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EVIDENCE FROM MUTATION SPECTRA THAT THE UV HYPERMUTABILITY OF XERODERMA PIGMENTOSUM VARIANT CELLS REFLECTS ABNORMAL, ERROR-PRONE REPLICATION ON A TEMPLATE CONTAINING PHOTOPRODUCTS

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By

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ABSTRACT

EVIDENCE FROM MUTATION SPECTRA THAT THE UV HYPERMUTABILITY OF XERODERMA PIGMENTOSUM VARIANT CELLS REFLECTS ABNORMAL, ERROR-PRONE REPLICATION ON A TEMPLATE CONTAINING PHOTOPRODUCTS

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Xeroderma pigmentosum (XP) variant patients are genetically predisposed to sunlight-induced skin cancer. Their fibroblasts have a significantly higher frequency of UV-induced mutations, as detected in the hypoxanthine (guanine) phosphoribosyltransferase (*HPRT*) gene, than do normal cells. In addition, their cells are abnormally slow in replicating UV-damaged DNA. However, in contrast to classic XP cells, XP variant cells are reported to excise UV photoproducts from their genome at a nearly normal rate. To investigate the cause of this UV hypermutability, I used antibodies to compare XP variant and normal cells for the rate of loss of the two major types of photoproducts specifically during S-phase. There was no difference. I also compared the kinds and location (spectrum) of mutations induced in the *supF* gene of UV-irradiated plasmids that had replicated in XP variant cells with that obtained when the same plasmid

replicated in normal cells. The two spectra differed significantly, suggesting that the replication complex of XP variant cells uses an abnormally error-prone process when bypassing photoproducts. To investigate whether what I found with the *supF* gene was also true for an endogenous gene, and whether replicational bypass differs significantly depending upon which strand of the target gene the photoproducts are located in, I determined the spectra of mutations induced in the coding region of the *HPRT* gene of XP variant cells irradiated in early S- or G_1-phase and compared them with those in normal cells similarly irradiated. The mutation spectrum in each strand of *HPRT* of XP variant cells differed very significantly from that of normal cells, strongly supporting the hypothesis that the XP variant cells' replication complex is abnormally error-prone. I also measured the rate of loss of the two types of photoproducts from each strand of the *HPRT* gene in XP variant and normal cells to determine if there was any difference. The results to date suggest that XP variant cells remove cyclobutane dimers more slowly than do normal cells.

This work is dedicated to:

my fiancé, Cheng-tsung Hsia

my father, Jui-hua Wang

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ABBREVIATIONS

^{3}H-TdR	tritiated thymindine
6-4's	6-4 pyrimidine-pyrimidone photoproduct
A	adenine
AAAF	N-acetoxy-2-acetylaminofluorene
ADA	adenosine deaminase gene
AP	apurinic/apyrimidinic site
BCC	basal cell carcinoma
bp	base pair
BPDE	(±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene
BS	Bloom's syndrome
C	cytosine
CHO	Chinese hamster ovary cell line
CPD	cyclobutane pyrimidine dimer
CS	Cockayne syndrome
DHFR	dihydrofolate reductase gene
E. coli	*Escherichia coli*
EM	enhanced mutagenesis
EMS	ethylmethanesulfonate
ENU	ethylnitrosourea
ERCC	excision repair cross-complementing repair dificiency
G	guanine
HCR	host cell reaction

HPRT	hypoxanthine(guanine)phosphoribosyltransferase gene
HSV	Herpes simplex virus
MM	malignant melanoma
NQO	4-nitroquinoline-1-oxide
SCC	squamous cell carcinoma
SV40	simian virus 40
T	thymine
TG	6-thioguanine
TRCF	transcription-repair coupling factor
TTD	trichothiodystrophy
UV	ultraviolet
XP	xeroderma pigmentosum

INTRODUCTION

Xeroderma pigmentosum (XP) variant patients inherit a predisposition to sunlight-induced skin cancer and develop the same clinical characteristics of the disease as do classic nucleotide excision repair-deficient XP patients (Robbins et al., 1974). However, in contrast to the classic XP patients, cells from XP variant patients are reported to excise UV photoproducts, including cyclobutane pyrimidine dimers (CPD) (Cleaver, 1972; Zelle and Lohmann, 1979; Roth et al., 1987) and 6-4 pyrimidine-pyrimidone photoproducts (6-4's) (Mitchell et al., 1987), at a normal or nearly normal rate in the genome overall. The excision repair process in XP variant cells appears to be error-free because when synchronized populations of XP variant cells are irradiated at various times prior to S phase to allow different lengths of time for excision repair before DNA replication, the mutant frequency decreases as a function of time for repair until it reaches background levels (Watanabe et al., 1985).

