Strand specificity for UV-induced DNA repair and mutations in the Chinese hamster HPRT gene

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ABSTRACT

DNA excision repair modulates the mutagenic effect of many genotoxic agents. The recently observed strand specificity for removal of UV-induced cyclobutane dimers from actively transcribed genes in mammalian cells could influence the nature and distribution of mutations in a particular gene. To investigate this, we have analyzed UV-induced DNA repair and mutagenesis in the same gene, i.e. the hypoxanthine phosphoribosyl-transferase (*hprt*) gene. In 23 *hprt* mutants from V79 Chinese hamster cells induced by 2 J/m^2 UV we found a strong strand bias for mutation induction: assuming that pre-mutagenic lesions occur at dipyrimidine sequences, 85% of the mutations could be attributed to lesions in the nontranscribed strand. Analysis of DNA repair in the *hprt* gene revealed that more than 90% of the cyclobutane dimers were removed from the transcribed strand within 8 hours after irradiation with 10 J/m^2 UV, whereas virtually no dimer removal could be detected from the nontranscribed strand even up to 24 hr after UV. These data present the first proof that strand specific repair of DNA lesions in an expressed mammalian gene is associated with a strand specificity for mutation induction.

INTRODUCTION

The presence of genotoxic agents in the environment poses a continual threat to the integrity of DNA in the cell. The interaction of such agents with DNA can result in cell death and mutation induction. Cytotoxicity may be caused by the interference of persisting DNA damage with nuclear processes such as DNA replication and transcription. Until recently, most types of DNA lesions caused by genotoxic agents could only be measured at the level of the genome overall, whereas the induction of mutations has always been measured in specific genes suitable for mutant selection. In order to understand the precise molecular mechanisms responsible for the nature of induced mutations in a particular gene, it is necessary to determine the induction and removal of DNA damage in the same gene.

Detection of damage in specific DNA sequences is now possible for some types of lesions, e.g. ultraviolet light (UV) induced cyclobutane dimers. During the past few years, it has been shown that repair of cyclobutane dimers occurs nonrandomly in the genome of mammalian cells (reviewed in (1)). Cultured rodent cells typically remove 10–20% of the cyclobutane dimers from the genome overall within 24 hr after UV-irradiation, but this is much more efficient in transcriptionally active genes such as dihydrofolate reductase (*dhfr*) and *c-abl* (2, 3). Although human cells exhibit a much higher removal of cyclobutane dimers from the bulk of the genome (60–70% after 24 hr), it was shown that at early times after UV-irradiation the active *dhfr* and adenosine deaminase (*ada*) genes are repaired faster in these cells than the genome overall (4, 5). Recently, it was shown that the preferential repair of the hamster and human *dhfr* genes is caused by the very rapid and efficient repair of the transcribed strand, whereas the nontranscribed strand is repaired to the same extent as the genome overall (6).

The efficient repair of the transcribed strands of active genes enables the cell to restore UV-induced inhibition of transcription well before the removal of the bulk of the DNA damage (7) and thereby to overcome the cytotoxic effect of cyclobutane dimers on gene expression. However, the effect of gene specific, and possibly strand specific, repair of UV-photoproducts on mutation induction has not been thoroughly studied. In this study, we investigated the effect of DNA repair on the induction of mutations in the *hprt* gene of Chinese hamster V79 cells. We have previously found that after irradiation at a relative high dose of 12 J/m^2 UV only 65% of the mutations were caused by photolesions in the nontranscribed strand (8). We analyzed the nature and distribution of *hprt* mutations isolated from V79 cells after a lower UV-dose of 2 J/m^2 to exclude possible effects of

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saturation of DNA repair processes. The effect of DNA excision repair on mutation induction was analyzed by measuring the removal of cyclobutane dimers in the same gene.

MATERIALS AND METHODS

Cell culture and HPRT mutant selection

V79 Chinese hamster cells were cultured under standard conditions (9). Twenty three independent HPRT mutants were isolated after a dose of 2 J/m^2 UV of short wavelength ultraviolet light (predominantly 254 nm) as described previously (8).

Mutation analysis

Total cytoplasmic RNA was isolated from *hprt* mutants and converted into cDNA. The *hprt* cDNA was then amplified *in vitro* using the polymerase chain reaction (PCR) procedure (10, 11), cloned in M13 sequencing vectors and sequenced as described previously (8, 12).

