gene and spanning the first four exons was probed with nick-translated pZH4 (4, 5), and the fragment downstream from the gene was probed with nick-translated pZH22. The pZH22 construct was prepared by Linus Ho (this laboratory) as a pGEM subclone of cs-14 (4) and was generously supplied by him. Strand-specific RNA probes were prepared from a pGEM-blue vector containing the pZH4 insert as described and supplied by Mellon *et al.* (5).

sisting of the bulk of the genome, and a nuclear matrix fraction. They found a uniform rate of removal of 7-methylguanine and 3-methyladenine in all four fractions. Nose and Nikaido (25) observed no differences between the introduction and removal of alkaline-sensitive sites in the active human type α2(I) procollagen gene and the inactive human β-globin gene when WI-38 normal human fibroblasts were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. In the case of unexpressed α-satellite DNA of African green monkey cells, the characteristic severe deficiency in repair of aflatoxin or furocoumarin damage was not observed when repair replication was measured in response to DMS treatment (3).

While repair of DNA containing *N*-methylpurines occurs at the same rate inside and outside the transcription unit of the DHFR gene, our data suggest that the repair rate for the total cellular DNA is somewhat slower. However, the alkaline sucrose sedimentation method used for determining bulk repair is perhaps less sensitive for this quantitation than the gel method.

Removal of pyrimidine dimers from the genome of mammalian cells is a very complex process, probably involving a multiprotein system for incision of the DNA near the site of damage. This step is followed by at least three others, excision, repair synthesis, and ligation. The loss of 7-methylguanine and 3-methyladenine, unlike pyrimidine dimers, can be due to either spontaneous base loss or active excision by a glycosylase. In isolated DNA, the reported half-lives for 7-methylguanine and 3-methyladenine in isolated DNA at 37°C and pH 7 are approximately 155 and 26 hr, respectively (14). If these values are accepted and if one also accepts that approximately 20% of the alkaline labile sites are due to 3-methyladenine and the remainder arise from the removal of 7-methylguanine, the fraction of the alkaline-labile sites due to spontaneous loss of these bases at 12 and 24 hr would be 10% and 18%, respectively. By subtracting these values from those obtained experimentally, the repair rate curves shift to the right, and the actual period necessary for the removal of 50% of the damage increases. Also, since the initial lesion frequency in the domain of the DHFR gene is indeed greater than that for the average over the entire genome (Table 1), the fact that a greater amount of damage is removed more rapidly from this region argues for intragenomic differences in repair rates. The half-lives of these adducts in various eukaryotes are much less, ranging from 10 to 26 hr for 7-methylguanine (26–29) and from 2 to 3 hr for 3-methyladenine (26, 30–33). The decrease in stability of the glycosidic bond of the *N*-methylpurines *in vivo* is attributed to the presence of 3-methyladenine-DNA glycosylase. However, these results were obtained by measuring the disappearance of the abnormal bases by HPLC and, therefore, represent average rates determined over the entire genome.