Several groups of investigators have shown that XP variant cells replicate DNA containing UV photoproducts with greater difficulty than do normal cells (Lehmann et al., 1975; van Zeeland and Filon, 1982; Boyer et al., 1990; Misra et al., 1993). The replication complex of XP variant cells is delayed at UV photoproducts much longer than that of normal cells. For a given dose of UV, the same number of UV photoproducts are formed in normal and XP variant cells, but the variant cells are three to four times more sensitive to inhibition of daughter strand growth. In addition, although XP variant cells are only slightly more sensitive than

normal cells to the cytotoxic effect of UV_{254nm}, they are significantly more sensitive to its mutagenic effect as determined from the frequency of cells with mutations in the hypoxanthanine (guanine) phosphoribosyl transferase (*HPRT*) gene after UV irradiation (Maher et al., 1976; Myhr et al., 1979). If as evidence suggests, mutations are causally involved in carcinogenesis, such UV hypermutability may account for the genetic predisposition of XP variant patients to develop skin cancer on sunlight-exposed parts of the body. However, the molecular mechanism(s) responsible for the UV hypermutability of XP variant cells has not been explained.

My dissertation research was designed to examine this question. The most obvious explanation for the UV hypermutability of XP variant cells is that, for some reason, their replication complex encounters more unexcised photoproducts than does that of normal cells, when the target gene for mutations, i.e., the *HPRT* gene, is being replicated. This would be the case if XP variant cells are significantly slower than normal cells in excision repair of UV-induced photoproducts specifically during DNA replication (S-phase). This had not been examined when I began my research. Another possibility is that XP variant cells are much slower than normal cells in repair of photoproducts specifically in active genes, such as the *HPRT* gene. This question had not been examined either. Another possible explanation for the UV hypermutability is that at a given dose of UV, the replication complex of normal and XP variant cells encounters the same number of unexcised UV photoproducts in the DNA, but the complex of the XP variant cells is defective and less likely than that of normal cells to incorporate the correct nucleotide opposite the photoproducts during replication. If true, this would not only result in

a high mutation frequency in XP variant cells, but also might result in significant differences between normal and XP variant cells in the kinds and locations (spectrum) of mutations induced.

Chapter I reviews the background literature about the studies on XP variant cells and the relationship of nucleotide excision repair and DNA replication to mutagenesis in mammalian cells. Chapter II consists of a manuscript by Wang et al. published in the September 1991 issue of **Proceedings of the National Academy of Science U.S.A.,** 88:7810-7814. It describes the results of the research I carried out to test the hypothesis that XP variant cells exhibit error-prone replication of DNA containing photoproducts. For this study I compared the spectrum of mutations induced in the *supF* tRNA gene of UV-irradiated plasmids that were allowed to replicate in XP variant cells with that obtained when the same plasmid replicated in normal cells. The data show that the spectra differ very significantly. Chapter III consists of a manuscript by Wang et al. published in the July 1993 issue of **Molecular and Cellular Biology,** 13:4276-4283. It describes the work I carried out to determine whether what I found using an exogenous gene carried on a shuttle vector plasmid was also true for an endogenous gene in the XP variant cells. To test this hypothesis, I examined the spectra of mutations induced in the coding region of the *HPRT* gene of XP variant cells irradiated in the beginning of S-phase or in early G_1-phase, 11 hours prior to the onset of S-phase, and compared them with those found in normal cells similarly irradiated. I also investigated whether the UV hypermutability of XP variant cells reflects a much slower than normal rate of excision of photoproducts during S phase of the cell cycle. The data indicate that the rate of excision is normal, but there are extremely significant differences in the