Removal of cyclobutane dimers

The removal of cyclobutane dimers from specific DNA sequences was analyzed essentially as described previously (5). Briefly, cells were irradiated with 10 J/m^2 UV and either lysed immediately or incubated for up to 24 hr in the presence of bromodeoxyuridine (to allow for separation of parental and replicated DNA). After incubation, cells were lysed and high molecular weight DNA was purified by phenol and chloroform extractions followed by ethanol precipitation. The DNA was restricted with EcoRI (Pharmacia) and centrifuged to equilibrium in CsCl density gradients. Gradients were fractionated and fractions containing parental density DNA were pooled, dialysed against TE (10 mM Tris pH 8.0, 1 mM EDTA) and ethanol precipitated. Equal amounts of DNA were either treated or mock-treated with T4 endonuclease V (13) and electrophoresed in 0.6% alkaline agarose gels. T4 endonuclease V specifically incises DNA at the site of a cyclobutane dimer resulting in a decrease of the amount of full-length DNA fragments. The DNA was transferred to Hybond N+ membranes (Amersham) by vacuum Southern blotting (Pharmacia-LKB Vacugene 2016) and hybridized with ^{32}P-labeled gene specific probes. After autoradiography the films were scanned using a Video densitometer (Biorad) and the number of cyclobutane dimers per fragment was calculated from the relative band intensities in the lanes containing DNA either treated or not treated with T4 endonuclease V, using the Poisson expression.

Preparation of strand specific probes

Two fragments comprising the Chinese hamster *hprt* cDNA (containing exons 1−5 and 6−9, respectively) were subcloned in M13 SSEV18/19 vectors (14). The orientation was confirmed by sequence analysis. The SSEV vectors contain a modified polylinker (when compared to M13mp18/19) which is able to form a stem-loop structure in the single stranded form. This stem-loop structure contains an EcoRI site and thereby allows for the separation of single stranded cloned inserts from vector sequences. Isolation and purification of inserts was performed essentially as described by Biernat et al. (15). Typically, 50 μg single stranded DNA was digested with 200 U EcoRI for 2 hr at 37°C. The EcoRI digest was size separated on a 1.5% agarose gel and the single stranded insert was excised from the gel and purified by electroelution. The fragment was then labeled by filling in the protruding EcoRI site using α-^{32}P-dATP and the Klenow fragment of DNA polymerase I. The specificity of the probes was checked by spotblots containing both orientations of the fragment used as a probe as well as the original SSEV vectors. Corresponding double stranded probes were prepared by random primer extension (16) of the purified double stranded cDNA fragments.

Table 1. Mutation analysis of 23 UV-induced (2 J/m^2) *hprt* mutants from V79 Chinese hamster cells. For every mutant the nature of the mutation, the amino acid change, the target sequence in the *nontranscribed* (−) strand, the position in the *hprt* coding region, the strand where a dipyrimidine photoproduct may have been formed (+ = transcribed strand, − = nontranscribed strand) are indicated. The exons missing from the cDNA of probable splice mutants are also indicated

mutant	change	amino acid change	target sequence 5′=>3′	position	strand
		Single base substitutions: Transitions			
UV2-V6	GC>AT	Pro>Leu	TATTC C TAATC	74	−
UV2-V4	GC>AT	Asp>Asn	TTGAG G ACATA	403	+
UV2-V13	GC>AT	Arg>stop	CCTCT C GAAGT	508	−
UV2-V8	AT>GC	Phe>Ser	TTTAT T TTGTA	65	−
UV2-V20	AT>GC	Phe>Ser	TAAAT T CTTTG	221	−
UV2-V5	AT>GC	Leu>Pro	TGACC T GCTGG	233	−
UV2-V1,V26	AT>GC	Leu>Pro	TCTGC T TTCCC	440	−
UV2-V18	AT>GC	Leu>Ser	GGATT T GAATC	605	−
		Transversions			
UV2-V9,V14	GC>TA	Leu>Phe	GATTT G AATCA	606	+
UV2-V15	GC>CG	Asp>Glu	CTTGA C TATAA	582	−
UV2-V3	AT>TA	Ile>Asn	TTACA T TAAAG	245	−
UV2-V19	AT>TA	Phe>Leu	TTCTT T GCTGA	225	−
UV2-V16	AT>TA	Leu>stop	GGATT T GAATC	605	−
UV2-V17	AT>TA	Ile>Asn	TGTCA T TAGTG	623	−
UV2-V24	AT>TA	Leu>stop	AACTT T AACTG	374	−
UV2-V27	AT>CG	Tyr>stop	GGCTC T AAATT	216	−
UV2-V10	AT>CG	Phe>Cys	AGATT T TATCA	296	−
UV2-V25	AT>CG	Val>Gly	AAAAG T TATTG	347	−
		Other mutations			
UV2-V1, V22	splice		exon 4		
UV2-V11	splice		exon 6		

