EFFECT OF STRAND-SPECIFIC EXCISION REPAIR ON THE SPECTRA OF MUTATIONS INDUCED BY BENZO[A]PYRENE-DIOL EPOXIDE AND ULTRAVIOLET RADIATION IN DIPLOID HUMAN CELLS

By

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ABSTRACT

EFFECT OF STRAND-SPECIFIC EXCISION REPAIR ON THE SPECTRA OF MUTATIONS INDUCED BY BENZO[A]PYRENE-DIOL EPOXIDE AND ULTRAVIOLET RADIATION IN DIPLOID HUMAN CELLS

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To study the effect of excision repair on the spectra of mutations induced in diploid human cells by UV and (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE), I synchronized repair-proficient cells, treated them at the beginning of S phase or in G_1 phase several hours prior to the onset of S phase, selected for thioguanine resistant cells, and determined the spectra of mutations in the coding region of the hypoxanthine(guanine)phosphoribosyl-transferase (*HPRT*) gene in the mutants. As a control, I compared the spectra of mutations similarly induced in repair-deficient xeroderma pigmentosum (XP) cells. There was no difference in the kinds of mutations observed in mutants derived from either cell strain treated with a particular mutagen either in S or in G_1. With BPDE, the majority were G.C->T.A transversions; with UV, they were mainly G.C->A.T transitions. However, the strand distribution of premutagenic lesions in mutants from repair-proficient cells treated in S or G_1 differed significantly. In mutants derived from cells treated with BPDE in S, such lesions were located in either stand, with 25% being in

the transcribed strand; with mutants derived from G_1, all of them were in the nontranscribed strand. With UV, the strand distribution of such lesions in the S-derived mutants was 71 transcribed: 29 nontranscribed, but in the G_1-derived mutants, this ratio was 20: 80. In contrast to what was found in repair-proficient cells, the ratio did not differ in mutants derived from XP cells treated in S or G_1. This ratio was very similar to that seen in repair-proficient cells treated in S. These results strongly support the hypothesis that human cells preferentially repair UV- and BPDE-induced lesions from the transcribed strand of the *HPRT* gene. To test this, I also measured the rate of repair of BPDE adducts from individual strands of the *HPRT* gene, using the UvrABC exinuclease and Southern hybridizations with strand-specific probes to detect lesions remaining. BPDE lesions were removed from the transcribed strand at a significantly faster rate than from the nontranscribed strand, consistent with my hypothesis. I also found that BPDE adducts were removed faster from either strand of the *HPRT* gene than from a transcriptionally inactive locus, indicating preferential repair of active genes. The results of these studies provide biochemical and biological evidence of strand-specific DNA repair of BPDE adducts in human cells.

This work is dedicated to:

my husband, Yuh-Shan Jou

my mother, Elaine Sha Chen

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ABBREVIATIONS

AAF	2-acetylaminofluorene
ADA	adenosine deaminase gene
AFB_1	aflatoxin B_1
B[c]PHDE	(±)-3α,4β-dihydroxy-1α,2α-epoxy-1,2,3,4-tetrahydroxy-benzo[c]phenanthrene
BPDE	(±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro-benzo[a]pyrene
BrdUrd	bromodeoxyuridine
CHO	Chinese hamster ovary cell line
DHFR	dihydrofolate reductase gene
EBV	Epstein-Barr virus
ENU	ethylnitrosourea
HPRT	hypoxanthine (guanine) phosphoribosyltransferase
HSV*tk*	Herpes simplex virus thymidine kinase gene
MNU	methylnitrosourea
MT	metallothionein
N-AAAF	N-acetoxy-2-aminofluorene
PBS	phosphate-buffered saline
py-py dimer	cyclobutane pyrimidine dimer
6-4 py-py	6-4 pyrimidine pyrimidone
RT	reverse transcription
Tet^R	tetracycline-resistant gene
TG	6-thioguanine

TG^r	6-thioguanine resistant
XP	xeroderma pigmentosum
XPRT	xanthine-guanine phosphoribosyltransferase

INTRODUCTION

The relationship of nucleotide excision repair and DNA replication to mutagenesis in diploid human fibroblasts has been examined by exposing synchronized cell populations to various carcinogens at different times across the cell cycle. Such studies showed that the frequency of 6-thioguanine-resistant (TG^r) mutants (resulting from the loss of the functional gene coding for hypoxanthine (guanine) phosphoribosyl-transferase, *HPRT*) induced by UV radiation (Maher et al., 1979; Konze-Thomas et al., 1982) or by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) (Yang et al., 1982; Watanabe et al., 1985) in repair-proficient cells is highest in cells treated in early S phase and much lower in cells treated in early G_1 phase under conditions that allowed at least 12-15 h. for repair to occur before the DNA replication. This difference in frequency cannot be explained by the difference in the physical state of the DNA during the carcinogen treatment, since no such difference in mutant frequency was found when cells derived from a xeroderma pigmentosum (XP) patient that are virtually incapable of nucleotide excision repair were treated in early S or early G_1 phase. These data suggest that nucleotide excision repair prior to the onset of S phase decreases the mutagenicity of such agents, and that S phase DNA replication is centrally involved in the conversion of DNA damage into mutations.

Recently, it has been shown that in cultured rodent and human cells,

nucleotide excision repair removes certain lesions, such as cyclobutane pyrimidine dimers, (py-py dimers) (Bohr et al., 1985; 1986; Madhani et al., 1986; Mellon et al., 1986), 6-4 pyrimidine-pyrimidone photoproducts (6-4 py-py lesions) (Thomas et al., 1989), and MNU-induced alkali-labile lesions (LeDoux et al., 1991), more rapidly from actively transcribed genes than from bulk DNA, or from nontranscribed genes. Furthermore, Mellon et al. (1989) showed that in these cells, py-py dimers are removed from the transcribed strand of an active gene much more rapidly than from nontranscribed strand. Information on strand-specific excision repair of BPDE adducts is not yet available. Futhermore, there are no data showing such repair of UV-induced damage from the human *HPRT* gene. But if strand specific repair occurred in that gene in human cells, TG^r mutants derived from the repair-proficient cells treated with BPDE or UV in early S phase should differ from those taken from cells treated in early G_1 phase in the strand distribution of the premutagenic lesions assumed to be responsible for the mutations. This difference in strand distribution should not occur in the XP12BE cells which are incapable of nucleotide-excision repair.

The extreme complexity of mammalian genome makes it very difficult to analyze mutations at the DNA sequence level in the endogenous genes of such cells. However, the recent development of polymerase chain reaction (PCR) (Saiki et al., 1988) has enabled researchers to selectively amplify a short segment of DNA more than 10^5-fold. This technique, used in conjunction with reverse transcription (RT) to produce cDNA, provides an efficient way to determine the sequence alterations in the coding region of human endogenous genes. Furthermore, Yang et al. (1989) modified this RT-PCR, so that sequence information can be obtained using a small clone

of only a few mutant cells. This makes it possible to study mutation spectra in endogenous genes of cultured human cells that have a finite life span.

The objectives of this present work were (1) to investigate the kinds and locations of mutations induced by BPDE and UV in the HPRT gene of diploid human fibroblasts treated at the onset of the S phase as well as in early G_1 phase; (2) to compare the strand distribution of BPDE- or UV-induced premutagenic lesions observed from cells treated at S phase with those from cells treated in G_1 phase, in order to determine if there were biological evidence of strand-specific repair of UV photoproducts and BPDE adducts in the human HPRT gene; (3) to determine directly the repair rates of BPDE adducts from the two DNA strands of the human *HPRT* gene using UvrABC exinuclease and Southern blot analysis. The latter study was undertaken to investigate whether there was biochemical evidence of strand-specific repair of BPDE adducts.

Chapter I reviews the background literature bearing on these questions. Chapter II consists of a manuscript published in the November 1990 issue of **Proceedings of the National Academy of Science U. S. A.**, *87*, 8680-8684. It describes the research I carried out to determine the kinds and locations (spectrum) of mutations induced by BPDE in the coding region of the HPRT gene of repair-proficient human fibroblasts treated in the S phase or in G_1 phase of the cell cycle, in order to investigate the effect of excision repair on the spectra of such mutations. Chapter III, which consists of a manuscript published in the May 1991 issue of **Cancer Research**, *51*, 2587-2592, describes comparable work I carried out with repair-deficient XP12BE cells treated with BPDE in S or G_1 phase and

compares the results obtained from this study with those I found in the repair-proficient human cells. Chapter IV is that a manuscript published in the April 1991 issue of **Molecular and Cellular Biology**, *11*, 1927-1934, which has been a collaborative work with my colleague Dr. W. Glenn McGregor. It describes a study of the UV-induced *HPRT* mutational spectra in repair-proficient cells and in XP12BE cells, as well as the investigation of the role of excision repair in such mutagenesis. In this study, the mutagenesis experiments and mutational analysis in repair-proficient cells were carried out by Dr. McGregor. The comparable study on the XP12BE cells was carried out by me (80% of the work) and by Dr. Lukash (20% of the work). Chapter V describes the biochemical study on the comparison of the repair rates of the BPDE adducts on the two strands of the *HPRT* gene of human fibroblasts. This chapter is written in the style of a manuscript for the **Proceedings of the National Academy of Science U. S. A.** and will be submitted for publication to this journal.

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CHAPTER I

LITERATURE REVIEW

A. Mechanisms of the DNA repair process responsible for removal of UV-induced damage and bulky chemical adducts

1. Model of nucleotide excision repair

The DNA repair processes are the cellular responses associated with the restoration of the normal nucleotide sequence of DNA after damage. The enzymatic pathways involved in DNA repair vary with the types of damage introduced, but the most general mechanism is the nucleotide excision repair pathway, which is responsible for removing UV-induced cyclobutane pyrimidine dimers (py-py dimers) and 6-4 pyrimidine-pyrimidone photoproducts (6-4 py-py), as well as adducts produced by benzo(a)pyrene-diol-epoxide (BPDE), mitomycin C, psoralen, acetylaminofluorene (AAF), cisplatin, 4-nitroquinoline-1-oxide, and many other carcinogens and/or chemotherapeutic agents.

During the mid-1960s, a series of important biological experiments were independently reported by Setlow et al. (1964) and by Boyce et al. (1964) that led to major insights into the nature of nucleotide excision repair in *E. coli*. They showed that when bacterial cells were UV-irradiated and then incubated, thymidine-containing pyrimidine dimers were lost from the acid-insoluble fraction of the DNA and could be recovered in the acid-soluble phase. These observations, together with the

demonstration of repair synthesis of DNA at about the same time (Pettijohn & Hanawalt, 1963; 1964) led to a general molecular model for the multi-step process of nucleotide excision repair. The sequential steps include (a) preincision recognition of damage; (b) incision of the damaged DNA strand at or near the site of the defect; (c) excision of a fragment containing the damaged site or exonucleolytic degradation of the affected strand; (d) repair replication to replace the excised region with a corresponding stretch of normal nucleotides; and finally (e) ligation to join the repair patch at its 3' end to the contiguous parental DNA strand.

For many years, researchers who studied excision repair in bacteria had believed that following the damage-specific DNA incising activities, the actual excision of the damaged nucleotides was performed by an exonucleolytic degradation of the DNA in the 5'-> 3' direction (Hanawalt et al. 1979; Lindahl, 1982). However, it has been demonstrated that *E. coli* contains a damage-specific DNA incising enzyme complex, UvrABC, which cuts the damaged strand both upstream and downstream of the damaged bases (Sancar & Rupp, 1983; Yeung et al., 1983). This dual incision facilitates the release of a 12 or 13 base oligonucleotide containing the lesion (Caron & Grossman, 1988; Kumura et al., 1985; Myles et al., 1987; Sancar & Rupp, 1983). This observation has led to the suggestion that this type of enzyme be designated an exinuclease.

2. Current understanding in the nucleotide excision repair process in mammalian cells.

The study of excision repair in mammalian cells was limited for many years by the lack of available mutant cells defective in their response to

DNA damage, until the observation that fibroblasts in culture derived from the skin of a human patient with the disease *xeroderma pigmentosum* (XP) were defective in UV-induced excision repair (Cleaver, 1968). XP is an autosomal recessive disease characterized clinically by a severe photosensitivity of the eyes and exposed regions of the skin, a very high incidence of skin cancers and frequent neurological abnormalities (Reed et al., 1969; Robbins et al., 1974; Cleaver & Bootsma, 1975; Robbins, 1978). Fusions between cells of different patients with this disease in various pairwise-combinations have established the existence of at least eight genetic complementation groups, designated A through H (Friedberg et al., 1979; Cleaver, 1983; Moshell et al., 1983). Members of each of them are defective or deficient in the excision repair of UV-induced photoproducts as well as various types of chemical damage to DNA (Setlow, 1978; Friedberg et al., 1979). It appears that much of the genetic complexity in XP is concentrated at very early steps in excision repair, specifically those involved in the incision of damaged DNA (Friedberg et al., 1979). Besides the eight complementation groups, approximately 10% of all cases of clinically typical XP do not have an apparent defect in the excision repair of DNA damage caused by UV radiation. These cases are referred to XP variants (Robbins & Kraemer, 1972; Cleaver, 1972; Cleaver et al., 1981).

Excision repair-deficient mutants of rodent cells have been isolated from a large population of mutagenized cells in culture (Busch et al., 1980; Thompson et al., 1980; 1981; Schiomi et al., 1982). Complementation groups have also been defined for these rodent mutants (the ERCC1-8 series) (Busch et al., 1989; Zdzienicka et al., 1988; Thompson et al.,

1988a). These mutants are similar in many respects to the human XP mutants. However, they differ quantitatively in the degree of deficiency of excision repair. Most XP groups have low levels of photoproduct excision, whereas rodent mutants tend to be either completely deficient or intermediate in repair (Clarkson et al., 1983). The rodent mutants are also distinctive in being sensitive to cross-linking agents, such as mitomycin C (Hoy et al., 1985), which is not the characteristic of XP cells (Fujiwara et al., 1977). Several types of complementation tests have been performed to establish a link between the members of these two species, but until now no overlap has been found (Stefanini et al., 1985; Thompson et al., 1985a). This implies the involvement of a substantial number of genes in excision repair in mammalian cells and a considerable complexity of this process at the biochemical level.

Why are so many genes apparently required for the excision repair in mammalian cells? At least two hypothetical possibilities have been raised. First, one might imagine that the enzymology of DNA excision repair in mammalian cells is strictly different from that in *E. coli*; i.e., a smaller number of genes is required in bacteria. Alternatively, some of these mammalian gene products may function specifically to create accessibility of sites of damage in chromosomes to a catalytically active endonuclease, since in eukaryotes, DNA in the form of chromatin is extremely tightly packaged and folded in the nucleus. Several lines of evidence that support the second possibility will be described in the next section.

Many of the details of excision repair in mammalian cells have yet to be characterized. One approach to the study of mammalian genes required

for the excision repair is to attempt the molecular cloning of these genes, with a view to expressing the proteins they encode in amounts suitable for direct biochemical study. The rodent mutants have proven to be more valuable as recipients for gene transfer than XP cell lines (Hoeijmakers et al., 1987). Cell fusion and DNA-mediated gene transfer have demonstrated that at least six (ERCC1-6) of these rodent complementation groups can be corrected by genes from normal human cells (Thompson et al., 1985b). Recently, the isolation of several human excision repair genes, ERCC-1, ERCC-2, ERCC-3, and ERCC-6 that are able to correct the defect in rodent complementation groups 1, 2, 3, and 6, respectively, has been reported (Westerveld et al., 1984; Weber et al., 1988; Weeda et al., 1990a; Troelstra et al., 1990).

The ERCC-1 gene was found to be the human equivalent of the *S. cerevisiaee* RAD10 repair gene (van Duin et al., 1986). In addition, this gene harbors two domains present in *E. coli* excision repair gene UvrA and UvrC (van Duin et al., 1988). However, it fails to correct the defects in any of the XP complementation groups (van Duin et al., 1989). Since the rodent mutants were derived *in vitro*, the absence of certain rodent complementation groups among XP groups may indicate that some mutations that remain viable in culture compromise development and viability in the whole organism (Cleaver, 1990).

The ERCC-2 gene product exhibits strong sequence similarity at the amino acid level to RAD3 of yeast (Weber et al., 1988; 1990). RAD3 is known to have ATPase (Sung et al., 1987a) and helicase activity (Sung et al., 1987b), suggesting a similar function for ERCC-2 (Weber et al., 1990). Mutational analysis has shown that the ERCC-2 protein is required

for cell viability as well as repair (Naumovshi & Friedberg, 1983; 1986), and it is also involved in recombination (Montelone et al., 1988). Weber et al. (1991) recently demonstrated that this gene complements the defect in XP-D cells.

The ERCC-3 protein harbors putative nucleotide, chromatin, and helix-turn-helix DNA binding domains and seven consecutive motifs conserved between two superfamilies of DNA and RNA helicases, suggesting that it is a DNA repair helicase (Weeda et al., 1990b). The gene was found to correct the excision defect in XP-B (Weeda et al., 1990b), which exhibits a complex phenotype having features of both XP and Cockayne's syndrome (Lehmann, 1987).

The ERCC-6 gene has been isolated (Troelstra et al., 1990) by correcting the excision defect in a chinese hamster cell line, UV61, which was found to harbor a deficiency in the repair of UV-induced py-py dimers but permit apparently normal repair of 6-4 py-py lesions (Thompson et al., 1988b). The open reading frame of this gene encodes a predicted protein of minimally 1500 amino acids with a putative chromatin binding domain and a serine phosphorylation site. In addition, it shows an almost perfect match with 7 domains conserved between the two superfamilies of helicase, which makes ERCC-6 the third putative helicase involved in excision repair (Troelstra et al., 1991).

Attempts to confer UV-resistance on XP cells by transfection with genomic DNA from normal cells had been unsuccessful, due to the spontaneous revertants appearing in the recipient cells as well as the difficulty in stably transfecting high-molecular-weight DNA into human cells (Lehmann, 1985). However, the mouse gene correcting SV40-transformed

XP-A fibroblasts has been cloned by extensive genomic DNA transfection experiments (Tanaka et al., 1989). Shortly thereafter, a human homolog of this XP-A-correcting cDNA has been isolated (Tanaka et al., 1990). This cDNA encodes a hydrophilic protein which contains a distinct zinc-finger motif, indicating that it interacts directly with DNA. The corresponding mRNA is greatly reduced in cell lines from several XP-A patients, due to a G->C transversion at a splice-acceptor site (Satokata et al., 1990).

Attempts to clone other XP genes are now in progress. A mouse DNA repair gene that partially corrects the defect in the XP-D cell line has been reported (Arrand et al., 1989). In addition, an apparent loss of a separate protein that binds to damaged DNA has been reported for the XP-E cells (Chu & Chang, 1988). This protein has many DNA-binding characteristics that resemble those of a yeast photolyase. However, it lacks the function of light-mediated photolysis (Patterson & Chu, 1989).

A soluble cell-free system that can carry out nucleotide excision repair *in vitro* has been developed (Wood et al., 1988). This system allowed the identification of the requirement for the human single-stranded DNA binding protein in excision repair (Coverley et al. 1991). In addition, it provides the method of functional assessments of purified XP gene products by biochemical complementation.

3. Heterogeneity of excision repair in the eukaryotic genome

Eukaryotic DNA is folded in a hierarchy of different structures to form chromatin. Most of the genome in a cell of a complex organism contains information unnecessary or inappropriate to its own specialized function, and is not expressed. It is possible that the requirement for

repair systems in such regions is very different from that in active genes where mutations or blockage of transcription could have direct effects on cell survival and functions. In addition, the various states of chromatin may modulate the introduction of certain types of damage into DNA and, as the proper substrate for the repair system, different chromatin states may play an important role in the kinetics or efficiency of DNA repair. To approach the issue of how repair systems might be influenced by different functional and structural aspects of the mammalian genome, considerable effort has been devoted in the past few years to investigating repair in particular genomic components. One approach is to use traditional methods to analyze repair or adduct frequency in DNA fractionated by procedures thought to separate it by structure or function, e.g. separating active and inactive chromatin fractions according to the nuclease accessibility (Mullenders et al., 1987), or isolating highly repetitive DNA species (Madhani et al., 1986a). Another approach is to study repair in specific DNA sequences, such as genes and their flanking regions, whose activity or chromatin organization can be analyzed and, in some cases, experimentally manipulated (Bohr, 1987; Hanawalt et al., 1989). With these approaches, a number of interesting and notable results indicating that excision repair is not uniform throughout genome have been reported.

3.1 The influence of chromatin organization on introduction of DNA damage and efficiency of excision repair

It is well recognized that DNA in mammalian cells exists in the form of chromatin which is organized and tightly packaged into higher order structure. In the first step of DNA packaging, the DNA is bound by histone

and non-histone proteins to form the repeating core and linker regions of the nucleosome structures (Moyne et al., 1981). This structure is further coiled into a "thick fiber" with 6-9 nucleosomes per turn (Thoma et al., 1980; Thomas & Butler, 1980). In the interphase nuclei and in metaphase chromosomes the thick fiber appears to be folded into loops (Cook & Brazell, 1975; Benyajati & Worcell, 1976) or domains (Paulson & Laemmli, 1977), and these repeating loops are constrained by attachment to a nuclear skeletal structure termed the nuclear matrix, or scaffold (Paulaon & Laemmli, 1977; Igo-kemenes & Zachau, 1977). There is increasing evidence that the nuclear matrix is associated with fundamental processes such as replication, transcription and repair (Nelson et al., 1986).

Several studies have been done to analyze DNA damage and repair processes at the level of nucleosomes in chromatin structure using micrococcal or staphylococcal nuclease, which preferentially digests linker regions of chromatin. With this approach, it has been shown that a number of bulky adducts are formed preferentially at linker regions, including those induced by the following agents: AAF (Baranyi-Furlong & Goodman, 1984), N-hydroxy-AAF (Metzger et al., 1977), N-acetoxy-AAF (N-AAAF) (Kaneko & Cerutti, 1980), dimethyl-N-nitrosamine (Ramanathan et al., 1976), methylnitrosourea (MNU) (Berkowitz et al., 1981), BPDE (Jahn & Litman, 1979; Arrand & Murray, 1982), and aflatoxin B_1 (Bailey et al., 1980). Studies have also been performed on the distribution of py-py dimers between nucleosome core DNA and linker DNA (Williams & Friedberg, 1979; Snapka & Linn, 1981; Niggli & Cerutti, 1982). The results of these studies indicated that there is essentially no difference between these two regions of the nucleosome unit in the distribution of py-py dimers at

low to moderate UV doses. However, at a finer level of resolution using the 3'->5' exonuclease activity of T4 DNA polymerase, Gale et al. (1987) showed that the distribution of UV-induced photoproducts (primarily py-py dimers) is not uniform throughout core DNA, but displays a striking 10.3 (± 0.1) base periodicity. The sites of maximum dimer formation in the core DNA mapped to positions where the phosphate backbone is farthest from the core histone surface. This pattern of dimer induction was not obtained from UV-irradiated core DNA either free in solution or bound tightly to calcium phosphate crystals, indicating that yield of dimers along the DNA may be modulated in a manner that reflects structural features of the nucleosome unit.