spectra between XP variant and normal cells, strongly suggesting an abnormal replication of DNA containing photoproducts. Chapter IV describes an on-going biochemical study investigating whether XP variant cells excise UV photoproducts, i.e., CPD and 6-4's, from the two strands of the *HPRT* gene more slowly than do normal cells. This research is not yet complete, but the chapter is written in the style of a manuscript by Wang et al. being prepared for submission to the **Journal of Biological Chemistry** or **Proceedings of the National Academy of Science U.S.A.** In addition to my research on the molecular mechanism(s) of UV hypermutability of XP variant cells, I participated in research by a team of scientists analyzing the spectrum of mutations induced when the shuttle vector plasmid containing adducts of 1,6-dinitropyrene was allowed to replicate in human cells. This work by Boldt et al. has been published in the journal **Carcinogenesis**, 12:119-126,1991. A summary of the research is presented in Appendix I. I also participated in research by a team of scientists analyzing the spectrum of mutations induced in human fibroblasts by ethylnitrosourea. This research by Maher et al. will be submitted for publication next month. Appendix II presents a summary of this work.

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CHAPTER I

LITERATURE REVIEW

A. Relationship Between Sunlight and Human Skin Cancer.

1. Epidemiological Evidence.

The earliest association between sunlight exposure and human skin cancer was the observation that the high incidence of skin cancer found in sailors was possibly related to prolonged exposure to the weather (Unna, 1968). Later, more epidemiological evidence, which involved intensive clinical studies and incidence surveys in different geographic areas world-wide, strongly supported the etiological significance of sunlight in the induction of human skin cancer. This section will discuss the three major types of human skin cancers.

1.1 Squamous Cell Carcinoma. The best correlation between sun exposure and skin cancer is seen in squamous cell carcinoma (SCC). Histological evidence suggests that there is a very close correlation between the sunlight exposure of selected anatomic sites and distribution of SCC. Pearl and Scott (1986) reported an extreme excess of SCC on exposed areas of the face, such as the nose. For other sites typically shielded from sunlight by hair or clothing, there is a much lower tumor incidence. A study that compared a group of patients with SCC with age- and sex-matched controls found that patients with SCC have significantly fairer skin,

lighter hair, lighter eyes, tanned less and sunburned more easily and more severely than the control group (Urbach et al., 1972). A similar conclusion was made by a case-control study in the Montreal region (Aubry and MacGibbon, 1984). The latter study also revealed that occupational sunlight exposure appears to be a risk factor for SCC. People in occupations in which the face and neck are repeatedly exposed, e.g., farmers and outdoor-construction workers, are most vulnerable.

1.2 Basal Cell Carcinoma. Basal cell carcinoma (BCC) is the most common skin cancer in the United States (Scotto et al., 1982). Anatomical examination suggests BCC also distributes mainly on the exposed areas of the skin, although some BCC are located in more shaded regions (Pearl and Scott, 1986). Vitaliano and Urbach (1980) compared different variables such as amount of life time exposure, ability to tan, and age with BCC, and showed that even with the safest host combination, a high level of exposure can yield a relative risk of BCC that is greater than a higher-risk host combination with a low level of exposure. In addition, the significant relationship between age and BCC may appear to be an artifact of the relationship between age and exposure. Although it has been believed that sun exposure is responsible for BCC, there is evidence that BCC is less closely associated with sun exposure than is SCC. For example, Urbach et al. (1972) have demonstrated that approximately one third of all BCC occurs on areas of the skin receiving little or no ultraviolet radiation, and a much lower fraction of BCC than of SCC appears on the areas receiving the most sunlight exposure. This lower effect of sunlight exposure on the development of BCC compared with SCC suggests that a higher exposure level is required for BCC to reach a similar relative risk as SCC (Vitaliano and Urbach, 1980).