RESULTS

Mutant isolation and characterization

Twenty three independent *hprt* mutants were isolated from Chinese hamster V79 cells irradiated with 2 J/m^2 UV (predominantly 254 nm). Although the UV dose employed is quite low, nearly all (>97%) of the recovered mutants were indeed caused by UV-irradiation: *hprt* mutants arose at a frequency of 1.3×10^{-4} mutants per viable cell after UV, whereas the background frequency for spontaneous *hprt* mutants was about 4×10^{-6}, i.e. 30-fold lower.

To determine the sequence alterations in these mutants *hprt* cDNA was amplified by the polymerase chain reaction (PCR) (10, 11) and cloned into M13 sequencing vectors. For all mutants, sequence analysis revealed a change in the *hprt* coding region (Table I). Among the 23 mutants isolated, 20 mutants showed a single base change. All types of single base substitutions were found among these 20 mutants, with about equal numbers of transitions and transversions (Table II). There did not seem to be a preference for a specific type of change, although transversions of GC base pairs were underrepresented. The other

Table 2. Types of UV-induced base substitutions in V79 Chinese hamster cells after 2 and 12 J/m^2 (8) UV

	V79 12 J/m^2	V79 2 J/m^2
Transitions	6 (30%)	9 (45%)
GC>AT	3	3
AT>GC	3	6
Transversions	14 (70%)	11 (55%)
GC>TA	6	2
GC>CG	0	1
AT>TA	4	5
AT>CG	4	3
Total	20	20

Table 3. Distribution of mutagenic photoproducts over the transcribed (+) and nontranscribed (−) strand of the hamster *hprt* gene

	V79 12 J/m^2	V79 2 J/m^2
+ strand	6	3
− strand	11	17

3 mutants recovered were probably mutated in a splice site because the sequence of a complete exon was missing from their amplified *hprt* cDNA.

All of the single base substitutions occurred at dipyrimidine sites, where UV photolesions can be formed. This allowed us to determine whether there was a difference between the transcribed and nontranscribed strand of the *hprt* gene in the frequency by which photolesions gave rise to mutations. According to this analysis, mutations were caused much more frequently by photoproducts in the nontranscribed strand than in the transcribed strand (Table III).

Removal of cyclobutane dimers

This was analyzed using the method originally developed by Bohr et al. (2). in which the frequency of cyclobutane dimers is measured in restriction fragments of the target gene. The presence of cyclobutane dimers in the fragment is visualized on a Southern blot as a decrease in the intensity of the full-length restriction fragments in the DNA treated with the cyclobutane dimer specific enzyme T4 endonuclease V. DNA from UV-irradiated cells was restricted with EcoRI which produces two fragments in the *hprt* gene of approximately 13 and 18 kb (see Fig. 1). The 13 kb EcoRI fragment is located entirely inside the coding unit, whereas the 18 kb fragment contains approximately 8 kb of 3′ flanking sequences. The cells were irradiated with 10 J/m^2 UV, since the dose of 2 J/m^2 used in the mutation experiments does not produce enough lesions in the *hprt* EcoRI fragments to allow for an accurate determination of the dimer frequency. It is important to note that V79 cells, derived from a male hamster, each contain a single active *hprt* allele.

When repair was examined in the 18 kb EcoRI fragment, a large difference in dimer removal between the two strands was observed (Fig. 2). The filter was successively hybridized with a probe that recognizes both strands and with probes specific for the transcribed and nontranscribed strand. When using a probe for both strands the intensity of the *hprt* band in the treated DNA increased with time when compared to the nontreated DNA (Fig.