Evidence of the repair heterogeneity at the level of the nucleosome has also been reported. In response to UV or chemicals that inflict damage randomly in chromatin, most of the repair synthesis in mammalian cells occurs in the staphylococcal nuclease-sensitive regions, but with increasing repair time the nucleotides inserted during repair synthesis become progressively more nuclease resistant (Smerdon & Lieberman, 1978; Lieberman et al., 1979). In addition, it was determined that the 5' and 3' end regions of core DNA are markedly enhanced in repair-incorporated nucleotides (Lan & Smerdon, 1985). Recently, Smerdon & Thoma (1990) examined excision repair of UV photoproducts in a small yeast plasmid (YRp-TRURAP), known to be folded into chromatin with positioned nucleosomes *in vivo*. Their results showed a correlation between the repair rate and the relative stability of nucleosomes on the DNA, with a more stable nucleosome exhibiting less accessibility to the repair enzymes.

Excision repair of a number of adducts has been examined in the

heterochromatic alpha DNA which can be physically isolated for analysis. It was shown that UV damage is repaired with similar efficiency in repetitive alpha sequences and in bulk DNA (Lieberman & Poirier, 1974), but that the repair of certain bulky adducts was markedly deficient in alpha DNA (Zolan et al., 1982; Leadon et al., 1983; Zolan et al., 1984; Smith, 1987). These studies suggest that the highly condensed chromatin structure of alpha DNA hinders access of the repair system that acts on bulky adducts.

The repair of UV-induced damage in human and rodent cells was investigated at the level of DNA loops attached to the nuclear matrix. These studies have demonstrated preferential repair in such matrix-associated DNA as compared to total nuclear DNA, particularly at low levels of UV exposure (McCready & Cook, 1984; Harless & Hewitt, 1987; Mullenders et al., 1988). This feature of preferential repair has been examined in some repair-deficient cells. The repair synthesis in XP-C cells, which carry out most of their residual repair activity in a limited portion of the genome, appears to be more closely associated with the nuclear matrix than that in normal cells (Mullenders et al., 1984). However, in contrast to the observations for XP-C and normal cells, the repair synthesis in Cockayne's syndrome fibroblasts is decreased in DNA associated with the matrix (Mullenders et al., 1988). The relationship between such heterogeneity in distribution of repair events in chromatin loops and the preferential repair of transcriptionally active genes will be discussed later.

3.2 Preferential repair of actively transcribed genes.

Repair of py-py dimers has been studied in individual genes and specific sequences using T4 endonuclease V which cleaves DNA at dimer sites (Bohr et al., 1985; 1986a; 1988). By Southern hybridization and probing for a sequence of interest, the fraction of molecules without dimers (zero class) can be quantitated. This value can be used with the Poisson equation to calculate the actual frequency of dimers in the fragment, and thereby measure its repair.

With this approach, removal of dimers from a number of genes in cultured mammalian cells has been reported. It was initially found that repair of dimers in the essential gene for dihydrofolate reductase (*dhfr*) in Chinese hamster ovary (CHO) cells was much more efficient than the repair in the overall genome (Bohr et al., 1985). This may provide an explanation for the paradox that rodent cells are deficient in overall genome DNA repair, but as resistant to UV damage as the repair-proficient human cells; it is possible that they survive by selectively repairing the vital sequences. It has been suggested that the determination of repair in essential genomic regions is an important feature if one is trying to correlate DNA repair and cell survival (Bohr et al., 1986a).

The fine structure of the preferentially repaired *dhfr* region in CHO cells has been characterized (Bohr et al., 1986b). Repair is most efficient in the 5' portion of the *DHFR* gene and in the 5' flanking sequence, while a distant upstream sequence and the 3' flanking downstream sequence were each repaired with an efficiency similar to that for the overall genome. Preferential repair of an active gene has also been found in human cells. Mellon et al. (1986) showed that dimers in the *DHFR* gene

in a human cell line, 6A3, were removed much more rapidly than those in the bulk DNA.

Besides the *DHFR* locus, preferential repair of transcriptionally active loci has been confirmed in several other genes. In a 21-Kb fragment encompassing most of the 25-Kb gene encoding *3-hydroxy-3-methylglutaryl coenzyme A reductase* in CHO cells, dimers are removed to about the same extent as in *dhfr* (Smith, 1987). In mouse 3T3 cells, Madhani et al. (1986b) observed efficient repair of the transcriptionally active *c-abl* gene in both growing and resting cells. However, very little repair was observed in 15- and 6-Kb fragments that contained portions of the mouse *c-mos* proto-oncogene, the transcription of which has not been detected in these cultured cells. In human fibroblasts, the preferential repair of the 32-Kb *adenosine deaminase (ADA)* gene was reported by Mayne et al. (1988). As a control, they analyzed repair in a 14-Kb fragment that lies in an unexpressed region of the X chromosome, termed the 754 locus. This fragment was repaired slowly, with the same kinetics as the overall genome.

Taken together, these results suggest that the preferential repair of py-py dimers from active genes is a general phenomenon in mammalian cells. Several possibilities have been suggested to explain this feature of repair. The chromatin structure in active genes could have a more "open" configuration to be easily accessible to repair complex, or fundamentally different repair systems with different efficiencies could operate on active or inactive DNA. Finally, the blockage of transcription by a lesion might make it a more attractive substrate to repair enzymes (Smith and Mellon, 1990).

Using the same approach, removal of dimers from transcriptionally active and inactive genomic DNA has been examined in repair-deficient human cells. In spite of the limited overall repair capacity in XP-C cells (Mansbridge & Hanawalt, 1983), py-py dimer removal from *ADA* and *DHFR* genes was found to be as efficient as that in normal human cells. However, although normal cells performed efficient repair from the 754 locus but at a slower rate than found for the active *ADA* and *DHFR* genes, the XP-C cells showed a very poor repair from the 754 locus (Venema et al., 1990a). In contrast to XP-C cells, Cockayne's syndrome cells are able to remove dimers from DNA but they are unable to repair the *DHFR* and *ADA* genes as rapidly and efficiently as normal cells (Venema et al., 1990b). These results correlate with the preferential removal of dimers from nuclear matrix associated DNA in normal and XP-C cells and its absence in Cockayne's syndrome cells. It was suggested that Cockayne's syndrome cells have a defect in excision of dimers from active genes located proximal to the nuclear matrix, whereas XP-C cells lack the ability to render the rest of the chromatin accessible to repair enzymes (Mullenders et al., 1988).

Little information has been available on the possible gene- or sequence-specific repair of photoproducts other than py-py dimers discussed above, or of DNA damage induced by chemical agents. More recently, techniques have been developed to study other lesions, for example by the use of the UvrABC excision nuclease to cleave the DNA strand specifically at damaged sites (Thomas et al., 1988), or by formation of strand breaks at sites of alkylation damage (Scicchitano & Hanawalt, 1989).

The *E. coli* enzyme complex UvrABC recognizes most DNA adducts that

create significant helical distortions (Sancar & Sancar, 1988). Thomas et al. (1989) measured repair of 6-4 py-py lesions in the *dhfr* gene of CHO cells using this enzyme complex in conjunction with another DNA repair enzyme from *E. coli*, DNA photolyase. The latter enzyme when bound to a py-py dimer in DNA and exposed to light causes the loss of the dimer affecting repair (Brash et al., 1985; Myles et al., 1987). The remaining photoproducts, which are almost exclusively 6-4 py-py lesions, are then digested with UvrABC. With Southern blot analysis and probing for specific sequences, the repair of 6-4 py-py lesions can be quantitated. It was found that these lesions are repaired more efficiently than py-py dimers and that there is preferential repair of these lesions in the *dhfr* coding region in CHO cells compared with its downstream, noncoding sequences.

With UvrABC treatment, Tang et al., (1989) examined the formation and repair of N-AAAF induced DNA damage in the DHFR coding and noncoding sequences. In contrast to that obtained for the repair of py-py dimers, the rates of removal of N-AAAF-induced damage from both sequences are similar in cells treated with 10uM N-AAAF. However, it is perhaps worth noting that the initial lesion frequency (3.1 adducts/14 kb) used in this study was considerably higher than in most experiments with UV; this might lessen the apparent differences in rate of repair between transcribed and nontranscribed sequences.

Evidence on heterogeneous repair of MNU-induced alkali-labile adducts has recently been provided (LeDoux et al., 1991). MNU spontaneously decomposes to form a carbonium ion which is capable of alkylating the nitrogens and oxygens of DNA bases. The predominant adducts are N^7-methyl-guanine and N^3-methyl-adenine which are removed by excision

repair (Montesano, 1981). The alkali lability of these two adducts was exploited to measure their formation and removal in the *dhfr* and *c-fos* loci in CHO cells. Rather than treat the isolated DNA with an endonuclease, they heated it to produce apurinic sites and then incubated it in alkali to cleave DNA at these sites. The remaining procedure used was the same as that developed for assaying the removal of py-py dimers. It was found that, although repair in the 3'-flanking sequence of *dhfr* gene was only slightly less efficient than that seen in its coding region, repair in the nontranscribed *c-fos*-containing fragment was markedly attenuated. This suggests that repair across the entire genome is heterogenous with different areas repairing at varying rates. However, the exact mechanisms governing the rate of repair in specific areas remain to be elucidated.

3.3 Effect of transcription on excision repair

The selective repair of transcriptionally active genes raises some interesting questions about the relationship between DNA repair and transcription. Mellon et al. (1987) found a dramatic difference in the efficiency of removal of dimers from the transcribed and nontranscribed strands of the *DHFR* gene in cultured hamster and human cells, with the transcribed strand repaired preferentially. This finding challenges the simple model of chromatin accessibility as the sole mediator of preferential repair, and further strengthens the possibility of the association between transcription and excision repair.

The relationship between repair and transcription has been studied in more detail using the approach allowing examination of the same gene

under different conditions. Gene activity can be modulated *in vitro* with inhibitors or natural effectors, such as gene specific inducers or suppressors. In such cases, it is usually considered that the rate of transcription of the gene is affected directly by trans-acting molecules, and that the chromatin structure of the gene is in some permissive state even when its transcription activity is low.

A number of mammalian genes are inducible after activation by external inducers and one of the most studied is metallothioneins (MT). Mammalian genomes encode two closely related metallothionein, MT I and MT II, which are transiently induced by exposure to heavy metals such as cadmium, zinc, and copper, and by various circulating factors including hormones and interferon (Brady, 1982; Kojima & Kaegi, 1978). Repair of dimers from the MT I gene in a CHO cell line has been examined comparatively with or without transcriptional activation of the gene by incubation in the presence of $ZnCl_2$ (Okumoto & Bohr, 1987). Whereas the repair efficiency was very low in the uninduced state, it increased about 50% after induction of the gene. Furthermore, no effect of $ZnCl_2$ was observed for repair in a noninducible gene, *dhfr*, or on overall repair synthesis in the cells.

Leadon and Snowden (1988) studied the repair of UV-induced damage in several members of the large MT gene family in human cells. They used an immunological method that allows isolation of DNA fragments containing bromodeoxyuridine (BrdUrd) in repair patches using a monoclonal antibody that recognizes BrdUrd (Leadon, 1988). Specific ^{32}P-labeled hybridization probes were used to detect and quantitate the sequences of interest in the repaired fraction of the DNA. Unlike methods that rely on the loss of an

endonuclease-sensitive site from a sequence, this technique allows comparative estimates of repair, but not actual lesion frequencies. It also provides a sensitive measurement of repair, since only one repair event will place an entire fragment into the "repaired" class, regardless of the lesion frequency on the fragment (Leadon, 1986). With this method, they showed that repair in fragments carrying the unexpressed MT-I_B and MT-II_B genes resembled repair in genome overall. About twice as much repair was found in the MT-I_A, MT-II_A, and MT-I_E genes which are expressed at a basal level. Furthermore, inducing transcription by cadmium or steroids increased repair about another twofold for the three expressed genes which are responsive to the inducers. The rates of repair in the unexpressed genes were not altered by these treatment. These results are consistent with the notion that the repair of these MT genes is correlated with their rates of transcription.

The findings of strand-specific repair and preferential repair of induced genes support a direct relationship between nucleotide excision repair and transcription. The recent demonstration that strand-specific repair occurs in the *E. coli* lactose operon (Mellon & Hanawalt, 1989) further indicates that this feature of transcription-dependent repair may be a general phenomenon, unrelated to eukaryotic chromatin structure. At least three models have been suggested to explain the effects of transcription on DNA repair: (i) the RNA polymerase antenna model, (ii) the twin supercoiling domain model, and (iii) the RNA-DNA heteroduplex model (van Houten, 1990).

RNA polymerase antenna model: There is strong evidence that transcription is blocked by py-py dimers in template DNA in both

prokaryotic and eukaryotic cells (Sauerbier & Hercules, 1978). It seems reasonable to suppose that only damage on the template strand produces this effect, whereas lesions on the other strand do not. It has been suggested that this lesion-stalled RNA polymerase acts as a damage antenna for the repair complex. The configuration of a lesion at the site of blockage may resemble the one brought about by preincision activities, thereby bypassing the need for some preincision processing. For example, DNA unwinding at the site of transcription bubble might circumvent the need for a repair helicase(s). The other possibility is that some specific factors in the transcription complex mediate the formation of preincision complex via direct protein-protein interactions.

Twin supercoiling domain model: It has been shown that passage of RNA polymerase through a gene leads to two waves of superhelical tension in the DNA: a positive supercoil wave in front of the transcription complex and a negative supercoil wave following behind the RNA polymerase (Tsoa et al., 1989; Wu et al., 1988). It is possible that these localized supercoiled waves could influence the repair of DNA lesions, although there is as yet no evidence of such an effect.

RNA-DNA heteroduplex model: Since the conformation of a DNA-RNA hybrid molecule in the transcription bubble is more A form than a DNA-DNA duplex, it is possible that a DNA lesion in close proximity to this RNA-DNA junction would alter the conformation. This might facilitate the recognition by repair enzymes.

B. Experimental systems that have been used to study the mechanisms of mutagenesis

1. Prokaryotic systems

A general strategy for studying mutagenic mechanisms is to determine the mutational specificity, i.e., the precise nature and locations of mutations produced by mutagens. The well-developed techniques of bacterial molecular genetics provide a simple way of obtaining precise mutational information on prokaryotic systems. Due to the simple organization of prokaryotic cells, extensive genetic studies have been done using prokaryotic genes. One of these is the *E. coli lacI* gene in which a large number of nonsense sites have been characterized and correlated with specific codons (Coulondre & Miller, 1977). This system has been widely used to investigate both spontaneous and induced mutations in bacteria (Calos & Miller, 1981; Miller, 1982). By comparing the profile of occurrences of nonsense mutations induced by different mutagens, the specificity of each mutagen has been determined.

In the 1970's, most of the methods for analysis of the specificity of mutagens were based on the production or reversion of nonsense mutations (Yanofsky, 1971; Prakash & Sherman, 1973; Ames et al., 1973; Lawrence & Christensen, 1979), and thus were biased to specific sites in the DNA. Only by analysis of a large number of such sites can one overcome this limitation (Coulondre & Miller, 1977). To eliminate the need for mutating specific sites in the DNA, a directed mutagenesis method has been developed for the analysis of the mutational specificity of mutagens. With this approach, Fuchs et al. (1981) analyzed forward mutations induced in

the tetracycline-resistance gene (Tet^R) of plasmid pBR322 by directing the chemical reaction of carcinogen N-acetoxy-AAF to a restriction fragment inside in this target gene. The AAF-modified restriction fragment was reinserted by *in vitro* ligation into the non-reacted pBR322 restriction fragment, and then the ligation mixture was used to transform *E. coli*. Mutants were selected for ampicillin resistance and tetracycline sensitivity. The plasmid DNA of such mutants was analyzed for sequence changes in the fragment where the AAF binding had been directed. Their results showed that most (90%) of the mutations induced by AAF adducts were frameshifts and were located within specific sequences, so called "hot spots" (Fuchs et al., 1981; Koffel-schwartz et al., 1984). This method is of general applicability to any mutagen or carcinogen that reacts with DNA. Livneh (1983) used a similar approach to examine UV-induced mutations in the Tet^R gene of plasmid pXf3. The data revealed that this system allowed the detection of various types of mutations, including transitions, transversions, frameshifts and deletions.

In addition to plasmid vectors, phage vectors were also used for analysis of mutational specificity. LeClerc & Isotock (1982) used M13*lac* hybrid phage, which contains the M13 single-stranded DNA with an insert of the regulatory region and the α-peptide of the *lacZ* gene of *E. coli* (Messing et al., 1977), as a mutation system to determine the UV-induced, forward mutations. Infection of *E. coli* JM103*lacZ M15* cells with this hybrid phage allows the production of a functional β-galactosidase by intracistronic complementation. However, mutations in the *lac* insert of phage DNA that inactivate the α-complementing activity give rise to colorless plagues of infected cells, easily distinguishable from the blue

plagues of M13*lac*$^+$-infected cells. With this system, they have determined the sequence changes in the 250-nucleotide region of *lac* operon from a large number of UV-induced phage mutants (LeClerc et al., 1984).

The cI repressor gene of lambda phage has also been used for determining the UV-mutational specificity (Skopek & Hutchinson, 1982; Wood et al., 1984). Mutants with the clear plaque phenotype were isolated, and the mutations were sorted into the cI, cII and cIII genes by complementation tests. Each cI mutation was mapped by genetic crosses. The appropriate DNA restriction fragment was isolated, and then sequenced. The spectra of UV-induced mutations observed in these studies are in good agreement with that obtained by LeClerc & Istock (1982) for the *E. coli lac* promoter cloned in M13.

2. Shuttle vector systems in mammalian cells.

Although the complexity of higher eukaryotic cells makes it very difficult to obtain information at the DNA sequence level on mutations in such cells, the development of recombinant DNA techniques during the last decade have enabled researchers to devise new approaches to the study of mutations in mammalian cells. One of these is the use of shuttle vectors (Calos et al., 1983; Lebkowski et al., 1985; Seidman et al., 1985), which can replicate in both mammalian and bacterial cells. Therefore, a target gene carried on this vector can be mutated in mammalian cells and then transferred to bacteria for rapid analysis of mutations. Although these SV40-based vectors must be supplied with T antigen for replication, they have to rely totally on the host cell DNA repair and DNA replication machinery, and therefore can be used to investigate the host cell's

mutagenesis activity based on the assumption that mutations are produced when DNA synthesis occurs on a damaged DNA template (Konze-Thomas et al., 1982; Grossman et al., 1985).

The *lacI* shuttle vector, which contains the entire *lacI* gene, the SV40 origin of replication and plasmid sequences required for replication in *E. coli*, was first used for determination of mutagenic specificity, due to the elegant identification system of *lacI* nonsense mutation developed by Coulondre & Miller (1977). However, this vector acquired mutations in its target gene at a very high frequency (1%), resulting simply from passage of the vectors through the mammalian cells (Calos et al., 1983; Razzaque et al., 1984). This high background frequency presented an obstacle to studies of mutagens that do not induce a very high mutant frequency. Later it was found that in human 293 cells, the frequency of transfection-associated mutations is lower (Lebkowski et al., 1984), and so these host cells have been used for transfecting the *lacI* shuttle. Using these cells, Lebkowski et al. (1985; 1986) found that the UV- and ethylnitrosourea (ENU)-induced mutant frequencies were significantly higher than the background frequency, and the mutational specificity of these two agents was analyzed.

Seidman et al. (1985) showed that the spontaneous mutations obtained in the SV40-based shuttle vectors can be minimized by judicious design of the vectors. They constructed the pZ189 vector, which contains the origin of replication and ß-lactamase gene from pBR327, a *supF* suppressor tRNA gene from *E. coli* as the target for mutational analysis, and the early region of SV40 DNA to allow replication in mammalian cells. Because of the small size of the target gene and its strategic location between two

essential genes, the ß-lactamase gene and the plasmid origin of replication, the spontaneous mutation frequency in the *supF* gene was greatly reduced (~0.04%). This vector, as well as its closely related plasmid, pS189 (Seidman, 1989), have been widely used for studying the spontaneous and carcinogen-induced mutations in mammalian cells (Hauser et al., 1986, Bigger et al., 1989; Kraemer & Seidman, 1989; Maher et al., 1989; Mah et al., 1990; Bodlt et al., 1991). These studies have demonstrated that a single base pair change at almost any site in the 85bp structure of the tRNA results in a mutant phenotype (Kraemer & Seidman, 1989), indicating that this gene is exceptionally responsive to mutagens and allows for very few silent mutations.

Another way to reduce the background mutation frequency of shuttle vectors has been the use of Epstein-Barr virus (EBV)-based vectors which are stably maintained in the cells and replicated synchronously with genomic DNA. Since most of the spontaneous mutations that occurr in the transiently replicating, SV40-based vectors are early events and are induced by transfection process (Razzaque et al., 1983; Lebkowski et al., 1984), the use of such stably maintained vector allows one to isolate rare clones of cells that contain only unmutated vector DNA, and thus circumvent the problem of high vectors' background frequencies. Two EBV-shuttles, pHET and pMc_i carrying the target genes of Herpes simplex virus *thymidine kinase* (HSV*tk*) and *E. coli lacI*, respectively, were constructed for this purpose, and have been used for the analysis of alkylating agent-induced mutations (DuBridge et al., 1987; Drinkwater & Klinedinst, 1986; Eckert et al., 1988).

3. Stably integrated exogenous gene system in mammalian cells

Shuttle vector systems have provided a rapid way for the study of sequence alterations in mammalian cells. However, one cannot be certain that mutations detected using these vectors reflect the normal nuclear replicative and repair mechanisms of the host cell, because the extrachromosomal DNA does not exhibit the normal chromosomal context. One approach to overcoming this problem is to study of the mutations induced in a small exogenous locus stably integrated into mammalian genome. For this approach, Tindall et al. (1984; 1986) constructed a cell line, AS52, which carries a single, functional copy of the *E. coli gpt* gene stably integrated into a HPRT$^-$ CHO cell line. Since the bacterial *gpt* gene product, xanthine-guanine phosphoribosyltransferase (XPRT), in the AS52 cell line is functionally analogous to HPRT of wild-type CHO cells, mutants at the *gpt* locus can be selected by their resistance to 6-thioguanine (6-TG). This cell line has been used in quantitative and molecular studies of mutations induced by several mutagens, including X-ray, UV, ENU, and ICR191 (Tindall et al. 1984; 1986; Stankowski et al., 1986). However, due to the difficulty to determine the sequence of mutated target gene, Southern blot analysis was used for their molecular studies of mutations, which had limited the identification of single base changes in this system.

Other systems have been devised to sequence mutated genes on a mammalian chromosome: the HSV*tk* gene on a shuttle vector that integrates into a chromosome of tk$^-$ mouse fibroblasts (Zakour et al., 1986), and the *supF* gene on the lambda phage DNA stably incorporated in a chromosome of mouse L cells (Glazer et al., 1986). Both systems allow the rescue of

integrated plasmid and phage DNA from mammalian cells by cloning and lambda DNA packaging, respectively, and therefore facilitate the determination of exact sequence changes on target genes.

4. Endogenous mammalian genes systems

The analysis of mutations at the DNA sequence level in the endogenous genes in mammalian cells requires a) a hemizygous target gene, b) a selection scheme to isolate mutant cells, and c) a method to retrieve and amplify the target gene for sequence analysis. The *HPRT* gene resides on the X-chromosome in all mammals studied (Thaker, 1985), and therefore, the locus is hemizygous in males and functionally hemizygous in females. Furthermore, the selection conditions are available to isolate *hprt*$^-$ mutants both *in vitro* (DeMars, 1974) and *in vivo* (Albertini et al., 1982). However, until recently the extremely large size of this gene had limited its application as a target for the generation of mutational spectra because of the technical difficulties associated with cloning and sequencing a large gene.