1.3 Malignant Melanoma. The relationship between sun exposure and the prevalence of malignant melanoma (MM) is complicated and in some respects obscure. The original evidence for the possible importance of exposure to sunlight as a causal factor for development of MM was the apparent influence of latitude of residence on the incidence and mortality of MM in the United States (Burhank, 1971) and Australia (Herron, 1969). A higher incidence of MM is found as one approaches the equator. An immigrant study of 511 patients and 511 matched control subjects in Western Australia suggested that MM is related to sun exposure (Holman et al., 1984). This is because of the positive association of MM with duration of residence in Australia, mean annual hours of sunlight received at residential location, and history of previous nonmelanotic skin tumors. In addition, immigrants arriving in Australia from less sunny parts of the world before the age of 10 years had the same risk as native-born Australians, whereas the incidence in immigrants arriving after the age of 15 years was one quarter the rate in native-born Australians. However, there are other indications of a more complicated relationship of sunlight to MM. Fitzpatrick and Sober (1985) reported that the most frequent sites for MM tend to appear on areas of the body generally not chronically exposed to sun. Furthermore, the majority of MM do not appear to be related to continual sun exposure but arise as a result of short exposures to high-intensity sunlight. An increased incidence of MM in renal transplant patients who are receiving immunosuppressive therapy suggested that immune surveillance might be involved in the prevention of MM (Gupta et al., 1986). It has also been proposed that dietary factors (Mackie et al., 1987) or unidentified chemical carcinogens (Rampen and Fleuren, 1987) are involved in the development of MM.

2. Experimental Evidence in Animals.

After the epidemiological data, the next best source of information on the relationship between sunlight and skin cancer is experimental animal studies. A number of genetic characteristics of the animals were also found to influence the induction of skin tumors, as is found in human epidemiological studies. These studies used particular rodent strains, including those with different degrees of pigmentation (Rush and Baumann, 1939; Forbes, 1981), different thickness of the stratum corneum (Hueper, 1941), and the presence or absence of hair (Blum et al., 1965). They showed that mice with lighter pigmentation, no hair, and thinner skin are more susceptible to ultraviolet (UV)-induced cancers than their counterparts. This following section is primarily concerned with the dose response and action spectrum of solar radiation, data that are unlikely to be acquired from human studies.

2.1 Dose Response. The earliest animal study demonstrated that skin cancer could be induced by UV radiation on the ears of albino mice and rats (Findlay, 1928). Later, Blum et al. (1965) showed a dose-dependent response to daily whole-body exposure to a sunlamp in hairless mice. As the daily dose increased, the mean latent period decreased, and tumor multiplicity increased. Blum and his associates (Forbes et al., 1981) also performed a large series of experiments quantitating the influence of pattern of dose-delivery in the production of skin tumors in hairless mice. For the six tolerated daily dose levels, all the animals were exposed to the same fluence, five days per week, but the length of daily exposure differed. A linear response in tumor median latent periods (time to 50% incidence) as a function of log dose was found. When another three groups of animals were treated with a weekly dose, either delivered

entirely on one day, or one-third on each of three days, or one-fifth on each of five days, the effectiveness of treatment increased directly with the number of fractions per week. Such dose response and dose-delivery studies imply that increasing the total dose or shortening the interval between exposure accelerated tumor formation.

2.2 Action Spectrum. The action spectrum, i.e., the relationship between the intensity of the response and the inciting wavelength can be determined by lethality, morphological transformation, and cyclobutane pyrimidine dimer (CPD) production. Some pioneer studies, using filters and various light sources, including sunlight, showed that the wavelength 300-320nm is the most effective range for producing tumors in albino mice (Griffin et al., 1958; Freeman, 1975).

Setlow and associates (Setlow et al., 1989) have developed an animal model from crosses and backcrosses of platyfish (*Xiphophorus maculatus*) and swordtails (*Xiphophorus helleri*). Such an animal model is useful for sunlight-induced melanoma because the hybrid offspring and succeeding backcross generations are sensitive to sunlight-induced melanoma. Two strains of these fish were susceptible to invasive melanoma induction by UV-B in the wavelength range 290-304nm. The melanomas induced are similar to those seen in mammalians. Most importantly, exposure of the fish to photoreactivating visible light after UV-B exposure reduces that tumor incidence to background level. This photoreactivation only works on CPD in DNA. It indicates that DNA is the propable target for the melanoma-inducing effect of UV-B and that these wavelengths cause their effect by direct absorption by DNA. A similar conclusion was made in another animal study using the small South American opossum (*Monodelphis domestica*) (Ley et al., 1989).