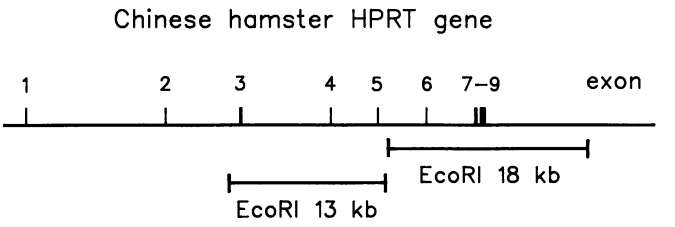

Figure 1. Genomic organization of the Chinese hamster *hprt* gene (25). The map shows the location of the exons and two EcoRI restriction fragments of 13 and 18 kb. The cDNA probes used to detect the EcoRI fragments comprised exons 1−5 and 6−9, respectively.

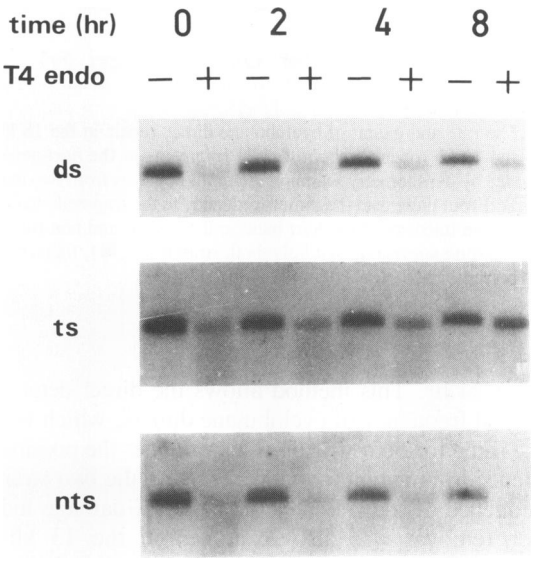

Figure 2. Autoradiograms showing removal of cyclobutane dimers in the 18 kb EcoRI fragment of the *hprt* gene. V79 cells were irradiated with 10 J/m^2 UV and incubated for up to 8 hr. DNA was isolated and restricted with EcoRI. Equal portions were either treated (+) or not treated (−) with T4 endonuclease V. The filter was consecutively hybridized with a cDNA probe which recognizes both strands (ds), and with probes specific for the transcribed (ts) and nontranscribed (nts) strand. Double stranded probes were prepared by random primer extension (16). Strand specific probes were made using the M13 SSEV vectors recently developed by Biernat et al. (14, 15).

2, top), indicating that dimers were removed from this fragment. However, the band in the treated DNA emerged much more rapidly when a probe was used which specifically recognized the transcribed strand (Fig. 2, center). Virtually no dimer removal could be detected when repair of the nontranscribed strand was examined (Fig. 2, bottom).

The results of this and similar independent experiments were quantified by densitometry scanning (Fig. 3). Dimer removal was clearly much more efficient from the active *hprt* gene than from the bulk of the genome: within 24 hr after UV-irradiation, approximately 60% of the cyclobutane dimers were removed from the *hprt* gene, compared to only 15% in the same time period from the bulk of the genome (17). The preferential repair of the *hprt* gene was caused entirely by efficient repair of only the transcribed strand. As much as 90% of the cyclobutane dimers were removed from this strand within 8 hr after treatment, whereas the nontranscribed strand was not significantly repaired

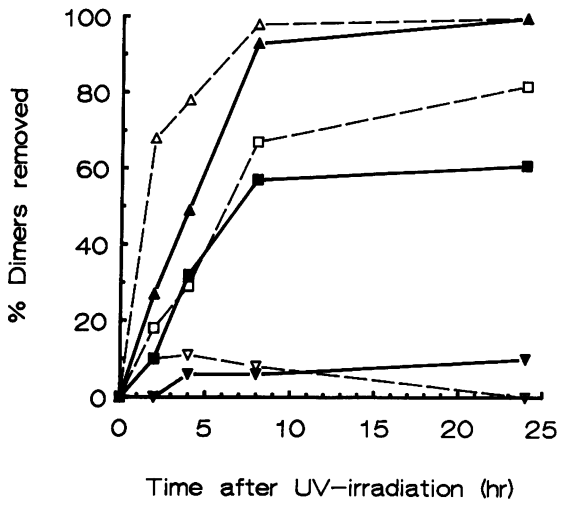

Figure 3. The rate and extent of cyclobutane dimer repair in the 18 kb (closed symbols) and 13 kb (open symbols) EcoRI fragments of the *hprt* gene. Results were obtained by densitometry scanning of autoradiograms from two independent experiments. From these data the dimer frequency in the fragment was calculated from the ratio in intensity of the *hprt* band in the treated and non-treated DNA, using the Poisson expression. Symbols: both strands (□,■), transcribed strand (△,▲), nontranscribed strand (▽,▼).

even after 24 hr. This method allows the direct determination of the initial frequency of cyclobutane dimers, which was found to be the same for both strands. This excludes the possibility that a difference in mutation induction between the two strands was a consequence of a different level of DNA damage induction.