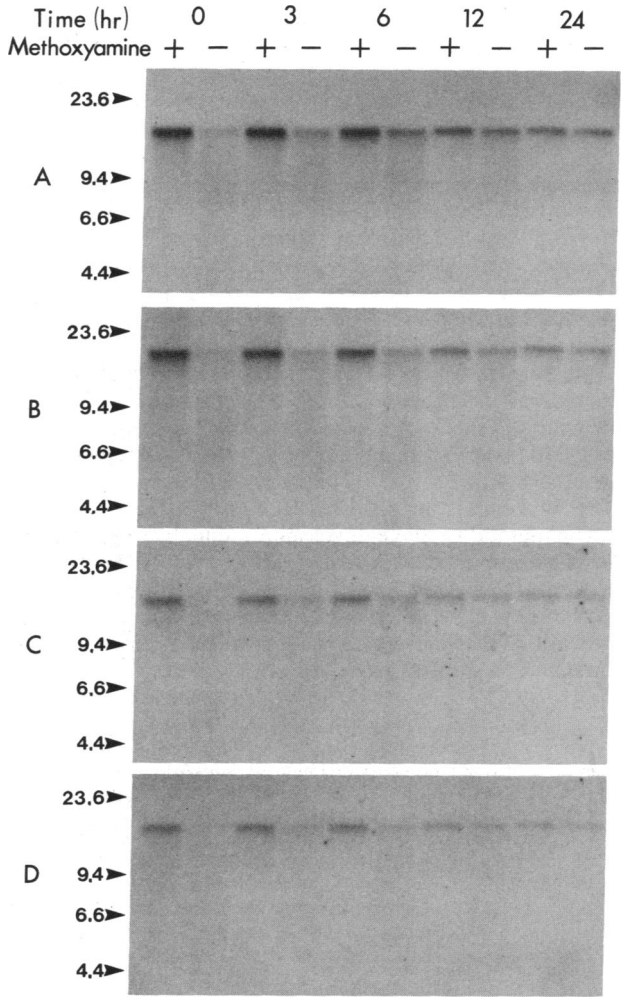

FIG. 4. Autoradiograms for analysis of repair in different regions of the CHO genome. DNA was isolated from CHO cells after exposure to DMS, and it was then digested with *Kpn* I. Samples containing 2 μg of DNA were treated with (lanes +) or without (lanes −) methoxyamine prior to electrophoresis. The same filter was used throughout, and ^{32}P-labeled probe was removed each time by heating the filter at 42°C in 0.4 M NaOH for 30 min and then neutralizing with 0.2 M Tris, pH 7.5/0.5% $NaDodSO_4$/18 mM NaCl/1 mM P_i/110 μM EDTA. The filter was hybridized with the DHFR gene (*A*), 3' flanking region (*B*), transcribed strand of the DHFR gene (*C*), and nontranscribed strand of the DHFR gene (*D*).

The data in the present study indicate that the half-life for the *N*-methylpurines might vary depending upon the genomic location of the lesion. If domains exist that are relatively inaccessible to the glycosylase, a greater reliance upon spontaneous depurination would be necessary for removal of the aberrant base to occur, thus increasing the half-life of the *N*-methylpurine to approach the characteristic value obtained for its hydrolysis from isolated DNA.

The molecular weights of 3-methyladenine-DNA glycosylases from human and rat are 25,000 and 24,000, respectively (11, 12). It is likely that the corresponding enzyme in CHO cells may be relatively small as well, permitting access to regions of DNA that might be inaccessible to larger enzymes. If different domains of the genome are not as readily repaired by the glycosylase due to steric factors, this could explain why the DHFR domain is repaired at a faster rate than the genome overall. This idea is supported by experiments showing that CHO cells deficient in the repair of pyrimidine dimers are phenotypically complemented to proficient repair in both transcribed and silent domains by the expressed *denV* gene from bacteriophage T4, which codes for a pyrimidine

dimer glycosylase with AP endonuclease activity (34). The molecular weight of this protein is only 16,000 (35), making it potentially accessible to many genomic regions.

These results indicate that repair of simple methylation damage in DNA proceeds at a faster rate in some specific sequences of DNA than in others. The data also show that the phenomenon of strand-specific repair in these cells is not a characteristic of all excision repair systems. It has been shown that pyrimidine dimers are blocks to both replication (36) and transcription (37); therefore, their removal from active genes may be necessary to ensure cell survival. *N*-methylpurines, on the other hand, do not completely block replication (38), and they have not been well characterized in terms of their effects on transcription in mammalian cells. If they have little effect on the process of gene expression, then their rapid repair in actively transcribed sequences would be less critical to survival. However, eventual repair may be necessary to alleviate the mutagenic consequences of the otherwise persisting AP sites.

The method we have developed should be of general value for studies on the fine structure of repair of any DNA adducts that can be modified to produce alkaline-labile apurinic or apyrimidinic sites. Such studies are important in view of the indications that these sites may be common intermediates in mutagenesis caused by a variety of chemical carcinogens (39).

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