The recent development of polymerase chain reaction (PCR) (Mullis et al., 1986; Saiki et al., 1988) has made it possible to selectively amplify a short segment of DNA more than 10^5-fold, so that DNA sequence analysis can be performed without the need for time-consuming library construction and screening. This technique, in conjunction with the utilization of cDNA as the source of sequence information, has provided a simple way to analyze mutations induced in the *HPRT* gene. Vrieling et al. (1989) have studied the UV-induced *hprt* mutations in a Chinese hamster cell line. Using reverse transcriptase, they synthesized *hprt* cDNA from total

cytoplasmic RNA isolated from 10-20 X 10^6 cells, amplified hprt cDNA by PCR, and then cloned it into an M13 vector for sequencing. To minimize the chance that the sequence alterations were due to nucleotide misincorporation by the Taq polymerase during amplification, at least two clones for each mutants were sequenced, and only mutations found in both clones were considered to be mutagen-induced. The same approach was used by Carothers et al. (1989) and Simpson et al. (1988) for analyses of mutations induced in the *HPRT* gene in human lymphoblasts and in the *dhfr* gene of CHO cells, respectively.

Yang et al. (1989) have developed a method allowing the rapid detection of sequence alterations in the endogenous genes of mammalian cells using only a few mutant cells, an improvement that is necessary for studying mutational spectra in endogenous genes of cultured mammalian cells that have a finite life span. This method involved synthesis of total cDNA directly from a lysate of a small number of human cells, selective amplification of the cDNA of the gene of interest 10^{10}- to 10^{11}-fold, and determination of the nucleotide sequence by direct sequencing of the amplified product. To avoid detecting the mutations randomly introduced by reverse transcriptase and Tag polymerase, 100-500 cells are subjected to reverse transcription and PCR. Since the amplified products were sequenced directly to obtain the consensus sequence, a random base substitution, even introduced in the reverse transcription process or in an early round of PCR, will not be seen on the DNA sequencing gel. This method is being used widely for studying mutational spectra in endogenous genes of mammalian cells.

C. The influence of DNA replication and nucleotide excision repair on mutagenesis

1. General roles of replication and repair in mutagenesis

The effect of nucleotide excision repair on the mutagenicity of various carcinogens in human diploid cells has been determined in two ways. One method made use of nucleotide excision repair-deficient fibroblasts derived from XP patients. Comparisons of these cells with cells obtained from normal donors showed that the XP cells are much more sensitive to the mutagenic effects of several carcinogens, such as UV (Maher et al., 1979; 1982), BPDE (Yang et al., 1980), 1-nitrosopyrene (Maher et al., 1988), and metabolic derivatives of several polycyclic hydrocarbon compounds (Maher et al., 1977). The second method investigated the frequency of mutant induction in cells treated with carcinogens and held in confluence. Such cells are able to carry out excision repair but are unable to replicate. After various periods of time in confluence, cells were replated at lower density, allowed to replicate, and after an appropriate expression period assayed for frequency of induced mutants. The repair-proficient cells released immediately following treatment exhibited the highest frequency. Furthermore, there was a gradual reduction of the frequency of induced mutants with time held in confluence. This was directly correlated with a gradual increase in survival. However, the repair-deficient cells showed no change in their survival and frequency of mutants throughout the entire holding period (Maher et al., 1979; Yang et al., 1980). These results indicate that excision repair can decrease the cytotoxic and mutagenic consequences of

carcinogen treatment, most probably by removing the DNA lesions responsible for the effects.

In order to determine the relationship of DNA replication to mutagenesis, synchronized human cells were used so that they could be exposed to carcinogens at various times in the cell cycle and then examined for the frequency of mutant induction. The results of these studies showed that the frequencies of mutants induced by UV (Konze-Thomas et al., 1982), BPDE (Yang et al., 1982), and 1-nitrosopyrene (Maher et al., 1988) were highest in cells treated in early S phase and much lower in cells treated in early G_1 phase, 12-15 hr. before the onset of DNA synthesis. Furthermore, the frequencies of mutant induction were directly correlated with the number of unrepaired lesions remaining in the cells' DNA when the target gene (*HPRT*) was replicated. These data support the hypothesis that DNA replication is the cellular event that converts unexcised DNA lesions into mutations, and that excision repair of such lesions in human fibroblasts protects them from potentially mutagenic lesions.

2. Role of heterogeneous repair in mutagenesis

Intragenomic heterogeneity in repair processes may influence mutagenesis. For instance, the apparent lack of repair of py-py dimers in unexpressed genes of rodent cells *in vitro* would be expected to result in higher mutation frequencies in those genes, compared to actively transcribed genes. However, since selectable phenotypes result only from expressed genes, it is not possible to select for mutants in such non-expressed genes. Therefore, the comparison of mutation frequencies in

expressed and silent genes would require development of a specific test system. One ideal system would be the use of an inducible gene whose loss of activity could provide cells with a phenotypic change suitable for selection under defined conditions, which would have to be applied after mutagen treatment and mutation fixation. So far, no such system has been available for mutational analysis.

Another important question raised by heterogenous repair is whether the preferential repair of the transcribed strand of a gene would result in a strand bias in mutations. Indeed, strand bias was reported for AAF- (Carothers et al., 1989), BPDE- (Carothers & Grunberger, 1990), and (±)-3α,4ß-dihydroxy-1α,2α-epoxy-1,2,3,4,-tetrahydrobenzo[c]phenanthrene (B[c]PHDE)- (Carothers et al., 1990) induced mutations in the rodent *dhfr* gene. However, since the identification mutants depends on the loss of the functional *dhfr* gene product, the sites at which a sequence change results in a non-functional protein may also be biased with respect to the strands. Notably, the selection of a $dhfr^-$ phenotype appears to be very stringent and tends to yield mostly nonsense and mRNA splicing mutations (Carothers et al., 1989). Therefore, such data can not readily be used to make inferences about the repair process, unless comparative studies in which mutations are induced in cells with different repair ability or with different lengths of time available for repair prior to mutation fixation have been performed.

Carothers et al. (1991) designed a nonsense-codon reversion assay, in which the targets for reversion are two nonsense codons at both ends of the *dhfr* gene. The sequence context for B[c]PHDE-induced base changes was similar (four tandem purines) on both DNA strands, and all R (purine) ->

T transversions primarily induced by B[c]PHDE at the two nonsense codons yield dhfr$^+$ phenotype. Thus in this system, mutational strand bias is independent of the selection for phenotypic change. Since each of the nonsense codons contains two purines on the nontranscribed strand, a 2:1 ratio of mutations at the purines on the nontranscribed strand versus the transcribed strand was expected if the DNA strands were mutated with equal probability. Instead, they found that >90% of the mutations were targeted to the purines on the nontranscribed strand, consistent with the model of preferential repair of the transcribed strand. However, preferential repair may not be the sole explanation for the discrepancy between the expected and observed ratio. For example, if the frequency of adduct formation on each of the purine targets were unequal, this would result in a strand-biased modification. Such data has not been yet determined in their system.

Another factor that could cause a strand specificity of mutagenesis is the fidelity of DNA replication of the two DNA strands. Studies on the replication of SV40 DNA using a reconstituted *in vitro* system implicated the requirement of DNA polymerase δ and polymerase α in the synthesis of the leading strand and lagging strand, respectively (Prelich & Stillman, 1988). If the ability of the two DNA polymerases to insert correct bases opposite the carcinogen-modified bases were different, this could result in a strand-specific mutagenesis. Indeed, it has been shown that during translesional DNA synthesis on an oligonucleotide template containing an 8-oxo-7-hydrodeoxyguanosine, DNA polymerase α is more error prone than polymerase δ (Shibutani et al., 1991). A difference in fidelity of DNA replication of the two strands has been suggested by Vrieling et al.

(1989) as the explanation for the strong strand bias of UV-induced *hprt* mutations in a repair-deficient Chinese hamster cell line (V-H1). Since the cell cannot repair UV damage, one would expect a random distribution of premutagenic lesions. However, ~90% of the mutations derived from such cells were targeted to photoproducts in the transcribed strand. Since this strand is also the leading strand for most part of the *hprt* gene, they suggested that polymerase δ is more error prone than polymerase α. However, this argument is contradictory to the results obtained by Shibutani et al. (1991). Furthermore, direct evidence of different error rates of the two polymerases during DNA replication bypassing UV-induced photoproducts has not been provided.

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CHAPTER II

Effect of excision repair by diploid human fibroblasts on the kinds and spectra of mutations induced by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene in the coding region of *HPRT* gene

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SUMMARY

To investigate the effect of nucleotide excision repair on mutations induced in diploid human fibroblasts by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE), a direct-acting carcinogen that forms bulky adducts predominantly (>90%) with guanine, we synchronized cells, exposed them to BPDE at the beginning of S-phase or in early G_1-phase, selected 6-thioguanine-resistant cells, and determined the nature of the mutations induced in the coding region of the hypoxanthine (guanine) phosphoribosyl- transferase (*HPRT*) gene. The mutant frequency in the populations treated in S was high (30 to 50 times background); for the G_1 cells, which had at least 12 hr to excise BPDE adducts before S-phase DNA replication, the frequency was 3-fold lower. To identify the mutations, the mRNA in lysates of 100 to 500 cells from each individual mutant clone was copied into cDNA, and the cDNA of the *HPRT* gene was amplified by polymerase chain reaction and sequenced directly from the product. The majority of the mutations in either population (20/24 and 19/23) consisted of base substitutions, predominantly G·C to T·A. But their location differed significantly, with one very strong "hot spot" found only in the G_1 cells. Assuming that the premutagenic lesion involves guanine, the strand distribution of the premutagenic lesions also differed significantly. In the cells treated in S-phase, 21% of such lesions were located in the transcribed strand, whereas in the G_1 phase-treated cells, none were. This suggests that in the *HPRT* gene of diploid human cells, excision repair of BPDE adducts occurs preferentially on the transcribed

strand.

INTRODUCTION

In previous studies, we and our colleagues investigated the relationship of DNA repair and DNA replication to mutagenesis induced by carcinogens in diploid human fibroblasts. We found that the frequency of mutants induced by UV radiation (1, 2), (±)-7β,8α-dihydroxy-9α,10α-expoxy-,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) (3, 4), or 1-nitroso-pyrene (5) in nucleotide excision repair-proficient cells is highest in cells treated at early S-phase and much lower in cells treated in early G_1-phase. The frequency per dose is higher still in cells from xeroderma pigmentosum (XP) patients that are virtually incapable of nucleotide excision repair i.e., and mutation induction in these cells is not cell cycle-dependent (2, 3). These data suggest that repair processes play a key role in the cell cycle-related sensitivity to mutagenicity observed in synchronized repair-proficient human fibroblasts, and that DNA replication is centrally involved in the conversion of DNA damage into mutations.

The effect of the cell cycle on the frequency of transformation has also been determined in diploid human cells (6) and rodent cells lines (7). Populations treated in early S-phase exhibit a higher frequency of transformed cells than populations exposed in early G_1-phase (6, 7). This difference was not seen with XP cells (6). A higher frequency of transformants might simply reflect a higher frequency of mutations induced in populations treated at the beginning of S-phase. However, it might result from a difference in the *kinds* or *spectrum* of mutations induced in

the two populations, so that a specific mutation required for transformation, e.g., leading to the activation of a cellular proto-oncogene, occurs preferentially when cells are treated in S-phase.

There are no published studies that have determined whether cells treated in S-phase and cells treated in G_1 differ in the *type* and/or *location* of mutations induced. However, Hanawalt, Bohr, and their colleagues showed that in certain mammalian cell lines the rate of nucleotide excison repair of pyrimidine dimers in transcriptionally active genes differs from that occurring in non-actively-transcribed genes (8-11). Mellon *et al.* (12) showed that in a Chinese hamster cell line and in human cells, the rate of excision repair of damage in the transcribed strand of the gene for dihydrofolate reductase (*dhfr*) is more rapid than in the non-transcribed strand. If such differential repair occurs in the *HPRT* gene of diploid human cells, and if mutations are introduced by the DNA replication which occurs in S-phase, significant differences should be seen between the mutational spectrum of synchronized cells exposed to DNA damaging agents just as S-phase DNA replication of a target gene is taking place or exposed in early G_1. To test this hypothesis, we treated repair-proficient human fibroblasts with BPDE in early S-phase or in early G_1-phase, and determined the kinds and location of mutations induced in the coding region of the hypoxanthine (guanine) phosphoribosyl-transferase (*HPRT*) gene. The results are consistent with the hypothesis. The two populations showed no difference in the kinds of mutations induced by BPDE but the location of the mutations differed significantly.

MATERIALS AND METHODS

Cells and media. Diploid human cells derived from neonatal foreskin material (13) were cultured in modified McM medium (14) prepared with Earle's salts and containing 10% supplemented calf serum (Hyclone, Logan UT) (culture medium). For selection of 6-thioguanine-resistant cells, the same medium, but lacking adenine and containing 5% supplemented calf serum (Hyclone), 5% fetal calf serum, and 40μM 6-thioguanine was used.

Cell synchronization. Cells were inoculated at a density 8- to 10-fold less than that attained at confluence and re-fed culture medium every other day to stimulate rapid growth to confluence. When the cells reached confluence, they were fed daily for 3 additional days to allow them to fill the dish to capacity. The medium was not changed for the next 72 to 96 hr so that the cells would cease proliferation and enter the G_0 state. Autoradiography studies showed that in such cultures DNA synthesis was virtually eliminated (2). To stimulate the cells to enter the cell cycle, they were plated in culture medium at a density of 10^4 cells/cm^2. Previous studies show that such cells begin DNA synthesis (S-phase) after 16 to 17 hours (4), and that at least 80% of the cells are at S-phase after 4 additional hours (2).

Exposure to mutagen and isolation of *HPRT* mutants. The details of the mutagenesis assay have been described (4,5). Briefly, a series of populations (1.5×10^6 cells each, plated in 150 mm-diameter dishes) was treated 17 hr after release from G_0 (at the beginning of S-phase) or 4 hr after release from G_0 (in early G_1-phase). The culture medium was replaced

with serum-free medium. BPDE, freshly dissolved in anhydrous dimethylsulfoxide, was delivered into the serum-free medium by micropipette. After 1 hr, the medium was removed, and the cells were rinsed with phosphate buffered saline (pH 7.4) and re-fed with fresh culture medium. The cells in one dish were assayed immediately for survival of colony-forming ability as described (15). The rest were allowed 8 days for expression of resistance to 6-thioguanine before 0.5 x 10^6 cells from each population were plated in selective medium. When macroscopic drug resistant clones developed 14 days later, these were located, isolated, and expanded to 10^4 to 5 x 10^5 cells.

Synthesis of first-strand cDNA directly from mRNA in cells. Cells were trypsinized and suspended in culture medium, the cell number was determined by electronic counting, and the cells were diluted in cold phosphate buffer saline (pH 7.4). From 100 to 500 cells were transferred to a 0.5 ml Eppendorf tube and centrifuged for 10 min at 4^0C. The supernatant was removed, and the cell pellet was resuspended in 5 μl of the cDNA cocktail described in detail by Yang *et al* (16). The reverse transcriptase reaction was performed at 37^0C for 1 hr to allow the cell membranes to be lysed by detergent and first-strand cDNA to be synthesized from total cytoplasmic poly A mRNA.

Amplification of *HPRT* cDNA and DNA sequencing. The experimental conditions, optimized for preparing second-strand *HPRT* cDNA, amplifying the cDNA 10^{11}-fold, using two 30-cycles of polymerase chain reaction sequencing the product directly using the three sequencing primers and a

modified Sanger dideoxynucleotide procedure have been described (16).

RESULTS

Cytotoxicity and frequency of mutants induced by BPDE in cells treated in the G_1-phase or S-phase of the cell cycle. Individual populations of cells were synchronized and exposed to various concentrations of BPDE in early S-phase or in early G_1. The cells were assayed for survival, and for the frequency of 6-thioguanine resistant cells (Fig. 1). As expected (3), there was little difference in the sensitivity of the two populations to the cytotoxic effects of BPDE indicating that the initial level of DNA adduct formation in the two populations was comparable (17). In contrast, the frequency of mutants induced in populations treated in early G_1-phase was 3-fold lower than that of cells treated in early S-phase. These results support the hypothesis that S-phase DNA replication is responsible for converting premutagenic BPDE-induced lesions into mutations since cells treated in G_1-phase will be able to excise a higher percentage of BPDE-induced adducts than will cells treated in S-phase. It should be pointed out that in each experiment, the frequency of 6-thioguanine-resistant cells in the populations treated at the onset of S was 30 to 50 times higher than background; in the cells treated in the G_1-phase, it was 10 to 16 times higher than background. Therefore, the chances of including a mutant containing a spontaneous mutation, rather than a BPDE-induced mutation, in the samples analyzed at the sequence level was very low.

Characterizaton of *HPRT* mutants. To determine the nature of the BPDE-induced mutations at the DNA sequence level, 20 drug-resistant clones were isolated from cells treated in S-phase and 20 from cells treated in

Figure 1. Cytotoxicity (upper panel) and frequency of mutations to 6-thioguanine resistance (lower panel) induced by BPDE in human cells treated in early S-phase (0) or 12 hr prior to the onset of S (0). The frequencies have been corrected for the cloning efficiency (40% to 60%) of the cells at the time of selection. The background frequencies which ranges from (5×10^{-6} to 15×10^{-6}) have been subtracted. Line drawn by the method of least squares.

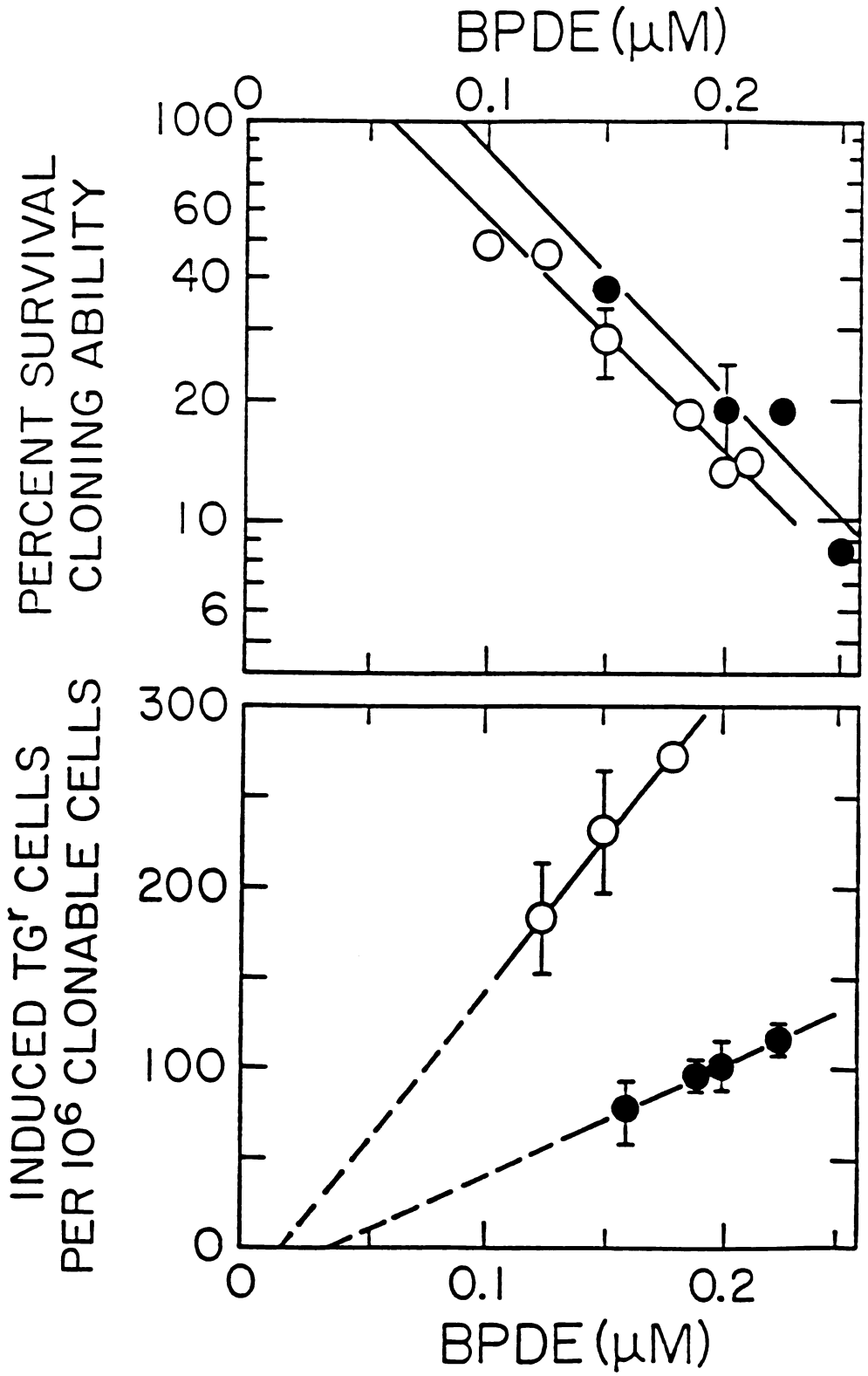
BPDE (μM)
0
0.1
0.2
100
60
40
20
10
6
PERCENT SURVIVAL
CLONING ABILITY
300
200
100
0
INDUCED TG^r CELLS
PER 10^6 CLONABLE CELLS
0
0.1
0.2
BPDE (μM)

Figure 1

G_1. The cells were tested for inability to grow in medium containing hypoxanthine (10^{-4} M), aminopterin (2×10^{-6} M), and thymidine (3×10^{-5} M), in order to confirm that they lacked a functional *HPRT* gene. Then cDNA was synthesized from the total mRNA of a lysate of 100 to 500 cells from each clone, and the cDNA of the *HPRT* gene was amplified 10^{11}-fold and sequenced directly. The results are shown in Tables 1 and 2. All 20 mutants listed from each phase represent unequivocally independent mutants, either because their alterations were unique or because they were derived from independent populations of treated cells.

There was no significant difference between G_1- and S-phase-derived mutants in the kinds of mutations they contained (Table 1 and 2). 16 mutants out of the 20 from each phase contained base substitutions, with 1 mutant from each phase exhibiting a second base substitution at a site distant from the first, and with 1 mutant from G_1-phase cells exhibiting a tandem base substitution. Of the remaining 4 mutants from each phase, 3 may also have contained base substitutions in a 5' or 3' consensus splice site, since one of the nine exons was completely deleted from their cDNA, but no other change was seen. The fourth G_1-phase mutant contained an A·T base pair insertion, and the fourth S-phase mutant exhibited a G·C base pair deletion.

As shown in Tables 1 and 2, in both populations the BPDE-induced mutations were not randomly distributed throughout the 9 exons of the coding region of the gene. The majority (16/24 for G_1-phase and 13/23 for S-phase) were located in exon 2 or the first half of the exon 3. Table 3 shows that there also was no significant difference in the kinds of base substitutions observed in the mutants obtained from the two populations.