Dimer removal was also examined in the 13 kb EcoRI fragment. In this part of the *hprt* gene, the induction of cyclobutane dimers was also found to be the same in both strands. With increasing repair time, cyclobutane dimers were only removed from the transcribed strand at a rate very similar to that observed for the 18 kb EcoRI fragment (Fig 3).

DISCUSSION

We have cloned and sequenced the coding region of 23 *hprt* mutants from V79 Chinese hamster cells after a low dose of UV irradiation (2 J/m^2). 20 mutations were single base substitutions giving rise to either missense (80%) or nonsense (20%) mutations. In the other 3 mutants, the sequence of precisely a single exon was missing from the amplified *hprt* cDNA, most likely indicating splice mutations in one of the *hprt* intron sequences.

Ultraviolet radiation induces two major classes of toxic and/or mutagenic photoproducts in the DNA i.e. cyclobutane dimers and (6-4) photoproducts, both of which are only formed at sites of adjacent pyrimidines. Assuming that these lesions are responsible for the mutations observed, we can determine in which strand the photoproducts responsible for the base changes were present. We found a very large strand specificity, i.e. almost 90% of the mutations were caused by photoproducts in the nontranscribed strand of the *hprt* gene (Table III). In a previous study, we have found a similar but less pronounced strand bias for UV-induced mutations at the Chinese hamster *hprt* gene after irradiation with a relatively high dose of 12 J/m^2 UV (8). Taken together, these results strongly suggest that cyclobutane dimers and/or (6-4) photoproducts were preferentially removed from the transcribed strand of the hamster *hprt* gene.

To investigate this, the rate and extent of cyclobutane dimer removal was determined for both strands of the *hprt* gene. Repair was monitored in two EcoRI restriction fragments using the method described by Bohr et al. (2). It was found that within 8 hr after UV-irradiation, 90% of the cyclobutane dimers were removed from the transcribed strand of the *hprt* gene, whereas less than 10% of the dimers were removed from the nontranscribed strand (Fig. 3). These results further extend previous observations by Mellon et al. (6) and Venema et al. (submitted) who observed strand specific repair of cyclobutane dimers in the human and Chinese hamster *dhfr* gene and the human *ada* gene, respectively. The proportion of mutations caused by photoproducts in either strand corresponds well with the relative extent of cyclobutane dimer removal from both strands, suggesting that preferential repair of cyclobutane dimers in the transcribed strand of the *hprt* gene causes a strand specificity for mutation induction. However, one should bear in mind that the mutation data were obtained after exposure to 2 J/m^2, whereas the repair data were obtained after 10 J/m^2. The good correlation observed between the strand bias for mutation induction and DNA repair is even more remarkable considering the fact that a much less extreme strand bias for mutation induction was found after 12 J/m^2. A possible explanation for this observation might be that most of the mutants isolated are from cells which were in late G1 or early S-phase of the cell cycle at the time of irradiation, by which little time was available for DNA repair. If removal of cyclobutane dimers is much faster at 2 J/m^2 than at 12 J/m^2, a much more pronounced effect of preferential DNA repair of the transcribed strand on mutation induction would be observed at the lower UV dose. Alternatively, it is possible that most of the mutations were not caused by cyclobutane dimers, but by (6-4) photoproducts, which are induced at these doses at a frequency of 20−30% of cyclobutane dimers (18). Recent studies have shown the relevance of (6-4) photoproducts for both cytotoxicity and mutagenesis in mammalian cells (19−21) and that (6-4) photoproducts are faster removed from the genome overall than cyclobutane dimers. Since at high UV doses (12 J/m^2) cell cycle progression is considerably delayed, more time for repair is available and (6-4) photoproducts could be removed from both strands in significant amounts resulting in a much less pronounced strand bias for mutation induction. This last explanation for the observed discrepancy in the extent of strand specificity between repair and mutation induction at higher UV doses would also imply that (6-4) photoproducts too are preferentially removed from the transcribed strand of the *hprt* gene. The methodology to determine a possible strand specific removal of (6-4) photoproducts at low doses and at early times is, however, not available yet.