The spectrum of sunlight at the earth is between 290nm and 330nm (Freeman, 1975). Environmental factors that decrease the ozone layer can increase the intensity of wavelengths shorter than 290nm. According to the action spectra obtained from these animal studies, the decreased ozone layer could contribute to the increased incidence of human skin cancer in the last two decades.

3. Evidence That Sunlight-Induced Mutations Are Involved in Human Skin Cancer.

Since the importance of sunlight in the human skin cancer was recognized by human epidemiological studies and animal experimental studies, substantial progress has also been made to establish a direct relationship at the *molecular* level between UV radiation and skin cancer. As discussed above, the earlier evidence suggests that DNA is the target for UV irradiation and the pyrimidine dimers in DNA can give rise to tumors (Hart et al., 1977). In the past years, attempts have been made to correlate the sunlight-induced mutations with the formation of human skin cancer by molecular and cellular studies on cells from patients with skin cancer.

3.1 Repair Deficient Disease With Skin Cancer. (A) Xeroderma Pigmentosum: Xeroderma pigmentosum (XP) is a rare autosomal recessive disease in which patients develop solar damage, pigmentation abnormalities, and malignancies in the area of skin exposed to sunlight (Robbin et al.,1974). Cells from the majority of XP patients tested have proven to be deficient in nucleotide excision repair of UV-induced damage (Cleaver, 1968; Zelle and Lohman, 1979; Francis and Regan, 1986). There is evidence that nucleotide excision repair is the major cellular event that eliminates

potentially cytotoxic and mutagenic lesions induced by various DNA damaging agents (McCormick and Maher, 1984). The increased sensitivity of XP cells to the killing effect, mutagenic effect (Patton et al., 1984) and transformation effect (Maher et al., 1982) of UV irradiation has been attributed to their defect in the repair of UV-induced damage. The hypothesis that mutations are involved in the process of tumor formation was further supported by observations of activation of various oncogenes in tumor cells isolated from XP patients. Keijzer et al. (1989) found that a melanoma cell line from an XP patient contained a mutated N-*ras* oncogene and the mutation occurred at a dipyrimidine site, i.e., a potential CPD sequence. Similarly, Suarez et al. (1989) reported multiple mutations in tumor cells from an XP patient, including a point mutation in a dyprimidine site of N-*ras* oncogene, while no changes were found in the non-tumor cells from the same XP patient.

(B) Other Diseases: Bloom's syndrome (BS) is an autosomal recessive disease. Individuals with BS have a high incidence of neoplasia including skin tumors (Bloom, 1966). Cells isolated from these patients exhibit a high incidence of chromosomal aberrations (Chaganti et al., 1974) and spontaneous hypermutability (Vijayalaxmi et al., 1983). An increased sensitivity to the killing effect of UV-irradiation was also found in these cells (Gianelli et al., 1977). Sirover and associates (Vollberg et al., 1987; Seal et al., 1988) observed an abnormal activity of uracil DNA glycosylase in BS cells by using a monoclonal antibody assay. Failure to excise uracil from DNA and repair the loss of the base by excision repair will certainly give rise to mutations and, perhaps, to chromosomal aberrations that may contribute to the development of cancer in this disease. Evidence that mutations are involved in sunlight induced skin

cancer was also found in a study measuring the rate of repair in peripheral leukocytes from seven patients with nevoid basal cell carcinoma syndrome. The patients with nevoid basal cell carcinoma syndrome had about 25% lower level of maximal DNA repair synthesis as compared to healthy individual (Ringborg et al., 1981). The nevoid basal cell carcinoma syndrome is an autosomal dominant disease with multiple BCC and the tumors arise sun exposed skin area (Howell and Anderson, 1976). A decreased UV-induced DNA repair synthesis may contribute to their malignancies when exposed to UV light.