Table 1. Kinds and locations of the mutations induced in the coding region of the *HPRT* gene in cells treated with BPDE in S phase of the cell cycle

Mutant	Position	Exon	Type of mutation	Surrounding sequence (coding strand)*	Amino acid change	Strand with the affected purine
			Base substitution			
BPS16†	88	2	G·C → T·A	GCT GAG GAT	Glu → Stop	NT
BPS87	97	2	G·C → T·A	TTG GAA AGG	Glu → Stop	NT
BPS28	119	2	G·C → T·A	CAT GGA CTA	Gly → Val	NT
BPS51	119	2	G·C → T·A	CAT GGA CTA	Gly → Val	NT
BPS4	130	2	G·C → T·A	ATG GAC AGG	Asp → Tyr	NT
BPS66	134	2	G·C → T·A	GAC AGG ACT	Arg → Met	NT
BPS3	149	3	G·C → T·A	CTT GCT CGA	Ala → Asp	T
BPS16†	178	3	G·C → T·A	GGC CAT CAC	His → Asn	T
BPS21	178	3	G·C → T·A	GGC CAT CAC	His → Asn	T
BPS23	178	3	G·C → T·A	GGC CAT CAC	His → Asn	T
BPS94	212	3	G·C → T·A	AAG GGG GGC	Gly → Val	NT
BPS47	229	3	G·C → T·A	GCT GAC CTG	Asp → Tyr	NT
BPS34	337	4	G·C → T·A	GGG GAC ATA	Asp → Tyr	NT
BPS78	438	6	G·C → C·G	ACT TTG CTT	Leu → Phe	NT
BPS83	447	6	G·C → C·G	TCC TTG GTC	Leu → Phe	NT
BPS75	451	6	A·T → G·C	GTC AGG CAG	Arg → Gly	NT
BPS13	471	6	G·C → T·A	AAG ATG GTC	Met → Ile	NT
BPS62	478	6	G·C → A·T	AAG GTC GCA	Val → Ile	NT
BPS95	531	7	G·C → T·A	CCA GAC TTT	Asp → Glu	T
BPS41	568	8	G·C → T·A	GTA GGA TAT	Gly → Stop	NT
			Putative splice site mutation			
BPS72	28–134	2	Exon 2 missing			NA
BPS32	319–384	4	Exon 4 missing			NA
BPS69	486–532	7	Exon 7 missing			NA
			Other			
BPS37	477 or 478	6	Deletion of a G	AAG GTC GCA	Frameshift	NT

T, transcribed; NT, nontranscribed; NA, not applicable.
*The base that was altered is underlined.
†Mutant with two base substitutions.

Table 2. Kinds and locations of the mutations induced in the coding region of the *HPRT* gene in cells treated with BPDE in G_1 phase of the cell cycle

Mutant	Position	Exon	Type of mutation	Surrounding sequence (coding strand)*	Amino acid change	Strand with the affected purine
			Base substitution			
BPG22	88	2	G·C → T·A	GCT GAG GAT	Glu → Stop	NT
BPG7	97	2	G·C → T·A	TTG GAA AGG	Glu → Stop	NT
BPG113	130	2	G·C → T·A	ATG GAC AGG	Asp → Tyr	NT
BPG29	134	2	G·C → T·A	GAC AGG ACT	Arg → Met	NT
BPG109	139	3	G·C → A·T	ACT GAA CGT	Glu → Lys	NT
BPG107	208	3	G·C → A·T	AAG GGG GGC	Gly → Arg	NT
BPG101	211	3	G·C → C·G	AAG GGG GGC	Gly → Arg	NT
BPG19	211	3	G·C → T·A	AAG GGG GGC	Gly → Cys	NT
BPG12	212	3	G·C → T·A	AAG GGG GGC	Gly → Val	NT
BPG13	212	3	G·C → T·A	AAG GGG GGC	Gly → Val	NT
BPG44	212	3	G·C → A·T	AAG GGG GGC	Gly → Asp	NT
BPG64	212	3	G·C → T·A	AAG GGG GGC	Gly → Val	NT
BPG77	229	3	G·C → T·A	GCT GAC CTG	Asp → Tyr	NT
BPG48	229	3	G·C → T·A	GCT GAC CTG	Asp → Tyr	NT
BPG8†	388	5	G·C → C·G	AAT GTC TTG	Val → Leu	NT
BPG76	393	5	G·C → C·G	GTC TTG ATT	Leu → Phe	NT
BPG18	419	6	G·C → T·A	ACT GGC AAA	Gly → Val	NT
BPG8†	529	7	G·C → T·A	CCA GAC TTT	Asp → Tyr	NT
BPG112	535	8	G·C → T·A	TTT GTT GGA	Val → Phe	NT
			Putative splice site mutation			
BPG10	28–134	2	Exon 2 missing			NA
BPG116	319–384	4	Exon 4 missing			NA
BPG9	403–485	6	Exon 6 missing			NA
			Other			
BPG17	98–100	2	Insertion of an A	TGG GAA AGG	Frameshift	NT
BPG100	535	8	Deletion of a G	TTT GTT GGA	Frameshift	NT

T, transcribed; NT, nontranscribed; NA, not applicable.
*The base that was altered is underlined.
†Mutant with two base substitutions.

Table 3 Types of base substitutions in *hprt* mutants cells treated with BPDE in G_1-phase and S-phase

Type of base substitutions	No. of substitutions observed	
	G_1-phase	S-phase
Transversions:		
G•C → T•A	13	15
G•C → C•G	3	2
A•T → C•G	0	1
T•A → G•C	1	0
Transitions:		
G•C → A•T	2	1
T•A → C•G	1	0
Total	20	19

All except one for an S-phase-derived mutant and two for G_1-phase mutants, involved G·C base pairs. Transversion of G·C to T·A occurred predominantly, viz.,13/20 (65%) for G_1-phase and 15/19 (79%) for S-phase.

Strand distribution of the premutagenic lesions induced by BPDE. Fig. 2 shows the location (spectrum) of mutations induced by BPDE in cells treated in G_1- and S-phase. BPDE binds DNA predominantly to the N^2 position of guanine (18, 19). If one assumes that the premutagenic lesion in these cells involves guanine, it is possible to distinguish the DNA strand in which the premutational lesion was located. In the population exposed in S-phase, of the 19 mutations which involved G·C base pairs, 4 affected guanine bases (21%) were located on the transcribed strand, and 15 (79%) were located on the nontranscribed strand (Table 2 and Fig. 2). However, in cells exposed in early G_1-phase, in all 18 cases, the affected guanine bases were located on the nontranscribed strand. The strand distribution of guanine bases involved in mutations induced in early G_1-phase differed significantly from that involved in mutations induced at S-phase mutants ($P, < 0.05$, by the Chi square test).

Mutational "hot spot" region obtained from HPRT mutants in cells exposed in G_1-phase. One very strong mutational "hot spot" region was found in the spectrum of mutations induced in cells treated in early G_1-phase (Fig. 2). Eight out of 24 mutations (33%) occurred within positions 208 to 212 (Table 1) in a region containing 6 consecutive guanine residues flanked by adenine on the 5' side and cytosine on the 3' side, a sequence which appears only once in the entire *HPRT* coding region. In contrast, only one out of 23 mutations (4%) induced in cells treated at S-phase occurred in this region (Table 2).

Figure 2. The location of mutations and the strand distribution of the guanine base involved in a base change induced by BPDE in the coding region of *HPRT* gene. The position of the ATG start codon, TAA stop codon, and the 9 exons are indicated. (Vertical lines, base substitutions; square, a deleted base pair; caret, an inserted base pair; shaded rectangles, deleted exons.)

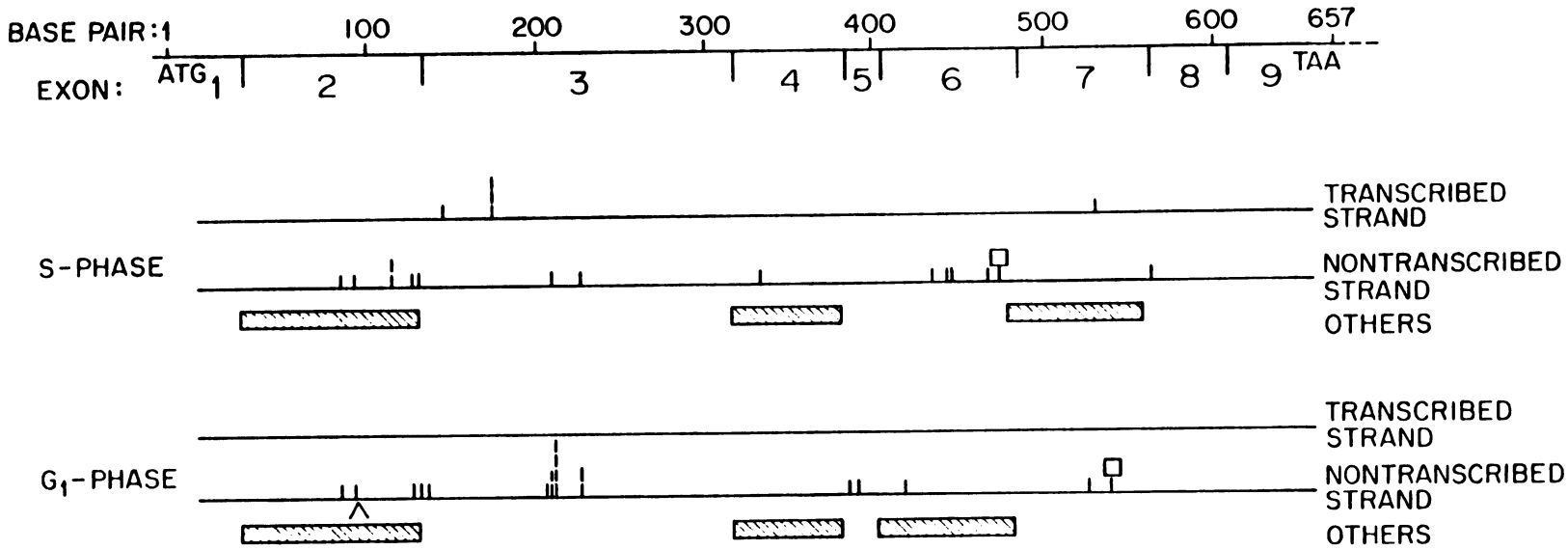
BASE PAIR:1
100
200
300
400
500
600
657
EXON:
ATG
1
2
3
4
5
6
7
8
9
TAA
S-PHASE
TRANSCRIBED STRAND
NONTRANSCRIBED STRAND
OTHERS
G1-PHASE
TRANSCRIBED STRAND
NONTRANSCRIBED STRAND
OTHERS

Figure 2

DISCUSSION

Although the mutant frequency in cells treated 12 hr prior to S-phase was 3 times lower than that in cells treated in early S-phase, the *kinds* of mutations induced were similar. Our finding that BPDE causes base substitutions mainly at G•C base pairs suggests that mutagenesis is targeted to adducts. The majority of the base substitutions were G•C to T•A transversions, which agrees with the results observed in the *lac* I gene of *E. coli* (20), in an extrachromosomal *supF* gene (21), and in several endogenous genes of rodent cell lines (22, 23). The predominance of G•C to T•A substitutions may be the result of the DNA polymerase preferentially inserting an adenine nucleotide opposite a non-instructional base containing a bulky BPDE adduct (24). Alternatively, the presence of a BPDE adduct on guanine may allow purine•purine base pairing to occur with some frequency and if this mispairing is not recognized, the result would be a mutation (20). Evidence of stable purine•purine base pairing in a model oligonucleotide has recently been provided (25, 26). Also, Norman *et al.* (27) have provided evidence that in a double-stranded oligonucleotide, stable base pairing can occur between adenosine and guanosine carrying a polycyclic aromatic adduct in the C8 position.

Of the 20 mutants derived from either phase of the cell cycle, 3 produced *HPRT* mRNA which lacked one of the nine exons completely. These mutants may be mutated in the 5' or 3' consensus splice site so that the splicing machinery cannot recognize the site, and splicing occurs using a nearest neighbor splice site. Splicing mutants resulting in exon skipping

have been found in mutants induced in the *dhfr* gene by UV-radiation or N-acetoxy-2-acetylaminofluorene (28, 29), as well as in UV-induced *hprt* mutants (30) in CHO cells.

Mellon *et al* (12) recently reported a significant difference in the efficiency of removal of UV-induced pyrimidine dimers from the two strands of the *DHFR* gene in cultured hamster and human cells, with the transcribed strand being selectively repaired in both species. Vrieling *et al* (30) investigated UV-induced *hprt* mutants in a repair-proficient and deficient hamster cell line and proposed preferential repair of the transcribed strand as one explanation for the strand specificity of the mutations observed in the repair-proficient cells. Little information has been available on differential or strand-specific repair of DNA damage induced by BPDE. However, if one assumes that the mutations we observed that involved a G•C base pair were targeted to a BPDE guanine adduct, the strand distribution of the premutagenic lesions induced in two populations differed significantly. Our data suggest that BPDE adducts are preferentially removed from the transcribed strand of *HPRT* gene of these human cells, so that at the time the unexcised adducts are converted into mutations, more adducts remain on the nontranscribed strand than on the transcribed strand. Our data further suggest that DNA replication is responsible for converting premutagenic lesions into mutations since it is the cells treated in G_1-phase that have the most time to repair before S-phase DNA replication takes place. It seems highly unlikely, that the strong strand-preference we observed for the mutations that occurred in the cells treated in G_1-phase could have resulted from preferential binding of BPDE to the transcribed strand of the *HPRT* gene in those cells. To

distinguish between strand-specific DNA repair and differential DNA strand binding as an explanation for the observed strand-specificity of mutations, we will carry out similar experiments in excision repair-deficient XP cells.

If DNA synthesis is the cellular event converting the BPDE adducts into mutations, cells treated in S-phase should have little or no time to excise BPDE adducts before replication. Therefore, in the S-phase cells, we expected little strand bias for premutational lesions above that caused by the distribution of guanines in the coding region of the gene, i.e., 160 guanines in the nontranscribed strand, but only 110 in the transcribed strand, a 59:41 ratio. However, only 21% of mutations in S-phase cells corresponded to a guanine located in the transcribed strand. A likely reason why this value was less than 41% is that there was time for excision repair. Since treatment of cells with BPDE temporarily stops DNA replication (31), by the time it resumes, some fraction of the adducts may have been excised. Furthermore, since our method of synchronizing cells does not yield 100% cell synchrony, some of cells may not have reached S-phase at the time they were exposed to BPDE, and so they had some time for repair.

Mazur and Glickman (22) showed that the "hot spot" region for mutations induced by BPDE in the adenine phosphoribosyltransferase gene of CHO cells involved a 5'-AG$(G)_n$A-3' sequence. In the coding region of the human *HPRT* gene, there are 9 such sequences which have not been interrupted by splicing junctions. However, analysis of our sequence context data indicates that only one mutation observed in cells treated in G_1-phase and two in cells treated in S-phase occurred in these sites. In

contrast, our study revealed a cell-cycle dependent "hot spot" in the sequence of 5'-A$(G)_6$C-3' (position 206-213). Eight out of the 24 mutations induced in G_1-phase (33%) were located in this region, compared with 1 out of 23 from S-phase cells (Fig. 2 and Tables 1 and 2). Although it has been reported that certain poly(dG) sequences are the preferred sites for BPDE binding (32, 33), preferential binding cannot explain the cell-cycle dependent feature of this very prominent "hot spot" which we observed, since it was not similarly prominent for the S-phase population. It is more likely that this "hot spot" resulted from inefficient repair of BPDE adducts in the run of 6 consecutive guanine residues, so that after excision repair had occurred for a certain period of time, the adducts remaining in this region represented a higher fraction than originally. Support for this hypothesis comes from several studies showing that the helix of poly (dG).poly (dC) prefers an A-like DNA helix geometry, rather than the traditional B form (34, 35). If this run of G's assumes a specific local DNA conformation, this could perhaps diminish the efficiency of excision repair of the BPDE adducts in this region. If inefficient repair accounts for the "hot spot" seen only in the G_1 cells, it should not be a prominent "hot spot" in XP cells.

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CHAPTER III

LACK OF A CELL CYCLE-DEPENDENT STRAND BIAS FOR MUTATIONS INDUCED IN THE *HPRT* GENE BY (±)-7β,8α-DIHYDROXY-9α,10α-EPOXY-7,8,9,10-TETRAHYDROBENZO[A]PYRENE IN EXCISION REPAIR-DEFICIENT HUMAN CELLS

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SUMMARY

We showed previously that in repair-proficient human cells the location of the premutagenic lesion induced by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE), viz., the guanine in a G•C base substitution, in mutants derived from cells treated at the beginning of S phase just when the hypoxanthine (guanine) phosphoribosyltransferase (*HPRT*) gene is replicated differs significantly from their location in cells treated 12 h prior to the beginning of S phase (early G_1 phase) (Chen, R.-H. *et al.*, Proc. Natl. Acad. Sci. USA, 87:8680-8684, 1990). This suggests that the cells preferentially remove BPDE adducts from the transcribed strand. We have now determined the kinds and location of independent mutations induced by BPDE in the coding region of the *HPRT* gene of synchronized repair-deficient xeroderma pigmentosum (XP) cells (XP12BE, complementation group A), treated at S or in G_1. Nineteen out of 25 mutants derived from S-treated cells and 23 out of 28 mutants from G_1-treated cells contained base substitutions. 89% of these involved a G•C base pair, primarily G•C --> T•A transversions. This is similar to the kinds of mutations we saw in the repair-proficient cells. However, in contrast to our earlier results, there was no change in strand distribution of premutagenic BPDE lesions. In both populations, ~ 26% of the base substitutions involving G•C base pairs had the G located in the transcribed strand, 5/18 in the S phase mutants and 5/21 in the G_1 phase mutants. These results support the hypothesis that the strong strand bias of induced mutations observed in the repair-proficient cells results from preferential repair of BPDE-induced DNA damage from the transcribed strand.

INTRODUCTION

Induction of mutations in mammalian cells by carcinogens is considered to play an important role in the multistep process of carcinogenesis. To understand the mechanisms by which carcinogens cause malignant transformation, we and our colleagues are investigating the kinds of mutations induced in diploid human fibroblasts by various carcinogens, as well as the role of DNA repair and DNA replication in this process. We showed that the frequency of mutants induced by UV radiation (1, 2), BPDE (3, 4), or 1-nitrosopyrene (5) in nucleotide excision repair-proficient cells is highest in cells treated in early S phase and much lower in cells treated in early G_1 phase. This difference cannot be explained by differences in the physical state of the DNA during treatment, since no such difference was found when xeroderma pigmentosum cells (XP12BE, complementation group A), which are virtually incapable of nucleotide excision repair (6), were used in such comparisons (2, 3). These data suggest that DNA replication is centrally involved in the conversion of DNA damage into mutations and that excision repair prior to the DNA replication decreases the frequency of mutants by eliminating DNA damage.

Recently, it has been shown that in cultured hamster and human cells, UV-induced pyrimidine dimers are excised more rapidly from an actively transcribed gene, dihydrofolate reductase, than from genome overall (7, 8), or from the 5' or 3' flanking region of the *DHFR* gene (9, 10). Mellon *et al.* (11) showed that in these cells such lesions are removed from the transcribed strand of the gene much more rapidly than from the nontranscribed strand. If this were also the case with BPDE-

induced DNA damage in the *HPRT* gene of diploid human cells, mutants derived from the repair-proficient cells treated in early S might well differ from those taken from cells treated in G_1 phase in the strand distribution of the premutagenic lesion assumed to be responsible for the mutations, i.e., the guanine-BPDE adduct.

We recently tested this hypothesis (12) by analyzing BPDE-induced mutants from the two populations for the kinds of mutations and their location in the coding region of the *HPRT* gene. There was no difference in the kinds of mutations, but in mutants from cells treated in early S, the premutagenic lesions were located in either strand, with 25% in the transcribed strand. In mutants derived from G_1-treated cells, all the premutagenic lesions were located in the nontranscribed strand. This difference could reflect preferential repair of BPDE-induced DNA damage from the transcribed strand of the *HPRT* gene, but other explanations are possible. If the observed difference reflects preferential excision repair, then there should be no such difference in mutants derived from populations of XP12BE cells exposed to BPDE in early S or 12 h prior to the onset of S phase. To test this hypothesis, as well as to gain information on the kinds and location of the BPDE-induced mutations in an endogenous gene of excision repair-deficient human cells, we have now determined the spectrum of such *HPRT* mutations in synchronized XP12BE cells. The results showed a transcribed: nontranscribed strand ratio of premutagenic lesions of 28:72 in S phase mutants and 24:76 in G_1 phase mutants, ratios similar to the 25:75 ratio found in repair-proficient cells exposed to BPDE in early S phase so that there would be little or no time for repair before the *HPRT* gene was replicated.

MATERIALS AND METHODS

Cells and Media. XP12BE cells obtained from the American Type culture collection (Rockville, MD) were cultured in modified MCDB-110 medium (13) prepared with Earle's salts and containing 10% supplemented calf serum (HyClone, Logan, UT) (culture medium). For selection of 6-thioguanine-resistant cells, the same medium, but lacking adenine and containing 5% supplemented calf serum (HyClone), 5% fetal calf serum and 40 μM TG was used.

Cell Synchronization. The method used has been described (4, 12). In summary, cells were inoculated at a density 8-fold less than that attained at confluence, fed culture medium every other day to stimulate rapid growth to confluence, and after reaching confluence, fed daily for 3 additional days. The medium was not changed for the next 72 h so that the cells would cease proliferating (G_0 state). To stimulate them to re-enter the cell cycle, the cells were released from confluence, and plated in fresh culture medium at a density of 10^4 cells/cm^2. Such cells begin DNA synthesis (S phase) after 16 h (14).

Exposure to Mutagen and Isolation of *HPRT* Mutants. The details of the BPDE treatment and mutagenesis assay has been described (12). Briefly, a series of independent populations was exposed to BPDE 17 h after release from G_0 (at the beginning of S-phase) or 5 h after release (in early G_1 phase). After 1 h, the medium was removed, and the cells were rinsed with phosphate-buffered saline (pH 7.4), and fed with fresh culture medium. The cells in one dish were assayed immediately for survival as described (12, 15). The rest were allowed 8 days for expression of

resistance to TG before 0.5 x 10^6 cells from each population were plated in selective medium. When TG-resistant clones developed 14 days later, these were located and isolated.

Synthesis of First-Strand cDNA Directly from mRNA in Cells. Mutant clones composed of 50 to 400 cells were detached from the dishes by 5 μl trypsin and suspended in 0.5 ml cold phosphate buffered saline (pH 7.4). The cell suspension was centrifuged for 10-min at 4°C. The supernatant was removed, and the cell pellet was resuspended in 5 μl of the cDNA cocktail described by Yang et al (16). The reverse transcriptase reaction was performed at 37°C for 1 h to allow the cell membranes to be lysed by detergent and first-strand cDNA to be synthesized from total poly(A)mRNA (16).

Amplification of *HPRT* cDNA and DNA Sequencing. The methods used to prepare second-strand *HPRT* cDNA, amplify the cDNA 10^{11}-fold using two 30-cycles of polymerase chain reaction, and sequence the product directly using three sequencing primers have been described (16). A fourth sequencing primer
(5'-^{500}CTTTTCACCAGCA^{488}-3') was also used to sequence three G_1 phase mutants (XBG23, XBG26, and XBG48).

RESULTS

Mutant Frequency. XP12BE cells were synchronized and exposed to 0.013 ~0.022 μM of BPDE in early S- or early G_1 phase, and assayed for survival and frequency of TG-resistant cells. Cell survival ranged from 12-34%, and the frequency of TG-resistant cells per 10^6 clonable cells ranged from 25 to 94. The frequency observed in untreated populations was ~ 5 mutants per 10^6 clonable cells. There was no significant difference in survival or mutant frequency between populations exposed in the two different phases of the cell cycle.

Characterization of Mutations. To decrease the possibility of including a mutant containing a spontaneous, rather than a BPDE-induced mutation, we only analyzed the mutational data from mutants derived from treated populations in which the frequency was at least 8 times higher than that of the untreated populations. The entire *HPRT* coding region of 25 such S phase-derived mutants and 28 such G_1 phase-derived mutants was amplified by polymerase chain reaction and sequenced directly. The mutants were recognized as being independent, either because their alterations were unique or because they had been derived from separate populations of treated cells. The kinds of mutations and their location in the coding region of the *HPRT* gene, and the consequence for the primary structure of the protein are listed in Tables 1 and 2.