The mutation spectrum observed here for cells irradiated with 2 J/m^2 UV light is quite similar to the spectrum previously determined after a 6-fold higher UV dose (Table II). In both cases, none of the six possible types of base changes predominated, although somewhat more transitions were recovered at the low dose. Comparison of the spectrum at the *hprt* locus after 2 J/m^2 in V79 cells with the spectrum determined at the *hprt* gene in a different Chinese hamster cell line (CHO AA8) after the same UV dose (Menichini et al., submitted) showed that in both cell lines comparable numbers of transitions and transversions were detected. CHO AA8 cells

also showed a similarly strong strand bias for mutation induction (Menichini et al., submitted) with in 19 out of 21 mutants the UV-induced *hprt* mutation being caused by a photoproduct in the nontranscribed strand.

UV mutation spectra have been reported for other chromosomal genes in hamster cells, i.e. in the endogenous hamster *aprt* gene (22) and in the *aprt* gene on a chromosomally integrated retroviral shuttle vector (23) after irradiation with 5 J/m^2 and in a stably incorporated and expressed *Escherichia coli* guanine phosphoribosyltransferase (*gpt*) gene (24) after a UV dose of 13 J/m^2. The spectra we determined for the *hprt* gene differ quite markedly from the spectra determined at the other target genes in Chinese hamster cells. In all three other spectra mentioned above a strong predominance (65−70%) of GC > AT transitions among the single and tandem double base substitutions was found, whereas at the *hprt* gene in Chinese hamster cells GC > AT transitions represent only 15−30% of the base changes in the UV mutation spectra (Table II). Moreover, no strand specificity for the induction of mutations was detected in these other studies. These differences in the nature and distribution of UV-induced mutations between different chromosomal target genes may result from the local repair pattern in these genes. For example, different efficiencies in removal of cyclobutane dimers as well as (6-4) photoproducts between target genes would result in the presence of different amounts of adducts at the time of mutation fixation. The presence or absence of strand specific repair in these loci could also strongly influence the mutation spectrum. The target genes used in the other studies are much smaller than the *hprt* gene, which could mean that the pattern of DNA repair is influenced by the presence of neighbouring genes.

Differences in mutation spectra between different target genes may also be related to differences in polarity of DNA replication. This is illustrated by the distribution of UV-induced *hprt* mutations in a DNA repair deficient Chinese hamster cell line, V-H1 (9), where *hprt* mutants were predominantly (>90%) caused by lesions in the transcribed strand of the *hprt* gene (8). We suggested that the extreme strand specificity for mutation induction found in the DNA repair deficient mutant results from differences in the fidelity with which the leading and lagging strand are synthesized when lesions are present in the template during DNA replication. Lesions in the template for the leading strand form a block to the DNA polymerase, which in its attempt to continue DNA synthesis beyond the lesion misincorporates a base opposite the DNA adduct. Synthesis of the lagging strand could just proceed by the generation of new Okazaki fragments beyond the lesion, without the necessity for the DNA polymerase to pass the lesion. This explanation would imply a large difference in pre-mutagenic potential between adducts in the template for the leading and lagging strands, respectively. The relevance of these notions with respect to the differences in mutation spectra determined for different cellular genes awaits the determination of the direction of replication in relation to the transcriptional organization of the genes.

In conclusion, the efficiency of removal of mutagenic lesions from either strand of a gene as well as the direction in which replication forks proceed through a gene may have a strong influence on the nature of induced mutations. The effect of DNA replication on the distribution of mutations over the two DNA strands may be either diminished or enhanced by preferential repair of the transcribed strand depending on the position of the origin of replication with respect to the direction of transcription of the gene. In this paper we have shown the importance of examining mutation induction and DNA repair in the same target gene. Our results show that the pattern of cyclobutane dimer removal correlates well with the distribution of mutations in the Chinese hamster *hprt* gene. It will be of interest to know if this correlation extends to other genes and cell lines as well. Furthermore, studies on the removal of (6-4) photoproducts will be of great importance in a further determination of the mutagenic potential of these adducts.

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