3.2 Other In Vitro Evidences. A number of *in vitro* studies have also indicated that there is causal relationship between UV-induced mutations and the development of human skin cancer. van't Veer et al., (1989) reported that 7 of 37 cutaneous melanomas from sun-exposed body sites contained mutations in N-*ras* oncogene and these mutations were all at or near diprimidine sites. Further investigation also identified a specific mutation in the H-*ras* oncogene in 30% of human keratoacanthoma, a benign skin cancer, and 13% of SCC (Corominas et al., 1989). The fact that most of the *ras* gene mutations in human skin cancers occurred at pyrimidine-rich sequences implies that unrepaired sunlight induced damage will eventually led to mutation and transformation. To determine whether UV-irradiation can also activate the *ras* proto-oncogene *in vitro*, van der Lubbe et al., (1988) irradiated naked N-*ras* proto-oncogene DNA and transfected it into rat cells. UV-irradiated N-*ras* proto-oncogene DNA, but not unirradiated DNA, resulted in the transformation of rat cells. Interestingly, most of these transformants contained mutations at dyprimidine sites. These mutations were similar to those found in skin tumors produced *in vivo*. In addition, when the UV-irradiated DNA was

treated with photoreactivating enzyme prior to transfection, the transformation frequency was reduced. This *in vitro* evidence strongly supports the hypothesis sunlight-induced mutations are involved in human skin cancer.

Mutations induced by sunlight exposure not only can activate specific oncogenes, which positively regulate cell growth, but also inactivate specific tumor suppressor genes, which act as negative growth regulators. Brash et al., (1991) have reported that 58% of SCC among 24 patients examined contained CC-->TT or C-->T substitutions in the p53 gene and these substitutions were found mainly in diprimidine sites. These investigators recently found similar p53 mutations in 27 BCC examined (Ziegler et al., 1993). When they combined the data obtained from BCC and SCC, they found several mutational hot-spots in skin cancer. In addition, measuring the frequency of UV photoproduct formation at hot-spots of either internal cancer or skin cancer revealed that no UV photoproduct was present at bases involved in hot-spots of internal cancer, whereas UV photoproducts formed at base involved hot-spots of skin cancer. Taken together these results suggest that UV-induced mutations in p53 gene play a role in sunlight-induced skin cancer.

4. Evidence That Mutations Are Not Involved In Human Skin Cancer.

4.1 Repair Deficient Disease Without Cancer. Cockayne Syndrome (CS) is an autosomal recessive disease. Cells isolated from these patients show an increased sensitivity to the killing effect of UV and high level of sister chromatid exchanges after UV irradiation (Bohr et al., 1989). In addition, CS cells are not only hypermutable to UV irradiation (Lehmann, 1982) but also defective in nucleotide excision repair (Klocker et al.,

1985) specifically of damage in transcriptionally active genes (Mullenders et al., 1988; Venema et al., 1991). However, CS patients do not have sunlight-induced skin cancers. Instead, they have completely different symptoms, e.g., neurological degeneration.

Another human disease with defective repair without developing cancer is Trichothiodystrophy (TTD) (Lehmann, 1987). TTD patients can be classified into three groups according to their ability of repair after UV (Lehmann et al., 1988). Type 1 cells have an almost normal repair ability; type 2 cells are deficient in excision repair with properties undistinguished from those of XP complementation group D which are defective in repair of both CPD and 6-4 photoproducts (6-4's); type 3 cells can repair CPD normally but deficient in repair of 6-4's (Broughton et al., 1990). In the case of CS and TTD, defective DNA repair is associated with hypermutability of UV, but this does not result in the skin cancer seen in XP. This implies that cellular event(s) other than mutations may also play a role in cancer formation.

4.2 Immune Control May Be Involved. It is well known that malignant transformation of normal cells is a multistep process. It would be an over-simplification to consider that sunlight-induced mutations play the *only* role in human skin tumor. Because the involvement of UV radiation in the etiology and pathogenesis of cutaneous MM has been a matter of controversy for some time and because of the existence of persons with DNA repair deficient diseases, but without cancer, along with the observation that patients receiving immunosuppressive therapy have an increased risk of developing skin tumors (Gupta et al., 1986), the hypothesis that immune surveillance may also be involved in the formation of skin cancer was postulated. This section presents evidence from immune studies in XP and

other animal systems that supports this hypothesis .