There was no significant difference between S- and G_1-derived mutants in the kinds of mutations they contained. Five mutants from S phase and 4 mutants from the G_1 phase were missing one or several consecutive exons from their cDNA. These exon deletions probably resulted from base changes

Table 1 *Kinds and locations of the mutations induced in the coding region of the HPRT gene in XP12BE cells treated with BPDE in the beginning of the S phase of the cell cycle*

Mutant	Position		Exon	Type of mutation	Surrounding sequence[a]	Amino acid change	Strand with the affected guanine[b]
Base substitutions							
XBS49	96		2	G·C → T·A	GAT TTG GAA	Leu → Phe	NT
XBS78	102		2	G·C → T·A	GAA AGG GTG	Arg → Ser	NT
XBS29	113		2	G·C → A·T	ATT CCT CAT	Pro → Leu	T
XBS76	118		2	G·C → T·A	CAT GGA CTA	Gly → Stop	NT
XBS39	119		2	G·C → C·G	CAT GGA CTA	Gly → Ala	NT
XBS17	166		3	G·C → A·T	AAG GAG ATG	Glu → Lys	NT
XBS59	178		3	G·C → T·A	GGC CAT CAC	His → Asn	T
XBS67	178		3	G·C → T·A	GGC CAT CAC	His → Asn	T
XBS39	208		3	G·C → T·A	AAG GGG GGC	Gly → Trp	NT
XBS63	208		3	G·C → T·A	AAG GGG GGC	Gly → Trp	NT
XBS30	233 234	Tandem	3	T·A → C·G G·C → T·A	GAC CTG CTG	Leu → Pro	NT
XBS38	416		6	G·C → A·T	GAC ACT GGC	Thr → Ile	T
XBS36	452 453	Complex	6	G·C → T·A G·C	GTC AGG CAG	Frameshift	NT
XBS1	486		7	C·G → G·C	aac agC TTG	Ser → Arg	T
XBS26	494		7	T·A → G·C	CTG GTG AAA	Val → Gly	NA
XBS24	569		8	G·C → T·A	GTA GGA TAT	Gly → Val	NT
XBS72	569		8	G·C → A·T	GTA GGA TAT	Gly → Glu	NT
XBS73	580		8	G·C → T·A	CTT GAC TAT	Asp → Tyr	NT
XBS83	605		8	G·C → T·A	GAT TTG AAT	Leu → Phe	NT
Deletions							
XBS58	83		2	Delete an A	CAT TAT GCT	Frameshift	NA
Putative splice site mutations							
XBS28	Exon 5 missing						
XBS32	Exon 5 missing						
XBS77	Exon 5 missing						
XBS62	Exon 5 missing						
XBS50	Exon 8 missing						

[a] Sequence of the nontranscribed strand. The sequence is in a 5′ to 3′ orientation. The small letters represent the sequence in an intron. The altered bases are underlined.

[b] T, transcribed; NT, nontranscribed; NA, not applicable.

Table 2 *Kinds and locations of the mutations induced in the coding region of the HPRT gene in XP12BE cells treated with BPDE in early G₁ phase of the cell cycle*

Mutant	Position	Exon	Type of mutation	Surrounding sequence[a]	Amino acid change	Strand with the affected guanine[b]
Base substitutions						
XBG26	69	2	G·C → T·A	TTT TGC ATA	Cys → Stop	T
XBG63	95	2	T·A → C·G	GAT TTG GAA	Leu → Ser	NA
XBG82	134	2	G·C → A·T	GAC AGg taa	Arg → Lys	NT
XBG102	152	3	G·C → T·A	GCT CGA GAT	Arg → Leu	NT
XBG50	170 Tandem	3	T·A → A·T	GAG ATG GGA	Met → Asn	NT
	171		G·C → T·A			
XBG95	178	3	G·C → T·A	GGC CAT CAC	His → Asn	T
XBG78	183	3	G·C → T·A	CAT CAC ATT	His → Gln	T
XBG13	197	3	G·C → T·A	CTC TGT GTG	Cys → Phe	NT
XBG65	197	3	G·C → T·A	CTC TGT GTG	Cys → Phe	NT
XBG20	208	3	G·C → T·A	AAG GGG GGC	Gly → Trp	NT
XBG55	209	3	G·C → C·G	AAG GGG GGC	Gly → Ala	NT
XBG101	393	5	G·C → T·A	GTC TTG ATT	Leu → Phe	NT
XBG97	429	6	G·C → T·A	ACA ATG CAG	Met → Ile	NT
XBG46	488	7	T·A → A·T	agC TTG CTG	Leu → Stop	NA
XBG81	508	7	G·C → A·T	CCA CGA AGT	Arg → Stop	T
XBG41	568	8	G·C → C·G	GTA GGA TAT	Gly → Arg	NT
XBG65	569	8	G·C → T·A	GTA GGA TAT	Gly → Val	NT
XBG37	569	8	G·C → A·T	GTA GGA TAT	Gly → Glu	NT
XBG91	569	8	G·C → T·A	GTA GGA TAT	Gly → Val	NT
XBG16	574	8	G·C → T·A	TAT GCC CTT	Ala → Ser	NT
XBG85	599	8	G·C → T·A	TTC AGG GAT	Arg → Met	NT
XBG52	606	8	G·C → T·A	GAT TTG AAT	Leu → Phe	NT
XBG45	610	9	G·C → C·G	tag CAT GTT	His → Asp	T
Deletions						
XBG35	578 or 579	8	Delete a T	GCC CTT GAC	Frameshift	NA
Putative splice site mutations						
XBG23	Exons 2, 3 missing					
XBG48	Exons 2, 3 missing					
XBG36	Exons 2, 3, 4 missing					
XBG38	Exon 5 missing					

[a] Sequence of the nontranscribed strand. The sequence is in a 5′ to 3′ orientation. The small letters represent the sequence in an intron. The altered bases are underlined.

[b] T, transcribed; NT, nontranscribed; NA, not applicable.

at splice consensus sequences, but the change in the *HPRT* gene has not been evaluated, since only the coding region of the gene was sequenced in this study. Besides these exon deletion mutants, all but one mutants from either group contained base substitutions. Most had only a single base substitution. One mutant from each phase exhibited tandem base substitutions and one S phase mutant carried a complex mutation (a base substitution in conjunction with a -1 frameshift). One mutant from each phase had a single base deletion. Table 3 shows that 89% of the base substitutions derived from either phase involved G•C base pairs. Among these, transversions of G•C to T•A predominated: 60% (12/20) for S phase and 60% (14/24) for G_1 phase. In both phases, the base alterations were distributed throughout the coding region, but a substantial fraction were located in exons 2, 3, and 8. The number of mutations analyzed was not large enough to tell if their locations varied with the phase of the cell cycle.

Strand Distribution of the Premutagenic Lesions. In analysis of the data for evidence of differences in location of premutagenic lesions that gave rise to the observed mutation, we used only the mutations involving G•C base pairs. This is because studies in mammalian cell lines in culture reveal that BPDE binds only to purines, with > 95% of the DNA adducts involving guanine (17, 18). As noted in Tables 1 and 2, and diagrammed in Figure 1, in mutants derived from S phase, 5 out of 18 such mutations (28%) had the guanine in the transcribed strand. A similar result was found with mutants derived from G_1 phase, i.e. in five out of 21 (24%) of the mutations, the guanine was in the transcribed strand. This result differs significantly from that found in repair-proficient

Table 3 *Types of base substitutions observed in the coding region of the HPRT gene in XP cells treated with BPDE*

Types of base substitutions	No. of substitutions observed	
	S phase	G_1 phase
Transversions:		
G•C --> T•A	12	14
G•C --> C•G	2	3
T•A --> G•C	1	0
T•A --> A•T	0	2
Transitions:		
G•C --> A•T	4	4
T•A --> C•G	1	1
Total	20	24

Figure 1. Location of independent HPRT mutations in XP12BE cells treated with BPDE in early S phase (27 mutations) or in early G_1 phase (29 mutations). Exon deletions are shown as open rectangles; base substitutions are indicated by vertical bars; tandem mutations by vertical arrows; and single base deletions by squares.

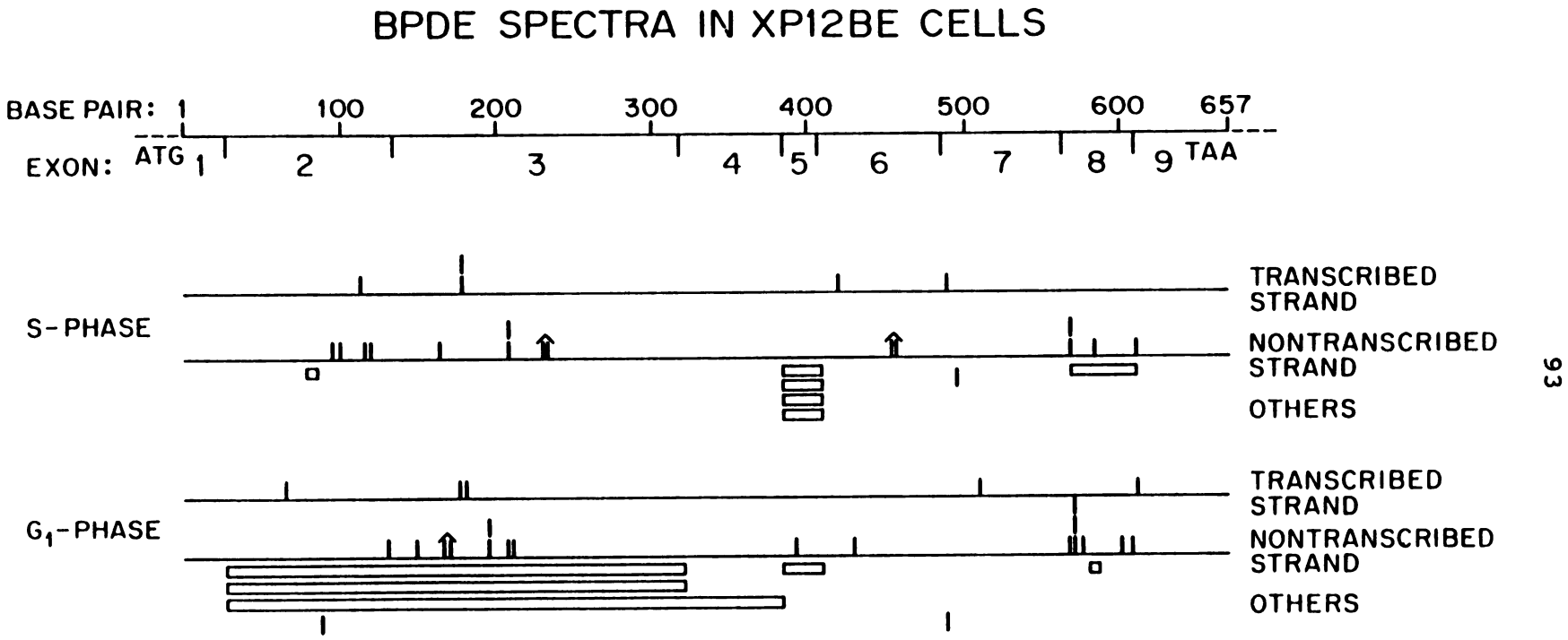
BPDE SPECTRA IN XP12BE CELLS
BASE PAIR: 1
100
200
300
400
500
600
657
EXON: ATG
1
2
3
4
5
6
7
8
9
TAA
S-PHASE
TRANSCRIBED STRAND
NONTRANSCRIBED STRAND
OTHERS
G1-PHASE
TRANSCRIBED STRAND
NONTRANSCRIBED STRAND
OTHERS

Figure 1

cells treated in G_1 phase, in which all the premutagenic lesions were located in the nontranscribed strand (12).

Seven out of 47 base pair changes identified did not involve a G•C base pair. However, among these, one from each phase was carried by a mutant containing a tandem base substitution occurring at a 5'-TG sequence (XBS30 and XBG50). It is very likely that each of these tandem base substitutions was caused by the presence of a single BPDE adduct formed at the 3' guanine. Therefore, only 5 mutations out of the 47 analyzed (<11%) did not involve G•C base pairs.

DISCUSSION

Our finding that the kinds and location of BPDE-induced mutations in the coding region of the *HPRT* gene of excision repair-deficient XP12BE cells treated in early G_1 phase or in early S phase were similar indicates that the physical state of the DNA in the cell during treatment did not affect the spectrum. The data support the conclusion from our previous study (12) that the difference in the strand distribution of premutagenic lesions observed between repair-proficient human cells treated in S phase and such cells treated in G_1 phase is caused by preferential repair of BPDE-induced DNA damage from the transcribed strand of the *HPRT* gene. This strand-specific repair of a bulky BPDE-DNA adduct is consistent with the finding of Mellon *et al.* (11) who showed that UV-induced pyrimidine dimers are preferentially removed from the transcribed strand of actively transcribed genes, such as the *DHFR* gene of human cells.

The kinds of mutations induced by BPDE in XP12BE cells are similar to those we found in repair-proficient cells (12), those induced in an extrachromosomal gene, *supF*, replicating in repair-proficient human cells (19), as well as those found in two other endogenous genes of repair-proficient mammalian cells (20, 21). In all the studies described above, G·C to T·A transversions predominate. This transversion would result if the DNA polymerase(s) preferentially inserted a deoxyadenosine triphosphate across from a non-instructional base containing a bulky BPDE-DNA adduct (22), or if the presence of a BPDE adduct on guanine allowed a stable purine·purine pairing to occur during replication, and this mispairing were not recognized and removed. There is evidence from model

oligonucleotides that unmodified guanine can pair stably with adenine (23, 24). Also, Norman *et al.* (25) showed that in a double-stranded oligonucleotide containing a single aminofluorene residue bound to guanine in the C8 position, adenine opposite the modified guanine produces a stable structure which places the aminofluorene in the B-DNA minor grove, with the guanine *syn*. Whether this occurs with BPDE-guanine adducts is not yet known.

The kinds of mutations we observed in XP12BE cells differ from the kinds found by Bernelot-Moens *et al.* (26) in the *lacI* gene of excision repair-deficient *E. coli* with BPDE. In addition to G·C to T·A transversions, A·T to T·A transversions and deletions of a G·C base pair (-1 frame shift) were frequently observed. This may reflect a difference in mutational mechanisms between procaryotic and eucaryotic cells.

We observed only five mutations involving A·T base pairs. Although BPDE binds to adenine at very low frequencies (17, 18), some of these mutations may represent pre-existing background mutations. Since the majority (89%) of the base substitutions analyzed in the coding region of the *HPRT* gene of mutants derived from untreated diploid human fibroblasts involved A·T pairs (27), and since the frequencies we obtained with BPDE in these XP cells were only 8 to 15-fold above background, we could have included one or two background mutants in our analysis.

Two tandem base substitutions and one complex mutation were recovered in the present study. These events have not been reported in other mammalian endogenous genes after BPDE treatment (20, 21), nor in BPDE-induced *HPRT* mutants derived from repair-proficient cells (12, 27). However, three mutants out of 86 generated when a shuttle vector carrying

BPDE-guanine adducts replicated in human cells contained such tandem base substitutions (19). The tandem base substitutions may be the result of a reduction of the fidelity of the DNA polymerase at the time of replication bypassing bulky lesions. Reduction of fidelity, followed by a strand slippage induced by BPDE adducts, could also account for the complex mutation observed in mutant XBS36.

The distribution of guanine nucleotides between the transcribed and nontranscribed strands in the coding region of the human *HPRT* gene is 41:59. The ratio becomes 38:62 if base substitutions that will not result in amino acid changes are excluded. Since XP12BE cells do not remove BPDE-guanine adducts, if binding of BPDE to the *HPRT* gene is random, and every amino acid change caused by a BPDE-induced mutation were to affect the activity of the HPRT enzyme, then one would expect 38% of the observed mutations to correspond to a BPDE-guanine adduct in the transcribed strand. Instead, only 26% did (28% for S phase and 24% for G_1 phase). This is very similar to the distribution we found in populations of repair-proficient cells exposed to BPDE in early S phase so that they had no time repair before replication of *HPRT* occurred (12). A 26:74 ratio is not significantly different from the theoretical 38:62 ratio ($P = 0.11$). However, we suggest that the 26:74 ratio reflects the strand distribution of guanines that cannot be mutated without affecting the activity of the protein. If this is the case, then studies in which the premutagenic lesions involved cytosine ought to show just the opposite strand distribution, i.e. 75% transcribed , 25% nontranscribed. No study of *HPRT* mutations induced in the absence of excision repair by a mutagen that causes only cytosine lesions has been reported. However, we and our

colleagues (14) recently carried out a study using UV radiation, an agent which induces the mutations at dipyrimidine sites and for which the affected pyrimidine is predominately cytosine. The results showed that the distribution of premutagenic lesions in repair-proficient cells treated in early S phase, as well as XP12BE cells irradiated in S or G_1, was 75% transcribed strand : 25% nontranscribed strand.

Vrieling *et al.* (28) attributed the strong strand bias of UV-induced *hprt* mutations observed in UV-sensitive Chinese hamster cells (V-H1), in which 90% of the mutations resulted from photoproducts in the transcribed strand, to a difference in fidelity of DNA replication between the leading and the lagging strand, with a more error-prone polymerase (e.g., polymerase delta) replicating the transcribed strand. Our finding that fewer mutations were targeted by BPDE-modified guanine in the transcribed strand of the *HPRT* gene than is predicted by the distribution of guanines in that strand argues against this explanation. Direct evidence that polymerase delta is not necessarily more error-prone than polymerase alpha comes from a study by Shibutani *et al.* (29) who showed that during translesional DNA synthesis on an oligonucleotide template containing an 8-oxo-7-hydrodeoxyguanosine, the correct nucleotide is less frequently inserted by polymerase alpha than by polymerase delta.

In our previous study (12), a cell cycle-dependent "hot spot region" was observed in *HPRT* mutants derived from repair-proficient cells treated with BPDE in G_1 phase and allowed 12 h for excision repair before S. Twenty-nine percent of the mutations induced in cells treated in G_1 phase were located in the sequence of 5'$A(G)_6C$-3' (positions 206-213), compared to 4% in the cells treated in S phase. If, as we suggested, this "hot

spot region" resulted from inefficient repair of BPDE adducts in the run of 6 consecutive guanine bases, so that after excision repair had occurred, the adducts remaining in this region represented a higher fraction than originally present, there should not be any such difference in the XP12BE cells. In the present study, only two mutations induced in each phase were located in this region, a significantly smaller percentage (8% for S and 7% for G_1) than the 29% seen in repair-proficient cells treated in G_1 phase. These results support the hypothesis that inefficient excision repair can account for the prominent "hot spot region" in the latter population. Several studies showed that the helix of poly(dG)-poly(dC) resembles an A-like helix geometry, rather than the traditional B form (30, 31). If a BPDE-adduct formed in the run of 6 G's were to produce a specific conformation at the site of the adduct, this could affect the recognition by repair enzymes. If localized unwinding is necessary for the formation of an excision repair complex at the site of the BPDE-DNA adduct, and the repair complex melts an A+T-rich region more easily than a G+C-rich region (32), this could also explain why repair is less efficient in this G+C-rich region of the *HPRT* gene.

In summary, we have shown that there is no significant difference in the kinds of mutations induced by BPDE in repair-deficient XP12BE cells, their location in the *HPRT* gene, or in the strand distribution of the premutagenic lesion that targeted the mutations. These results support the hypothesis that the strong strand bias of induced mutations observed in the repair-proficient cells (12) results from preferential repair of BPDE-induced DNA damage from the transcribed strand.

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CHAPTER IV

Cell Cycle-Dependent Strand Bias for UV-Induced Mutations in the Transcribed Strand of Excision Repair-Proficient Human Fibroblasts, But Not in Repair-Deficient Cells

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SUMMARY

To study the effect of nucleotide excision repair on the spectrum of mutations induced in diploid human fibroblasts by UV_{254nm}, we synchronized repair-proficient cells and irradiated them when the *HPRT* gene was about to be replicated (early S phase) so there would be no time for repair in that gene before replication, or in G_1 phase 6 h prior to S, and determined the kinds and location of mutations in that gene. As a control, we also compared the spectra of mutations induced in synchronized populations of xeroderma pigmentosum (XP) cells (XP12BE cells, which are unable to excise UV-induced DNA damage). Among the 84 mutants sequenced, base substitutions predominated. Of the XP mutants from S or G_1 and the repair-proficient mutants from S, ~62% of these were G·C --> A·T. In the repair-proficient mutants from G_1, 47% were. In mutants from the repair-proficient cells irradiated in S, 71% of the premutagenic lesions (10/14) were located in the transcribed strand; with mutants from such cells irradiated in G_1, only 20% were (3/15). In contrast, there was no statistically significant difference in the fraction of premutagenic lesions located in the transcribed strand of the XP12BE cells; ~ 75% of the premutagenic lesions (24/32) were located in that strand, i.e., 15/19 (79%) in the S phase cells and 9/13 (69%) in the G_1 cells. The switch in strand bias supports preferential nucleotide excision repair of UV-induced damage in the transcribed strand of the *HPRT* gene.

INTRODUCTION

Populations of diploid human fibroblasts, synchronized by release from the density-inhibited G_0 state and plated at 10^4 cells/cm^2, begin DNA synthesis after approximately 16 h (26). If populations of nucleotide excision repair-proficient cells are irradiated with UV_{254nm} light 17 h after release from confluence, the frequency of 6-thioguanine-resistant (TG^r) mutants (resulting from loss of function of the gene coding for hypoxanthine (guanine) phosphoribosyltransferase, HPRT) is significantly higher than in cells irradiated in early G_1 phase (9). This difference in frequency cannot be attributed to differences in the physical state of the DNA since no such difference in mutation frequency is found when diploid xeroderma pigmentosum (XP) cells (XP12BE, complementation group A), which are virtually devoid of nucleotide excision repair (18), are irradiated in early S phase or in early G_1 phase (9). These data suggest that S phase replication is centrally involved in the conversion of potentially mutagenic DNA damage into mutations ("fixation"), and that excision repair prior to the onset of S phase decreases the frequency of mutants by eliminating such lesions.

Of the two major classes of potentially mutagenic UV photoproducts, i.e., cyclobutane pyrimidine dimers (Py-Py dimers) and 6-4 pyrimidine-pyrimidone (6-4 Py-Py) lesions, the latter have been shown to be removed from human genomic DNA more rapidly than the former (15). It has also been shown that Py-Py dimers are excised more rapidly from an actively transcribed gene of human cells, dihydrofolate reductase (*DHFR*), than from

bulk DNA (1, 12). What is more, Mellon et al. (14) demonstrated that there is a strand bias in the rate of removal of Py-Py dimers from the *DHFR* gene in human cells. The majority of such lesions are removed within 24 h, but dimers in the transcribed strand are excised much faster than those in the nontranscribed strand. Information on strand-specific excision repair of UV-induced damage from the *HPRT* gene of human cells is not yet available. But if such repair occurs, *HPRT* mutations induced by UV in excision repair-proficient human cells irradiated at the onset of S phase should arise from 6-4 Py-Py lesions and Py-Py dimers located in either strand of DNA. In contrast, the lesions responsible for the mutations induced in such cells allowed 6 h for repair before the onset of S phase should arise mainly from Py-Py dimers, and these should be located primarily in the nontranscribed strand. This switch in strands should not occur in XP12BE cells.

There are no published data on the kinds and locations (i.e, the spectrum) of UV-induced mutations in an endogenous gene of human cells, nor data showing whether excision repair in such cells alters the spectrum. The present study was designed to examine the spectrum of mutations induced by UV in the *HPRT* gene of diploid human cells and to determine, at the DNA sequence level, the effect of nucleotide excision repair of UV-induced photoproducts on the induction of such mutations. We were particularly interested to see if there were biological evidence of strand-specific repair of UV photoproducts in diploid human fibroblasts and to compare the results with what was found in V79 Chinese hamster cell lines by Vrieling et al. (25).

Although the time of *HPRT* replication has not been measured directly,

studies of the mutation frequency of TG-resistant mutants induced by UV irradiation at different times across the S phase of synchronized populations of human fibroblasts showed that the highest frequency occurred when cells were irradiated in the first 1/4 of S phase (7). A similar finding was reported for Syrian hamster cells by Tsutsui et al. (23). To investigate the effect of nucleotide excision repair on the spectrum of UV-induced lesions, we irradiated repair-proficient and repair-deficient diploid human cells in early S phase, just before the *HPRT* gene is replicated, or in G_1 phase, 6 h before replication and determined the kinds and location (spectrum) of the mutations in the coding region of the gene. We then analyzed the data to determine in which strand the premutagenic dipyrimidine photoproduct was located. The distribution of such sites in the coding region of the human *HPRT* gene is: 59% transcribed strand, 41% nontranscribed. If their mutagenic potential is comparable and their distribution in *HPRT* sequences coding for critical parts of the protein is random, then the mutations induced in repair-proficient cells irradiated in early S phase and in XP12BE cells ought to be 59% arising from lesions in the transcribed strand, and 41% from lesions in the nontranscribed strand. Mutations in repair-proficient cells irradiated in G_1 phase might not show this pattern.

The XP12BE cells showed a transcribed:nontranscribed strand ratio of premutagenic lesions of 79:21 in S phase and 69:31 in G_1 phase mutants. The repair-proficient cells also showed a 71:29 ratio in the S phase mutants, but the G_1 mutants showed a 20:80 ratio. These data indicate bias toward UV-induced mutations resulting from lesions in the transcribed strand of the *HPRT* gene in the absence of repair, and preferential repair

of such lesions in that strand.

MATERIALS AND METHODS

Cells and media. Finite life span diploid human fibroblasts, designated NFSL89, were explanted from the foreskin of a normal newborn (11). These cells have normal nucleotide excision repair capacity. XP12BE cells, were obtained from the American Type Culture Collection (Rockville, MD). Early-passage cells were used and were routinely cultured in a modified MCDB-110 medium (20) containing 10% supplemented bovine calf serum (Hy Clone, Logan, Utah) (culture medium). For selection of TG^r cells, the same medium, but lacking adenine and containing 5% fetal bovine serum, 5% supplemented calf serum and 40 μM TG was used. For thymidine incorporation experiments, the medium was changed to Eagle's minimal essential medium (MEM) to eliminate thymidine.

Mutagenesis and Isolation of $HPRT^-$ Mutants.

Cell Synchronization. Cells were driven into the G_0 state as described (26). To stimulate the cells to enter the cell cycle, they were plated in culture medium at a density of 10^4 cells/cm^2. The time of onset of S phase following release from G_0 was determined by the incorporation of ^{3}H-thymidine into acid-insoluble material as described (26).

Exposure to UV Light and Isolation $HPRT^-$ of Mutants. A series of independent populations (1.5 x 10^6 cells each, plated in 150 mm-diameter plastic dishes) was irradiated 17 h after release from G_0 (at the beginning of S phase) or 11 h after release from G_0 (in G_1 phase). The culture medium was aspirated, and the cells were washed with sterile phosphate

buffer saline (PBS, pH 7.4). The cells were irradiated as described (17) using a UV fluence of 6.5 J/m^2 for repair-proficient cells and 0.5 J/m^2 for repair-deficient cells and re-fed with culture medium. Cells plated at cloning density were similarly exposed and used to determine survival of colony-forming ability. The target cells were allowed an 8 day expression period before selection of 2.5 X 10^5 cells from each population with TG was begun as described (11). When macroscopic drug-resistant clones had developed 14 days later, these were located, isolated, and expanded into cultures composed of ~ 2.0 X 10^4 cells, or the cDNA was amplified directly from the original clone.

Synthesis of first-strand cDNA directly from mRNA in cells. Cells were trypsinized and suspended in culture medium and if the cells in a colony had been expanded, the cell number was determined by electronic counting, and the cells were diluted in cold PBS (pH 7.4). From 100 to 500 cells in PBS were transferred to a 0.5 ml Eppendorf tube and centrifuged for 10 min at 4°C. The supernatant was removed, and the cell pellet was resuspended in 5 μl of the cDNA cocktail described in detail by Yang et al. (28). The reverse transcriptase reaction was performed at 37°C for 1 h to allow the cell membranes to be lysed by detergent and first-strand cDNA to be synthesized from total cytoplasmic poly(A)mRNA (28).

Amplification of HPRT cDNA and DNA sequencing. The experimental conditions, optimized for preparing second-strand *HPRT* cDNA, amplifying the cDNA 10^{11}-fold using two Polymerase Chain Reaction (PCR) stages of 30-cycles each, and sequencing the product directly using three sequencing

primers and a modified Sanger dideoxynucleotide procedure have been described (28).

RESULTS

Determining Onset of S Phase. Repair-proficient NFSL89 fibroblasts and repair-deficient XP12BE fibroblasts were synchronized by release from confluence (density-induced G_0 state) and plated into a series of culture dishes in Eagle's MEM at 10^4 cells/cm^2. At various times post-release, the cells were pulse-labeled with tritiated thymidine (5 μCi/ml medium) for 20 min and the acid-precipitable counts were determined. As shown in Fig. 1, DNA synthesis in both cell lines began 15-16 h following release. By 17 h both cell populations were actively incorporating tritiated thymidine.

Survival and mutation frequency. Synchronized populations of XP12BE cells were irradiated with 0.5- 0.6 J/m^2 of UV light 17 h post-release from confluence at the beginning of S phase, or in G_1 phase 6 h prior to S, and assayed for survival and the frequency of TG^r cells. Eight replicate cultures in either S or G_1 phase of the cell cycle were irradiated in each experiment, together with non-irradiated controls, and three experiments were conducted for a total of 24 independent populations of cells irradiated at the beginning of S phase and 24 populations irradiated 6 h before the onset of S phase. Cell survival ranged from 10-34%. The frequency of TG^r mutants per 10^6 clonable cells in populations irradiated in early S phase ranged from 223 to 324; that in populations irradiated 6 h prior to the onset of S ranged from 253 to 323. The background frequency (in the absence of UV treatment) was less than 10 TG^r mutants per 10^6 clonable cells. The cloning efficiency of the cells at the time of selection was 15-22%.

Figure 1. Incorporation of ^{3}H-thymidine into acid-insoluble material following a 20 min pulse-label at the indicated times. Counts have been normalized to 10^5 cells, and the background (300 cpm/10^5 cells) has been subtracted. Populations were released from density-induced G_0 at T = 0 h.

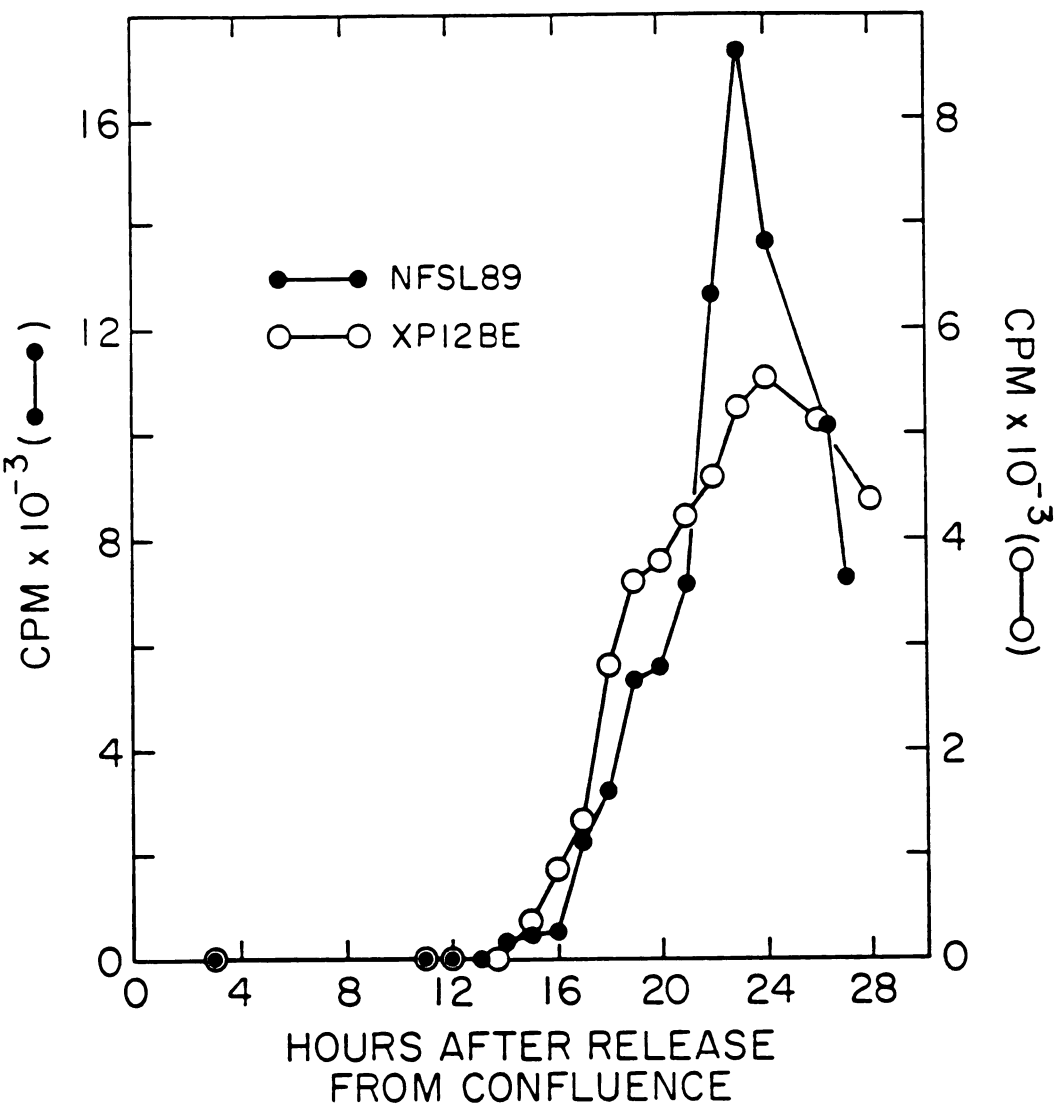
NFSL89
XP12BE
CPM x 10⁻³(●—●)
CPM x 10⁻³(○—○)
HOURS AFTER RELEASE
FROM CONFLUENCE
0
4
8
12
16
20
24
28

Figure 1

Repair-proficient diploid human fibroblasts were synchronized and irradiated as described above, but using a fluence of 6.5 J/m^2. Their survival, which was the same whether irradiation was in early S phase or in G_1 phase, ranged from 18-22%. The frequency of TG^r mutants per 10^6 clonable cells in populations irradiated in early S phase ranged from 145 to 162; that in populations irradiated 6 h prior to the onset of S phase, ranged from 67 to 76. The frequency observed in unirradiated cells was 5-10 mutants per 10^6 clonable cells. The cloning efficiency of the repair-proficient cells ranged from 50-60%.

Nucleotide sequence alterations in XP mutants. The coding region of the *HPRT* gene of 42 independent TG^r mutants was amplified by PCR and the product was directly sequenced. Of this number, 22 were derived from populations of XP12BE cells irradiated at S phase and 20 from cells in G_1 phase. The mutants could be distinguished as independent either because the sequence alterations were unique, or they had been generated in separate populations. The kinds of mutations seen in mutants derived from cells irradiated in S phase, their location in the coding region, and the consequence for the primary structure of the protein are tabulated in Table 1. The majority were base substitutions. Among these, transitions predominated, accounting for 68% (15/22) of the total. One mutant had an insertion of 62 bp from intron 5 which very probably resulted from inactivation of the 5' splice site of intron 5 by a G·C --> A·T transition and use of a downstream cryptic splice donor site (16). Three mutants had an exon completely deleted. These deletions probably resulted from base substitutions in splice sites, but because we sequenced only the

TABLE 1. Kinds and locations of mutations induced in the coding region of the *HPRT* gene of XP12BE cells irradiated in S phase

Type of change and mutant	Position	Exon	Deleted exon	Type of mutation	Surrounding sequence	Amino acid change	Strand with affected dipyrimidine[a]
Base substitution							
XUS39	74	2		C · G→T · A	ATA CCT AAT	Pro → Leu	NT
XUS27	118, 119 tandem	2		G · C→A · T G · C→A · T	CAT GGA CTA	Gly → Lys	T
XUS9	125	2		T · A→A · T	CTA ATT ATG	Ile → Asn	NT
XUS53	126	2		T · A→G · C	CTA ATT ATG	Ile → Met	NT
XUS15	135	3		G · C→T · A	GAC AGG ACT	Arg → Ser	T
XUS42	209	3		G · C→A · T	AAG GGG GGC	Gly → Glu	T
XUS75	403	4		G · C→A · T	GAA GAT ATA	Asp → Asn	T
XUS40	471	6		G · C→A · T	AAG ATG GTC	Met → Ile	T
XUS61	478	6		G · C→T · A	AAG GTC GCA	Val → Phe	T
XUS19	532	7		T · A→C · G	GAC TTT GTT	Phe → Leu	NT
XUS51	539	8		G · C→A · T	GTT GGA TTT	Gly → Glu	T
XUS70	544	8		G · C→C · G	TTT GAA ATT	Glu → Gln	T
XUS77	568, 569 tandem	8		G · C→A · T G · C→A · T	GTA GGA TAT	Gly → Lys	T
XUS20	580	8		G · C→A · T	CTT GAC TAT	Asp → Asn	T
XUS35	599	8		G · C→A · T	TTC AGG GAT	Arg → Lys	T
XUS8	600, 601 tandem	8		G · C→A · T G · C→A · T	TTC AGG GAT TTC AGG GAT	No change Asp → Asn	T T
XUS52	601	8		G · C→A · T	AGG GAT TTG	Asp → Asn	
XUS69	649	9		A · T→C · G	TAC AAA GCC	Lys → Gln	T
Splice site mutation							
XUS3	Splice donor site, intron 5			G · C→A · T	GAAgtaagt	62-bp insertion	T
Putative splice site mutation							
XUS31			8				
XUS44			5				
XUS47			5				

[a] NT, Nontranscribed; T, transcribed.

coding region of the gene, the nature of the presumed intron mutations which led to such exon deletions has not been evaluated.

As shown in Table 1 and diagrammed in Fig. 2, the base substitutions were distributed throughout the exons, but with more located in exon 8 than would be expected on a random basis. The premutagenic dipyrimidine photoproducts presumed to be responsible for the observed base substitutions were primarily located in the transcribed strand (79%, 15/19).

Table 2 lists the sequence alterations found in 20 mutants isolated from XP12BE cells irradiated 6 h before the onset of S phase. The kinds of mutations did not differ significantly from those induced at S phase, nor did their location in the gene (Fig. 2). The majority contained base substitutions, predominantly G·C --> A·T, i.e., 56% (9/16), and the corresponding dipyrimidine photoproducts were primarily located in the transcribed strand, 69% (9/13). There were 6 exon deletions resulting from putative splice site mutations. One mutant (XUG20) contained a mutation that did not occur at a dipyrimidine site, but involved a base substitution and a deletion at an ACA site. This sequence has been reported to form a complex photoproduct following UV radiation (2), but we cannot be certain that this unusual photoproduct was the premutagenic lesion, and therefore this mutation has been excluded from analysis of the strand location of the premutagenic lesions.

Nucleotide sequence alterations in mutants from repair-proficient cells. Our hypothesis predicts that the mutations induced in the repair-proficient cells irradiated at the onset of S will resemble those found in

Figure 2. Location of independent *HPRT* mutations in XP12BE cells irradiated with 0.5 J/m^2 UV light (254 nm) in early S phase (22 mutants) or 6 hr before S phase, in G_1 phase (19 mutants). Mutation XUG20, which did not occur opposite a dipyrimidine sequence, has been excluded. Exon deletions are shown as open rectangles; base substitutions are indicated by vertical lines; and tandem mutations are indicated by vertical arrows. A base substitution in intron 5 of the transcribed strand is indicated by an asterisk. Strand assignments were made on the basis of which strand contained the dipyrimidine sequence which presumably targeted the mutation. There was no statistically significant difference between cells irradiated in S phase or in G_1 phase in the kinds of mutations induced or in the strand distribution of the potentially mutagenic photoproducts.

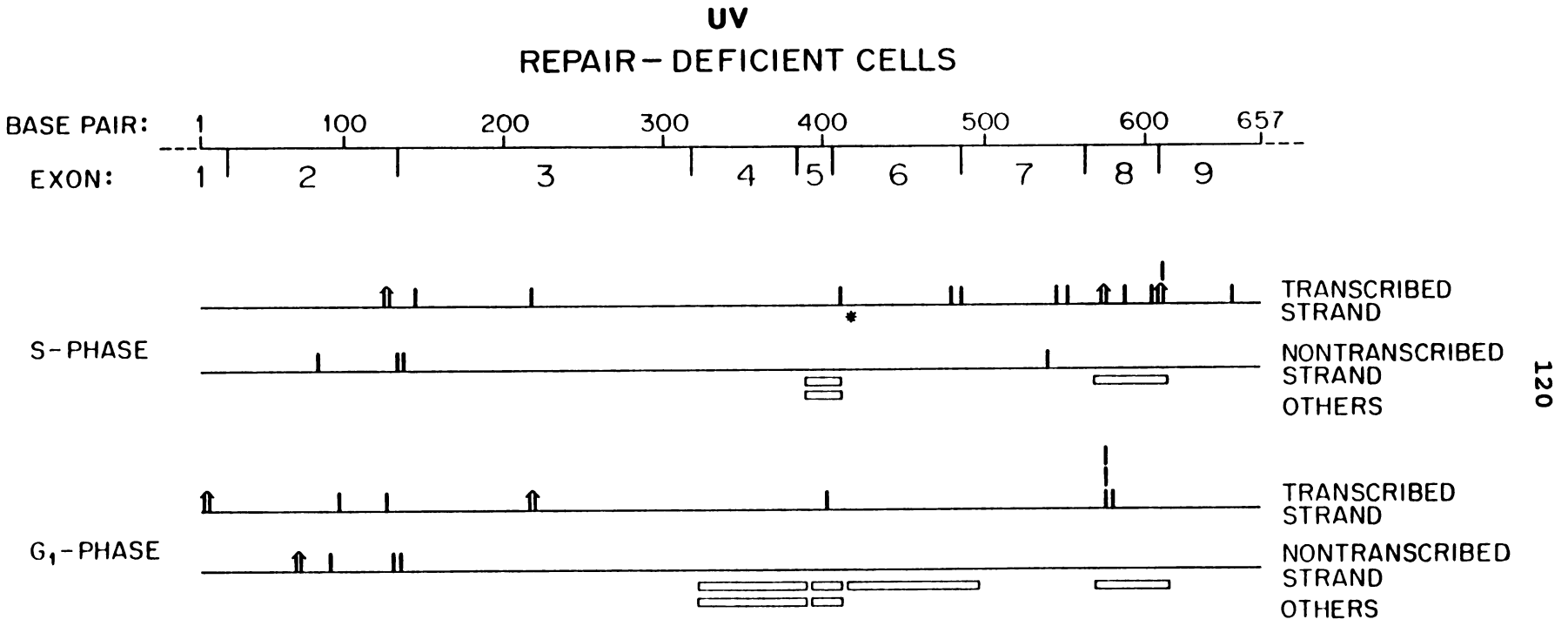
UV
REPAIR – DEFICIENT CELLS
BASE PAIR:
1
100
200
300
400
500
600
657
EXON:
1
2
3
4
5
6
7
8
9
S-PHASE
TRANSCRIBED STRAND
NONTRANSCRIBED STRAND
OTHERS
G_1-PHASE
TRANSCRIBED STRAND
NONTRANSCRIBED STRAND
OTHERS

Figure 2.

TABLE 2. Kinds and locations of mutations induced in the coding region of the *HPRT* gene of XP12BE cells irradiated in G_1 phase

Type of change and mutant	Position	Exon	Deleted exon	Type of mutation	Surrounding sequence	Amino acid change	Strand with affected dipyrimidine[a]
Base substitution							
XUG44	3 tandem	1		G · C → A · T	ATG GCG ACC	Met → Ile	T
	4			G · C → A · T	ATG GCG ACC	Ala → Thr	
XUG22	61 tandem	2		T · A → A · T	GAT TTA TTT	Leu → Lys	NT
	62			T · A → A · T			
XUG23	82	2		T · A → G · C	CAT TAT GCT	Tyr → Asp	NT
XUG21	88	2		G · C → T · A	GCT GAG GAT	Glu → Stop	T
XUG33	119	2		G · C → A · T	CAT GGA CTA	Gly → Glu	T
XUG42	122	2		T · A → C · G	GGA CTA ATT	Leu → Pro	NT
XUG31	125	2		T · A → A · T	CTA ATT ATG	Ile → Asn	NT
XUG11	208 tandem	3		G · C → A · T	AAG GGG GGC	Gly → Lys	T
	209			G · C → A · T			
XUG20	243	3		C · G / A · T → T · A	GAT TAC ATC	Frameshift	—[b]
	244						
XUG16	393	5		G · C → T · A	GTC TTG ATT	Leu → Phe	T
XUG4	568	8		G · C → A · T	GTA GGA TAT	Gly → Arg	T
XUG13	568	8		G · C → A · T	GTA GGA TAT	Gly → Arg	T
XUG14	568	8		G · C → A · T	GTA GGA TAT	Gly → Arg	T
XUG10	569	8		G · C → A · T	GTA GGA TAT	Gly → Glu	T
Putative splice site mutation							
XUG34			4				
XUG5			4				
XUG27			5				
XUG12			5				
XUG28			6				
XUG40			8				

[a] T, Transcribed; NT, nontranscribed.

[b] We cannot be certain that the premutagenic lesion involved ACA; therefore, the strand involved in the mutation cannot be determined. The nature of the base substitution also cannot be determined since either base pair could have been involved.

the XP12BE cells treated in S or G_1 phase. As shown in Table 3, which presents the *HPRT* sequence alterations found in 22 mutants derived from such populations, this is the case. The majority of the mutants (15/22) contained single or tandem base substitution, for a total of 18 base substitutions in the 15 mutants; of this number 11(61%) were G·C --> A·T transitions. With the exception of the A·T --> C·G base substitution in mutant NUS13, all base pair substitutions occurred at dipyrimidine sites where photoproducts could form, and 71% of these photoproducts (10/14) were located in the transcribed strand. The substitution in mutant NUS13 may represent a pre-existing background mutation since 8 of the 9 base substitutions we found in unirradiated background mutants involved A·T base pairs (27).

One S phase mutant had a 10 bp deletion in exon 8; the premutagenic lesion resulting in this deletion could not be determined. Seven putative splice site mutations which resulted in complete exon deletions were found. The strand distribution of these mutations, which is shown in Fig. 3, resembled that found in mutants from either population of XP12BE cells.

Table 4 lists the spectrum of mutations analyzed from 20 mutants isolated from populations of repair-proficient fibroblasts irradiated in G_1 phase, 6 h before the onset of scheduled DNA synthesis. The majority were base substitutions, mainly G·C --> A·T (47%), but the fraction of the premutagenic photoproducts located in the transcribed strand was greatly reduced, i.e., 20%, rather than 71%. As shown in Fig. 3, there were few mutations resulting from lesions in the transcribed strand. For convenience, Table 5 compares the frequency of the various kinds of base substitutions observed in the 4 different populations.

TABLE 3. Kinds and locations of mutations induced in the coding region of the *HPRT* gene of repair-proficient diploid cells irradiated in S phase

Type of change and mutant	Position	Exon	Deleted exon	Type of mutation	Surrounding sequence	Amino acid change	Strand with affected dipyrimidine[a]
Base substitution							
NUS13	84	2		A · T → C · G	CAT TAT GCT	Tyr → Stop	—[b]
NUS8	122	2		T · A → C · G	GGA CTA ATT	Leu → Pro	NT
NUS1	123	2		A · T → G · C	GGA CTA ATT	No change	T
	568	8		G · C → A · T	GTA GGA TAT	Gly → Arg	T
NUS21	208, 209 tandem	3		G · C → A · T G · C → A · T	AAG GGG GGC	Gly→ Lys	T
NUS9	447	6		G · C → C · G	TCC TTG GTC	Leu → Phe	T
NUS16	463, 464 tandem	6		C · G → T · A C · G → T · A	AAT CCA AAG	Pro→ Leu	NT
NUS14	471	6		G · C → A · T	AAG ATG GTC	Met → Ile	T
NUS19	498, 499 tandem	7		A · T → G · C A · T → G · C	GTG AAA AGG	No change Arg →Gly	T
NUS7	500, 501 tandem	7		G · C → A · T G · C → A · T	AAA AGG ACC	Arg → Lys	T
NUS4	505	7		C · G → T · A	ACC CCA CGA	Pro → Ser	NT
NUS15	596	8		T · A → C · G	TAC TTC AGG	Phe → Ser	NT
NUS5	599 600	8		G · C G · C → A · T	TTC AGG GAT	Frameshift	T
NUS6	600	8		G · C → C · G	TTC AGG GAT	Arg → Ser	T
NUS3	601	8		G · C → A · T	AGG GAT TTG	Asp → Asn	T
Putative splice site mutation							
NUS2			4				
NUS18			5				
NUS17			5				
NUS22			7				
NUS11			7				
NUS10			8				
NUS12			8				
Other							
NUS20				10-bp deletion, 536–545[b]			

[a] NT, Nontranscribed; T, transcribed.
[b] The strand containing the premutagenic lesion cannot be determined since this mutation did not occur at a dipyrimidine site.

Figure 3. Location of independent *HPRT* mutations in repair-proficient human fibroblasts irradiated with 6.5 J/m^2 UV light in early S phase (21 mutants) or in G_1 phase, 6-7 hr before S phase (20 mutants). Mutation NUS13, which did not occur opposite a dipyrimidine sequence, has been excluded. Mutations are indicated as in Fig. 1. In 71% of the mutants from cells irradiated in S phase, the premutagenic lesion was located in the transcribed strand; in only 20% of the mutants from cells irradiated in G_1 phase was the premutagenic lesion located there. This switch in strand bias indicates preferential repair of the transcribed strand of the *HPRT* gene.

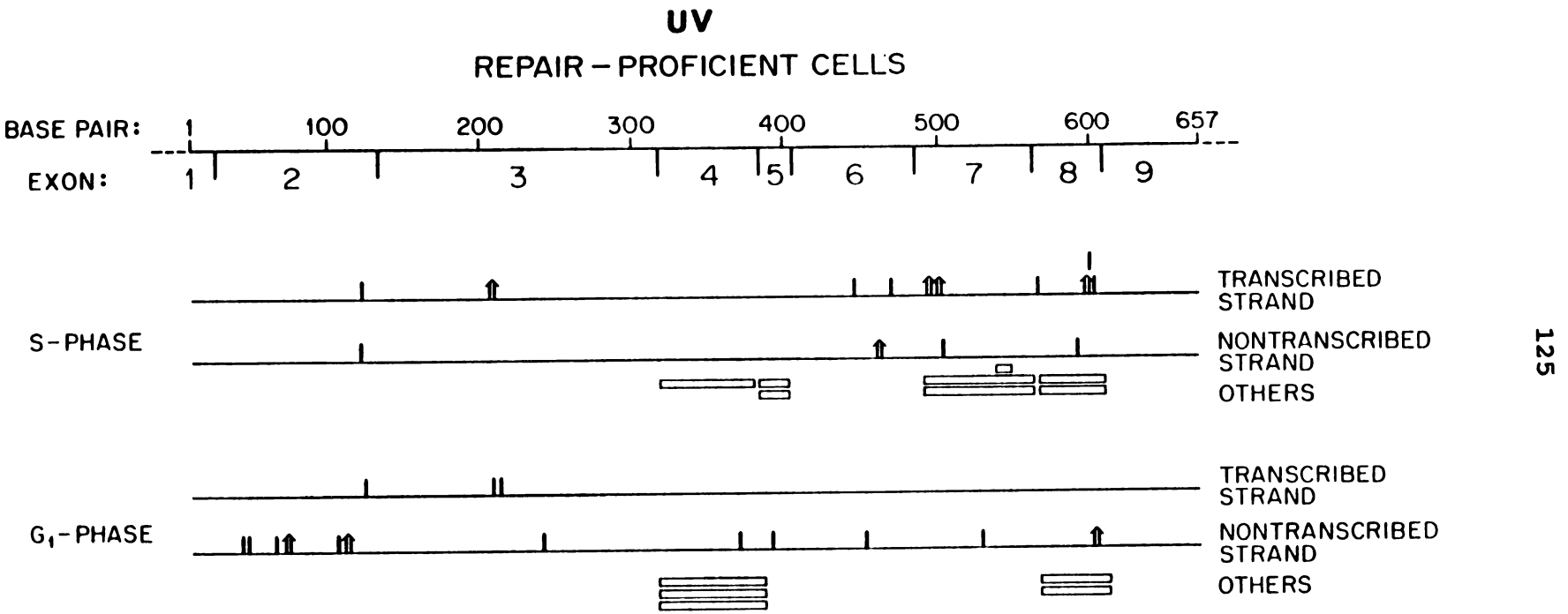
UV
REPAIR – PROFICIENT CELLS
BASE PAIR:
1
100
200
300
400
500
600
657
EXON:
1
2
3
4
5
6
7
8
9
S-PHASE
TRANSCRIBED STRAND
NONTRANSCRIBED STRAND
OTHERS
G1-PHASE
TRANSCRIBED STRAND
NONTRANSCRIBED STRAND
OTHERS

Figure 3.

TABLE 4. Kinds and locations of mutations induced in the coding region of the *HPRT* gene of repair-proficient diploid cells irradiated in G_1 phase

Type of change and mutant	Position	Exon	Deleted exon	Type of mutation	Surrounding sequence	Amino acid change	Strand with affected dipyrimidine[a]
Base substitution							
NUG2	43	2		C · G → G · C	GAA CCA GGT	Pro → Ala	NT
NUG15	44	2		C · G → T · A	GAA CCA GGT	Pro → Leu	NT
NUG10	67	2		T · A → A · T	TTT TGC ATA	Cys → Ser	NT
NUG3	73, 74 tandem	2		C · G → T · A C · G → T · A	ATA CCT AAT	Pro → Phe	NT
NUG7	108	2		T · A → A · T	GTG TTT ATT	Phe → Leu	NT
NUG14	112, 113 tandem	2		C · G → T · A C · G → T · A	ATT CCT CAT	Pro → Phe	NT
NUG4	124	2		A · T → G · C	CTA ATT ATG	Ile → Val	T
NUG12	209	3		G · C → A · T	AAG GGG GGC	Gly → Glu	T
NUG5	212	3		G · C → T · A	GGG GGC TAT	Gly → Val	T
NUG11	241	3		T · A → C · G	GAT TAC ATC	Tyr → His	NT
NUG6	371	4		C · G → T · A	TCA ACT TTA	Thr → Ile	NT
NUG18	392	5		T · A → C · G	GTC TTG ATT	Leu → Ser	NT
NUG19	464	6		C · G → T · A	AAT CCA AAG	Pro → Leu	NT
NUG20	532	7		T · A → C · G	GAC TTT GTT	Phe → Leu	NT
NUG1	603, 604 tandem	8		T · A / T · A → C · G	GAT TTG AAT	Frameshift	NT
Putative splice site mutation							
NUG16			4				
NUG8			4				
NUG9			4				
NUG13			8				
NUG17			8				

[a] NT, Nontranscribed; T, transcribed.

TABLE 5. Types of base substitutions induced in the coding region of the *HPRT* gene in repair-deficient XP12BE fibroblasts and repair-proficient cells irradiated in S phase and in G_1 phase

Type of base substitution	Number of substitutions observed			
	XP12BE cells		Repair-proficient cells	
	S phase	G_1 phase	S phase	G_1 phase
Transitions:				
G•C --> A•T	15	9	11	8
A•T --> G•C	1	1	5	5
Transversions:				
G•C --> C•G	1	0	2	1
G•C --> T•A	2	2	0	1
A•T --> C•G	2	1	0	0
A•T --> T•A	1	3	0	2
TOTAL	22	16	18	17

DISCUSSION

The mutation and survival data for the repair-proficient fibroblasts and the XP12BE cells are consistent with previously published reports from this laboratory (9). As discussed by those investigators, the higher survival of the colony-forming ability in the repair-proficient cells than in XP12BE cells indicates that following irradiation, there is time for DNA repair before "reproductive death" occurs. But the lack of variation in survival with the cell cycle indicates that the time available for such repair is not time prior to S phase. Instead, it probably reflects time before the cells experience a need for certain critical proteins that cannot be produced because of blocks in transcription.

The present data show that in human cells that have no time (or no ability) to excise UV photoproducts, the strand distribution of potentially mutagenic lesions averages 75% transcribed:25% nontranscribed, but in repair-proficient cells given 6 h for repair before S, the distribution is reversed to 20:80. We argue that such a switch in strand distribution is best explained by preferential repair of lesions from the transcribed strand of the *HPRT* gene. There was no statistically significant difference in the frequency or kinds of mutations induced, or in the strand distribution of the premutagenic lesions in the mutants derived from populations of XP12BE cells irradiated in either phase of the cell cycle. The data in Fig. 2 suggest that in S phase cells, mutations induced by UV are more likely to occur in the 3' half of the *HPRT* gene (72%) and in G_1 phase cells, they are more evenly distributed (47% in the 3' end). This apparent change cannot be due to DNA excision repair since

XP12BE cells are virtually devoid of such repair. This difference was not seen in XP12BE cells following exposure to benzo(a)pyrene diol epoxide (BPDE) (3) and so it might reflect differential accessibility of the gene to UV damage during S and G_1 phase. Nevertheless, it is important to note that there was no cell cycle-related change in the strand distribution of UV-induced premutagenic lesions in these XP12BE cells. Furthermore, there was no statistically significant difference between the XP12BE cells in S or G_1 and the NFSL89 cells in S, in the strand distribution of premutagenic lesions. Therefore, the switch in strand bias observed in mutants derived from the repair-proficient cells irradiated in S or G_1 phase can be attributed to preferential repair of the transcribed strand.

This conclusion, which is based on a comparison of repair-proficient diploid human fibroblasts irradiated in two different phases of the cell cycle, agrees with that of Vrieling et al. (25). These investigators based their conclusion on a comparison of mutants from asynchronous cultures of a repair-proficient and a repair-deficient Chinese hamster cell line. Both studies support the work of Mellon et al. (14), indicating that UV-induced dipyrimidine dimers are preferentially removed from the transcribed strand of an actively transcribed gene in a human cell line. Although it is not yet clear how such targeting of the excision repair complex to a particular strand occurs, preferential repair of the transcribed strand is abolished in mammalian cells by inhibition of RNA polymerase II with α-amanitin (4). Conversely, in *E. coli*, induction of a gene has been shown to induce preferential repair of the transcribed strand (13). These studies suggest that there is a coupling of transcription with nucleotide excision repair of UV-induced lesions. As

suggested by Mellon et al. (14), it is possible, for instance, that the stalled RNA polymerase acts as a signal to target the repair complex to the site of the transcription-blocking lesion on the transcribed strand.

Glickman and colleagues (5, 6) have suggested from data in *E. coli* and in CHO rodent cells that UV-induced G·C --> A·T transitions result predominantly from 6-4 Py-Py lesions. However, Vrieling et al. (25) suggest that such transitions result primarily from Py-Py dimers because they found that 100% (15/15) of the UV-induced base substitutions were G·C --> A·T in a Chinese hamster cell line (VH-1) that is incapable of repairing Py-Py dimers, but repairs 6-4 Py-Py lesions at a rate 50% that of their repair-proficient V-79 hamster cell line. Since in human cells virtually all 6-4 Py-Py lesions are repaired within 6 h (15), if 6-4 Py-Py lesions uniquely cause a particular kind of base substitution, and if these substitutions constitute a substantial fraction of the UV-induced mutations seen in these cells, we should have observed a significant difference in the kinds of base substitutions in mutants from repair-proficient cells irradiated in S and those in G_1. This was not observed in our study. Therefore, we conclude that in human cells G·C --> A·T transitions result from both unexcised Py-Py dimers and 6-4 photoproducts. This could explain why such transitions are the most common mutations induced by UV radiation in a variety of organisms (5, 8, 10, 21, 24).

As noted above, the distribution of dipyrimidine sites between the transcribed and the nontranscribed strands in the coding region of the human *HPRT* gene is 59:41. If all lesions were equally mutagenic, and if each mutation resulted in a nonfunctional protein, 59% of the mutations in XP12BE cells or repair-proficient cells irradiated in S should have arisen

from photoproducts in the transcribed strand, and 41% from lesions in the complementary strand. Instead, we found a distribution of 75:25. Vrieling et al. (25) found a still larger shift, 90:10, and suggested that the bias reflected a more error-prone polymerase (i.e., polymerase delta) replicating the transcribed strand of the gene. As discussed by these investigators, the origin of replication of the *HPRT* gene is putatively located in the first intron (22), implying that the transcribed strand of most of this gene is also the leading strand during DNA replication and, therefore, is synthesized by DNA polymerase delta (19). If polymerase delta were more error-prone than polymerase alpha, lesions in the transcribed strand would be more likely to result in mutations than lesions in the complementary strand. However, there is at least one other possible explanation. If photoproducts involving cytosine were more mutagenic than those involving thymine, and if in the coding region of the *HPRT* gene, mutations involving a C·G base pair with the C in the transcribed strand were particularly likely to result in a nonfunctional enzyme, this would explain the observed bias. We found that 71% of the UV-induced base substitutions (52/73) involved C·G base pairs, and among mutations targeted to lesions in the transcribed strand, 89% (40/45) involved C·G base pairs. In contrast, only 43% (12/28) of the mutations targeted to lesions in the nontranscribed strand involved C·G base pairs. Furthermore, Chen et al. (3), using the same system, recently showed that when guanine substituted with a bulky residue of BPDE was the premutagenic lesion in the transcribed strand of *HPRT*, the distribution of premutagenic lesions was lower than predicted, i.e., 25% instead of 40%. This argues against an error-prone delta polymerase causing the increase above the

expected strand distribution observed in the UV studies. Rather, the increase seen in the XP12BE cells and the repair-proficient cells in S more likely reflects selection for nonfunctional HPRT protein.

In summary, we have shown that if diploid human fibroblasts are able to excise UV-induced photoproducts before DNA replication occurs, they exhibit a significant switch in the strand distribution of premutagenic lesions. The ratio of lesions in the transcribed to nontranscribed strand is reversed from 75:25 to 20:80, consistent with preferential excision repair of UV photoproducts from the transcribed strand of the *HPRT* gene.

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CHAPTER V

Preferential repair and strand-specific repair of benzo[a]pyrene diol epoxide adducts from the *HPRT* gene of diploid human fibroblasts

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SUMMARY

We showed previously that if excision repair-proficient human cells are allowed time for repair before onset of S phase, the premutagenic lesions formed by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) are lost from the transcribed strand of the hypoxanthine(guanine) phosphoribosyltransferase (HPRT) gene faster than from the nontranscribed strand. No change in strand distribution was seen with repair-deficient cells. These results suggest strand-specific repair of BPDE-induced DNA damage in human cells. To test this hypothesis, we measured the initial number of BPDE adducts formed in each strand of the actively transcribed *HPRT* gene and their rate of repair, using the UvrABC exinuclease in conjunction with Southern hybridization and strand-specific probes. We also investigated the possibility of preferential repair of such damage by comparing these rates with the rate of loss of damage from the inactive 754 locus located in the same X-chromosome. Following exposure to 1.0 or 1.2 uM BPDE, the frequency of adducts formed in either strand of a 20 kb fragment that lies entirely within the transcription unit of the *HPRT* gene was similar. The frequency of adducts formed in the 14 kb 754 fragment was slightly lower (~20%) than in the *HPRT* gene. In contrast to the frequency of adduct formation, the rate of repair in the two strands of the *HPRT* fragment differed significantly. Within 7 hr after treatment with 1.2 uM BPDE, 55% of the adducts had been removed from the transcribed strand, while only 26% had been removed from the other strand; after 20 hr of repair, these values were 87% and 58%, respectively, confirming the prediction of our mutagenesis studies. In contrast to the rapid rate of repair in the *HPRT* gene, only ~14% of the

BPDE adducts were lost from the 754 locus, a value even slower than the rate of loss from the overall genome (i.e., 38%). Our results demonstrate strand-specific and preferential repair of chemically-induced BPDE bulky adducts in human cells. They suggest that the heterogeneous repair of BPDE adducts cannot be accounted for merely by the greatly increased rate of the repair specific to the transcribed strand of the active genes, and point to a role for the chromatin structure.

INTRODUCTION

Recent studies have demonstrated that UV-induced cyclobutane dimers are repaired more rapidly (i.e., preferentially) from the actively transcribed genes of rodent and human cell lines in culture than from inactive genes or from nontranscribed segments of DNA or from the overall genome of the cells (1-4). Furthermore, Mellon et al. (5) showed that in these cells such lesions are excised faster from the transcribed strand of the actively transcribed dihydrofolate reductase (*dhfr*) gene than from the nontranscribed strand of that gene. These results led to the investigators to hypothesize that nucleotide excision repair is coupled to gene transcription (5). Indeed, a factor responsible for such coupling has recently been partially purified from *E. coli* cell extract (6).

Although the existence of such heterogeneous repair of cyclobutane dimers from the DNA of mammalian cells is well documented, the applicability of this model to bulky chemical residues covalently bound to DNA (adducts) is unclear and controversial. The results of our studies(7, 8) of the effect of excision repair in diploid human cells on the strand distribution of the principal premutagenic lesions induced by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) showed that in the absence of repair, such lesions are located in both strands of the coding region of the hypoxanthine (guanine) phosphribosyltransferase (*HPRT*) gene, but after 12 hr of excision repair, no premutagenic lesions remains in the transcribed strand. These results provide strong biological evidence of strand-specific repair of BPDE adducts from the human *HPRT* gene.

Such strand-specific repair could account for the strand bias for

BPDE-induced premutagenic lesions observed by Mazur and Glickman (9) in the adenine phophoribosyltransferase gene and by Carothers and Grunberger (10) in the dhfr gene of excision repair-proficient Chinese hamster cell line (CHO cells), although these investigators did not demonstrate that in the absence of excision repair premutagenic BPDE lesions were distributed in both strands of their target genes. However, using the technique originally developed by Bohr et al. (1) to detect lesions initially present or remaining in specific genes and using the *E. coli* UvrABC exinuclease to recognize and incise bulky DNA adducts, Tang et al. (11) demonstrated that there is no difference in the rate of repair of DNA damage induced by a structurally related compound, N-acetoxy-2-acetylaminofluorene, from the active and nonactive (nontranscribed) regions of the dhfr gene of CHO cells. More recently, Tang and his colleagues (12) reported that BPDE induced DNA adducts are also not preferentially removed from such regions of the *dhfr* gene of CHO cells.

The present study was designed to directly measure the ability of human cells to remove BPDE adducts from either strand of the *HPRT* gene to see if strand-specific repair occurred. We also wanted to test for preferential excision repair of BPDE adducts by comparing the rate of their removal from an active gene *(HPRT)* and an inactive locus (13) on the same X-chromosome (the 754 locus). To investigate these questions, we synchronized normal human fibroblasts and treated them with BPDE in early G_1 phase so that there could be a long period for excision repair before the onset of semi-conservative DNA replication. The cells were harvested immediately or after various times after treated, and the DNA was extracted and assayed for lesions in the *HPRT* gene. It has been

extracted and assayed for lesions in the *HPRT* gene. It has been demonstrated that the UvrABC exinuclease is able to specifically and quantitatively incise at least 80% of BPDE-DNA adducts in human genomic DNA (14). Using this enzyme, in conjunction with the Southern blotting and hybridization with probes specific for the individual strands of the *HPRT* gene, we quantified the initial numbers of BPDE-DNA adducts formed in the two strands and the numbers remaining at various times post treatment.

The results showed that there was no significant difference in the initial frequencies of BPDE-DNA adducts formed in the two strands of the HPRT fragment, but the rate of removal of adducts from the two strands was markedly different. The transcribed strand was repaired significantly faster than the complementary strand. Furthermore, the rate of excision repair of BPDE adducts from the 754 locus was slower still.

MATERIALS AND METHODS

Cell culture and cell synchronization. Diploid human male fibroblasts, derived from foreskin material of a neonate (15), were cultured in Eagle's medium containing 10% supplemented bovine calf serum (HyClone, Logan, UT). Such cells were driven into the G_0 state by density inhibition and nutrient depletion as described (16). To stimulate the cells to re-enter the cell cycle, they were released from confluence, and plated in fresh culture medium at a density of 10^4 cells/cm^2. The time of onset of S phase following release from G_0 was determined by the incorporation of ^{3}H-thymidine into acid-insoluble material as described (16).

BPDE treatment and post-treatment incubation. Cells were treated 5 hr after release from G_0 (in early G_1 phase) to allow them to have a long repair period before DNA replication began. They were rinsed with phosphate-buffered saline (PBS) and then treated with BPDE as described (7), using a dose of 1.0 or 1.2 uM. After treatment, cells were either lysed immediately or incubated for up to 24 hr in fresh culture medium.

Isolation and purification of DNA. After a specific repair period, cells were washed with PBS and lysed in lysis buffer containing 50 mM Tris.HCl (pH 8.0), 10 mM EDTA, 100 mM NaCl, 0.5% sodium lauryl sarkosine, and 100 ug/ml proteinase K. The cell lysates were incubated at 50^{0}C for 8-16 hr. The DNA solution was extracted with a equal volume of phenol, phenol/chloroform (1:1), and chloroform. DNA was then precipitated with ethanol, resuspended in TE buffer (10mM Tris.HCl, pH7.5 and 1mM EDTA), and treated with RNase A (50 ug/ml) for 2 hr at 37^{0}C. The DNA was re-extracted as above and precipitated with ethanol. After centrifugation, the DNA

pellet was dissolved in TE buffer at a concentration of 0.2 ug/ul. The purified DNA was digested with restriction enzyme *Bam*HI or *Eco*RI (5 units/ug DNA) at 37^{0}C for 3 to 5 hr. The completion of digestion was verified by electrophoresis of the DNA samples on agarose minigels. The digested DNA was purified by extraction and precipitation as above.

UvrABC excision reaction. The UvrA, UvrB and UvrC proteins were prepared as described (17). 10 ug of DNA derived from cells which had been allowed various lengths of time to repair damage was used for repair analysis. To serve as an internal standard, 5 pg of linearized plasmid DNA containing a fragment of the gene to be probed was added into each DNA sample. This mixture was then separated into two equal parts; one was exposed to UvrABC exinuclease, the other to the exinuclease buffer alone. The exinuclease reaction mixture was composed of 5 ug restricted genomic DNA plus 17 pmoles of each of the three subunits of UvrABC in a final volume of 250 ul buffer containing 50 mM Tris.HCl (pH7.5), 10mM $MgCl_2$, 75 mM KCl, 2mM ATP, 1 mM dithiothreitol, and 1 ug/ul bovine serum albumin (BSA). At the end of 1 hr incubation at 37^{0}C, the reaction was stopped by adding 0.1 ug/ul proteinase K and 0.1% Sodium dodecyl sulphate (SDS) to each sample and incubating for 1 hr at 42^{0}C. The DNA was purified by ultrafiltration using Centricon-30 tubes and precipitated with ethanol.

DNA denaturation, gel electrophoresis and Southern blot analysis. The DNA samples were dissolved in 90% formamide containing 0.025% bromophenol blue and incubated at 37^{0}C for 30 min to denature the DNA. Immediately after incubation, the DNA samples were electrophoresed at 2.5 V/cm for 16 hr in a 0.6% agarose gel. After electrophoresis, the DNA in the gel was stained and depurinated with acid using standard procedures

(18). The DNA was then transferred to a Zeta-probe GT membrane (Bio-Rad, Richmond, CA) using the conditions recommended by the manufacturer. Hybridizations were performed in 20 ml of solution containing 50% formamide, 5X SSPE (10mM NaCl, 10mM $NaPO_4$ (pH 7.8), 1mM EDTA), 0.5% SDS, 0.02% Ficoll, 0.02% polyvinylpyrolidone, 0.02% BSA, denatured salmon sperm DNA (50 ug/ml), *E. coli* t-RNA (100 ug/ml) and 2-3 X 10^7 cpm ^{32}P-labeled probe at 42^0C for 20-24 hr. The riboprobes used for strand-specific hybridizations to the *Bam*HI fragment covering the 5'-half of the *HPRT* genomic DNA were generated using the method described by Melton et al. (19), with the modifications suggested by Boeringer-Mannheim Biochemicals. DNA probes were labeled with ^{32}P-dNTPs by random primer extension (20). After hybridizations, the membranes were washed at 60^0C with a final wash step at 0.1X SSPE, 0.1% SDS and exposed to Kodak XAR-5 X-ray films using intensifying screens.

Hybridization probes. Plasmid pG2Pa (21), which was constructed by subcloning a 1.4 kb *Eco*RI-*Xho*I fragment containing sequences from the first intron of the human *HPRT* gene into the vector pGEM2, was kindly provided by Dr. A. C. Chinault (Baylor college, Houston, TX). This plasmid contains the SP6 and T7 promoters. RNA transcripts synthesized by the SP6 and T7 polymerases hybridize with the transcribed and nontranscribed strands of *HPRT* genomic fragment, respectively. The 2.0 kb *Hind*III fragment of the 754 locus (22) was a gift from Dr. L. H. F. Mullenders (Leiden University, The Netherlands).

Quantitation. The intensities of bands were quantified using the Bio-Image Visage 110 system (Millipore). The intensity of the full-length fragment band was normalized with the internal standard band referred to

above. The average number of UvrABC sensitive sites per fragment was calculated by the Poisson distribution equation as described by Bohr et al. (23). These calculations took into consideration the nonspecific incisions produced by the UvrABC enzyme complex, which ranged from 0.05 to 0.15 incision per 10 kb in the present experiments.

RESULTS

Determining delay in onset of DNA replication induced by BPDE. Repair-proficient human fibroblasts were synchronized by release from confluence and plated into a series of culture dishes at 10^4 cells/cm^2. The density was used because it corresponded to the experimental conditions we had used previously in our study of the "effect of excision repair on the strand distribution of premutagenic BPDE adducts in the human *HPRT* gene" (8, 9). Before beginning biochemical measurement of the rate of removal of such adducts from cellular DNA, we determined the earliest time of onset of semiconservative DNA synthesis in cells treated with a 1.0 or 1.2 uM dose of BPDE. For this measurement, cells were plated into a series of 60 mm-diam dishes and assayed as described for the incorporation of tritiated thymidine into acid-insoluble material at various times after treatment. Although in untreated populations plated at this density DNA replication begins 16 hr following release from confluence (16), the cells exposed to 1.0 or 1.2 uM BPDE showed no evidence of incorporating tritiated thymidine even when assayed 24 hr after treatment, i.e., 29 hr after release from confluence (data not shown). These results indicated that it would be possible to harvest DNA from cells after allowing various lengths of times for excision repair up to at least 24 hr posttreatment without the need to separate newly-synthesized DNA from parental DNA.

Determining the rate of excision repair of BPDE adducts. For these assays, cells were plated at 10^4 cells/cm^2 into a series of 150 mm-diam dishes, 10-15 dishes per repair time period to be assayed. After 5 hr, the cells were treated with BPDE and populations were harvested immediately or after various repair periods. For each time point determination, DNA was

extracted from treated or control populations, digested with an appropriate restriction enzyme, and then treated with UvrABC exinuclease or with incubation buffer. The DNA samples were then denatured under neutral conditions as described by Tang et al. (11) to avoid the possibility of DNA strand breaks formed at the sites of the alkali-labile N^7-guanine adducts through a nonenzymatic mechanism (24). They were then separated on agarose gels and analyzed by Southern hybridizations using gene- or strand-specific probes.

Evidence of strand-specific repair of BPDE adducts in human cells. Using this method, we measured the initial number of BPDE-induced lesions formed in the individual strands of the 20 kb *Bam*HI fragment of the human *HPRT* gene, as well as their rates of removal. This fragment is located in the 5'-half of the gene and is entirely within the transcription unit (Fig. 1). To avoid detection of homologous pseudogenes containing *HPRT* exon sequences (25), we used riboprobes complementary to intron sequences of the gene. Representative autoradiograms of such repair studies are shown in Fig. 2A and 2B. The intensities of the full length fragments were determined by densitometric scanning, and the numbers of incisions and the percentage of adducts removed were calculated as described. The data obtained from two separate experiments are given in Table 1, and plotted in Fig. 3. The level of adduct formation induced by BPDE was found to increase with dose, and to be similar in each strand of the *HPRT* fragment at the two doses used. However, the rates of their removal from the transcribed and nontranscribed strand were significantly different. Within 7 hr following the treatment, 55% of the adducts had been removed from the transcribed strand, compared to a level of 26% removed from the

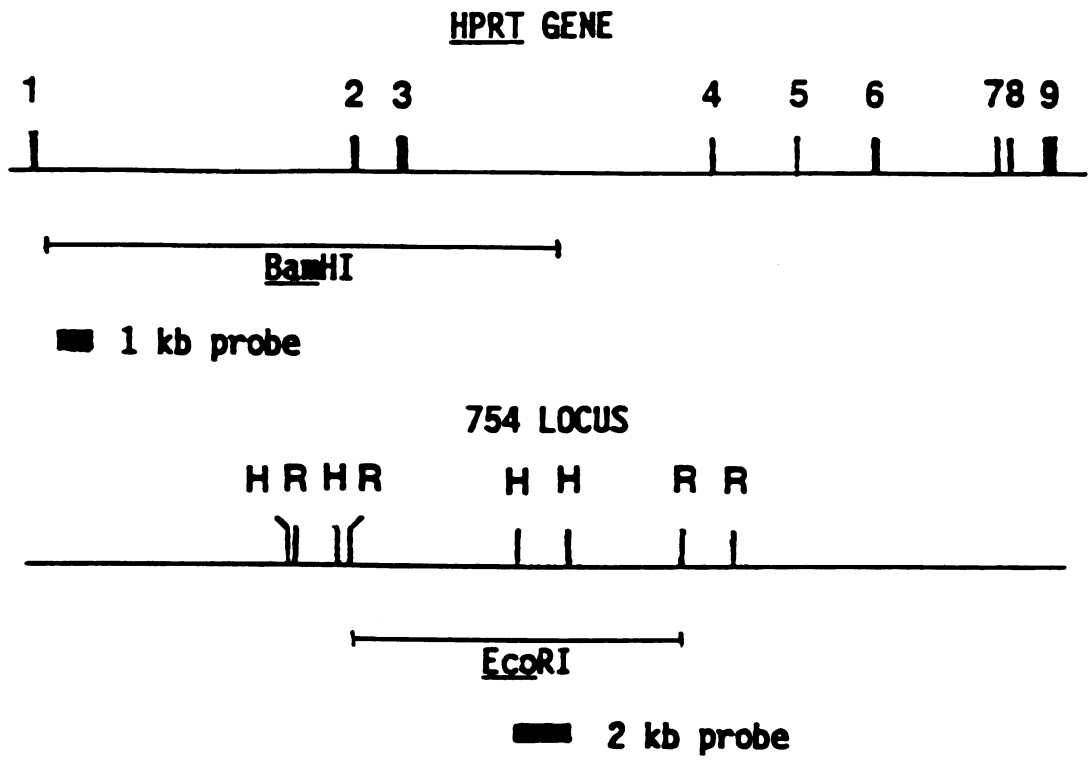

Figure 1. Molecular organizations of the human *HPRT* gene and the 754 locus. The *Bam*HI and *Eco*RI restriction fragments analyzed for repair are indicated. Vertical bars in the *HPRT* and 754 maps indicate exons and restriction sites, respectively. The locations of the probes are shown as rectangles. H, *Hind*III; R, *Eco*RI.

Figure 2. Autoradiograms illustrating extent of repair in the transcribed (A), nontranscribed (B) strand of the *HPRT* gene and in both strands of the 754 locus (C). DNA was isolated from untreated human fibroblasts (first two lanes of each panel), or from cells incubated for the indicated repair period posttreatment with BPDE at a dose of 1.2 uM. DNA samples were restricted with the appropriate enzyme. DNA fragment containing the sequences to be probed was included in each DNA sample as an internal marker. DNA samples were then treated or not treated with UvrABC and subjected to electrophoresis and Southern hybridizations with ^{32}P-labeled probes as described. The upper panel shows the bands corresponding to the 20 kb fragment of the *HPRT* gene or the 14 kb fragment of the 754 locus. The bottom panel contains bands corresponding to the DNA fragments serving as internal markers.

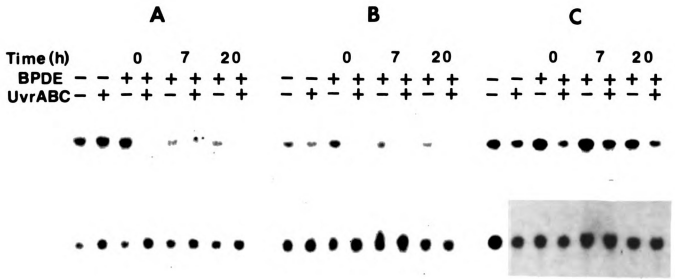

Figure 2

Table 1. Formation of BPDE adducts in the individual strands of the 20 kb *Bam*HI fragment and their rates of removal

Dose	Repair time	Transcribed strand		Nontranscribed strand	
μM	(hr)	Incisions/fragment[a]	% repair	Incisions/Fragment[a]	% repair
1.2	0	1.67	0	1.77±0.1	0
	7	0.75	55	1.31±0.02	26
	20	0.22	87	0.75±0.07	58
1.0	0	1.30±0.02	0	1.34±0.07	0
	8	0.58±0.02	55	0.93±0.02	30
	24	0	100	0.15±0.02	89

[a]The numbers are calculated from the densitometric scanning as described. The nonspecific incisions which ranged from 0.09 to 0.3 have been subtracted.

Figure 3. Rates of removal of BPDE adducts from the transcribed (closed symbols), nontranscribed (open symbols) strand of the *HPRT* gene, and from both strands of the 754 locus (half closed symbols). Circles and squares indicate data obtained from cells treated with 1.0 & 1.2 μM BPDE, respectively.

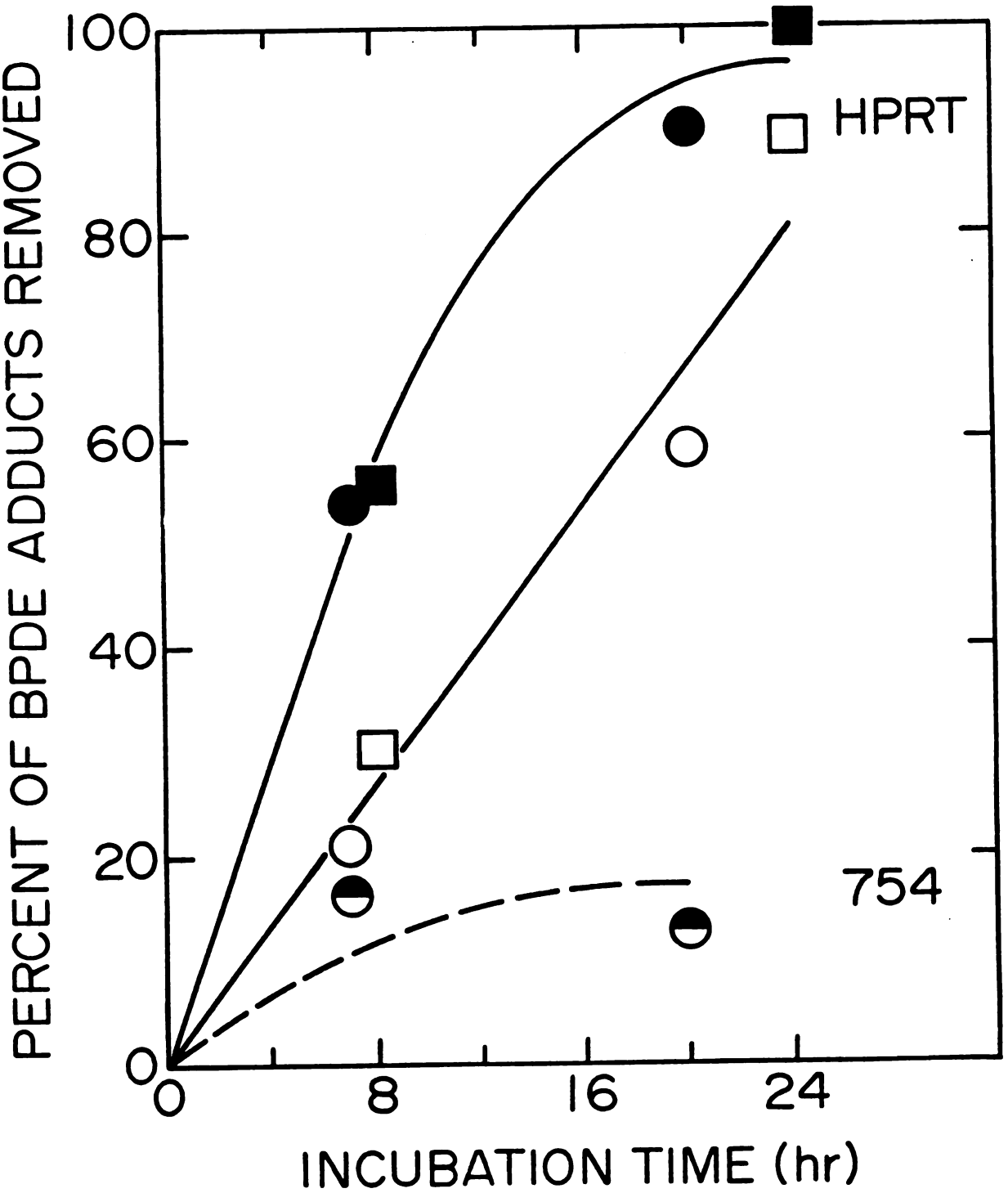
PERCENT OF BPDE ADDUCTS REMOVED
100
80
60
40
20
0
0
8
16
24
INCUBATION TIME (hr)
HPRT
754

Figure 3

nontranscribed strand. By 20 hr, the vast majority (87%) of the adducts had been removed from the transcribed strand, whereas approximately half (58%) of them still remained in the nontranscribed strand of the *HPRT* gene.

Evidence of preferential repair of BPDE adducts in human cells. To investigate whether there was a difference in the rate of excision repair of BPDE adducts from a nontranscribed (inactive) genomic sequence compared to the actively transcribed *HPRT* gene, the formation of such adducts and their rate of repair were determined in a 14 kb *Eco*RI fragment (Fig. 1) of the transcriptionally inactive 754 locus, using a 2.0 kb probe labeled by the random priming method. The autoradiogram of that study is shown in Fig. 2C. The analyzed data are given in Table 2, and plotted in Fig. 3. The initial frequency of adducts formed by treatment of 1.2 uM BPDE was 0.92/14 kb fragment or 1.31/20 kb, which is slightly (~20%) less than that observed in either strand of the *HPRT* fragment. However, the efficiency of repair in the 754 locus was markedly reduced. Few adducts (12%) were removed from this region in the 20 hr period after treatment, while 90% and 59% of them were repaired in the transcribed and nontranscribed strands of the *HPRT* gene at this time point, respectively.

Table 2. Formation of BPDE adducts in both strands of the 14 kb *Eco*RI fragment of the 754 locus and their rate of removal

Dose μM	Repair time (hr)	Incisions/fragment[a]	% repair
1.2	0	0.92	0
	7	0.77	16
	20	0.81	12

[a]The numbers are calculated from the densitometric scanning as described. The nonspecific incision (0.05/fragment) has been subtracted.

Discussion

We have determined the formation and rate of repair of DNA damage induced by a chemical carcinogen, BPDE, in the individual strands of the single copy, single allele human *HPRT* gene. The results of this study show biochemical evidence of strand-specific repair of BPDE-induced bulky adducts, with the transcribed strand being repaired faster than the other strand. This is consistent with the hypothesis derived from our mutagenesis studies, namely, that strand-specific repair is the explanation for the difference in strand distribution of premutagenic lesions observed in repair-proficient cells treated in S phase and such cells treated in G_1 phase. There is biochemical evidence of strand-specific repair of DNA damage induced by UV (5, 26), psoralen plus near UV light (6), and cisplatinum (6) in *E. coli* and/or mammalian cells. Such repair has been suggested to account for the fact that essentially all of the premutagenic lesions induced by (±)-3α,4β-dihydroxy-1α,2α-epoxy-1,2,3,4,-tetrahydrobenzo[c]phenanthrene in the *dhfr* gene of CHO cells were located in the nontranscribed strand (27). Such strand-specific repair, which was first reported by Hanawalt and his colleagues (5), has suggested a specific coupling between transcription and repair. Recently, an in vitro system carrying out strand-specific repair has been developed using cell-free extract derived from *E. coli*, and indeed a candidate "transcription-repair coupling factor" has been partially purified from this extract (6). There are at least two ways that this coupling factor could lead to a faster rate of repair in the transcribed strand than in the opposite strand. This factor may associate with component(s) of the transcriptional complex via protein-protein interactions. The presence of this factor in

the transcriptional complex may facilitate the assembly of the repair complex in the vicinity of the transcriptional complex, so that repair enzymes can scan the strand that is being transcribed. Another possibility is that this factor directly or indirectly recognizes a unique DNA structure or other signal(s) generated by the stalled transcriptional complex at the site of DNA damage, and targets the repair enzymes to the template strand.

In the present study, the removal of BPDE adducts from the transcriptionally inactive 754 locus was found to be much less efficient than removal from either strand of the *HPRT* gene. This indicates that there are at least three different rates of repair of BPDE adducts within the genome of human cells. The slow repair rate observed in the 754 locus agrees with the hypothesis suggested by Hanawalt and his colleagues (1, 3) that DNA damage located in the condensed, inactive chromatin is less accessible to repair enzymes, compared to that in the active chromatin. Our finding that repair of BPDE adducts in both strands of the inactive gene is even slower than that observed in the nontranscribed strand of the active gene is consistent with what was recently found for the repair of aflatoxin B_1-induced adducts in the active and inactive human metallothionein genes (28). However, in rodent and human cell lines the removal of cyclobutane pyrimidine dimers from the nontranscribed strand of the *DHFR* gene occurs at a rate equal to the rate of removal from both strands of the nonactively transcribed region (5). Little is known about the mechanism(s) of repair in the compact chromatin where the inactive genes are located. However, the lack of repair of cyclobutane dimers in the inactive genes of xeroderma pigmentosum cells of complementation group

C (22, 29), which have been shown to repair transcriptionally active genes at a normal rate, is consistent with a requirement for an additional factor to enable the repair enzymes to reach DNA damage in such chromatin. If so, our results with BPDE suggest that this factor functions less efficiently for processing some bulky adducts, such as the BPDE-adducts, than it does for cyclobutane dimers.

In contrast to the results we obtained in the present study, Tang and Zhang (12) reported that the rate of removal of BPDE adducts from the transcriptionally active and the 3' nonactive regions of the *dhfr* gene of CHO cells was equal. The reason for the discrepancy between these two studies is not known, but the cell types used and the genes examined are not the same, and the concentration of BPDE they used was higher than ours. Furthermore, we compared the rate of repair in an active gene to that in an inactive locus, and they assayed the actively transcribed segment of the *dhfr* gene and its 3' nontranscribed region. It has been reported that in certain genes the region in which the preferential repair occurs is larger than the entire transcription unit (30). It is therefore possible that, unlike repair of cyclobutane dimers (31), repair of BPDE adducts extended beyond the transcribed section of the *dhfr* gene into its nontranscribed flanking regions. Another possible reason for the discrepancy is that double-stranded probes were used in their analysis. This gives information on the average rate of repair in the two DNA strands, and that would lessen the difference detected in their study.

In order to use the Southern hybridization technique for studies of repair rates, one needs to introduce an average of at least one UvrABC efficiently recognizable lesion per fragment analyzed and not more than

two such lesions. Under our experiment conditions, i.e., diploid human cells plated at a density of 10^4 cells/cm^2, this was accomplished using 1.0uM BPDE, a dose that yielded a mean of 1.4 UvrABC sites per 20 kb fragment. This level of BPDE-induced damage reduced the colony forming ability of the population to less than 0.1% of the untreated control. However, during the 20 to 24 hr following BPDE treatment, the cells still remained attached to the dishes. It is important to point out that such cells were capable of removing almost all the UvrABC sites from the *HPRT* gene within the 24 hr following BPDE treatment, even though the majority of the cells in the population would eventually fail to replicate.

The level of BPDE adduct formation we observed in each strand of the *HPRT* gene was very similar, indicating that there was no strand-specific modification by this agent. However, the frequency of adduct formation in the 754 locus was only 80% of that in the *HPRT* gene. Certain chemical carcinogens including BPDE have been found to react preferentially with regions of DNA that are sensitive to DNase I (32, 33). Sucrose gradient sedimentation analysis has also demonstrated a preferential binding of various polycyclic aromatic hydrocarbons to the slowly-migrating euchromatin fractions (34, 35). These studies suggest a binding preference of such chemicals to actively transcribed genes. But, it has also been reported that the microsomally activated parental compound, Benzo[a]pyrene, binds preferentially to the DNase I-resistant regions of chromatin (36). Additionally, use of Southern transfer and hybridization with gene-specific probes indicated that a transcriptionally active gene and an inactive gene were similarly modified by a serious of polycyclic aromatic compounds (37). Nevertheless, chromatin accessibility may not be

the only explanation for the slightly lower binding frequency we observed in the 754 locus. It should be pointed out that the G.C content of a fragment could also affect this frequency, since BPDE binds predominantly to guanine (38). Information on the G.C content of the 754 fragment used in this study is not yet available.

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