(A) Reflections on XP: Earlier, Dupuy and Lafforet (1974) had found an abnormal cell-mediated immunity in some XP patients and hypothesized that this might contribute to their development of skin cancer. Bridges (1981) proposed that in addition to an increased frequency of mutations after UV irradiation, an impaired immune system may be necessary for the development of skin cancer in XP patients. A number of studies later demonstrated various defects of immunity in XP patients. For example, Wysenbeek et al. (1986) performed different immune tests in 9 XP patients and found a significant decrease in the T4 positive lymphocyte subpopulation and a delay of skin antitumor responses. Norris et al. (1988) examined both adaptive and natural immune responses in five XP patients. They found the natural-killer-cell activity in XP patients is depressed. Although direct evidence has not been provided, these studies suggest that in addition to the defective nucleotide excision repair, mutation in the locus controlling the immune system is also required for clinical symptoms of XP.

(B) Evidence From Animal Studies: The ability of UV radiation to alter immune response came originally from studies on the immunobiology of skin cancers induced by UV radiation in inbred mice (Fisher and Kripke, 1977). During the transplantation of UV-irradiated skin samples into synergistic recipients, the samples could only be propagated in immuno-deficient recipients. Such study was further examined and led to a conclusion that UV irradiation produced a systemic alteration that somehow interfered with normal immunological response (DeWitt, 1981). Furthermore, suppressor T lymphocytes were shown to be an important factor in the development of primary skin cancers. This was demonstrated by experiments showing that

when normal mice were injected with T lymphocytes from UV-irradiated or non-irradiated donors and then exposed to UV radiation to induced primary skin cancer, tumors developed much earlier in mice injected with T lymphocytes from UV-irradiated donors than in the mice that received non-irradiated lymphocytes (Fisher and Kripke, 1982). Other evidence that supports the involvement of the immune system in the formation of skin cancer came from the observation that higher dose of UV radiation can cause tumors at unexposed sites and this is accompanied by the development of abnormal suppressor T lymphocytes (Noonan et al., 1981).

B. Role of Nucleotide Excision Repair in Protecting Human Cells from Mutations, Especially UV-Induced Mutations.

1. Nature of Nucleotide Excision Repair.

Nucleotide excision repair was first found in the mid-1960's in *Escherichia coli (E.coli)* (Setlow and Carrier, 1964). It is the most versatile repair process in terms of the variety of DNA lesions it can handle. The pathway involves sequential recognition of the damage, incision of the damaged DNA strand at or near the lesion, excision of a stretch of nucleotides surrounding the lesion, repair synthesis in the resultant gap using the undamaged complementary strand as a template and, finally, ligation of the repair patch to the contiguous parental DNA strand (Hanawalt and Sarasin, 1986; Sancar and Sancar, 1988). In the ensuing years, these processes have been studied in detail. This section presents information in nucleotide excision repair in prokaryotes and eukaryotes as well as progress in cloning of genes involved in nucleotide excision repair in mammalian cells.

1.1 Model in Prokaryotes. In the past ten years, there has been significant progress in understanding the proteins that mediate individual steps involved in nucleotide excision repair. Three proteins encoded by the *uvrA*, *uvrB*, and *uvrC* genes in *E.coli* act in sequential steps to first recognize and bind to the damaged site and then hydrolyze two phosphodiester bonds, one 7 nucleotides 5' and the other 3 or 4 nucleotides 3' of the modified nucleotide (Sancar and Rupp, 1983; van Houten, 1990). The resultant gap of 12-13 nucleotides is filled in by DNA polymerase I with the help of DNA helicase I and sealed by DNA ligase (Caron et al., 1985; Husain et al., 1985). The UvrA, UvrB, and UvrC

proteins, when considered as subunits working in cooperation, are further named "UvrABC excinuclease".

UvrABC excinuclease has been shown to remove UV-induced CPD and 6-4's, as well as nucleotide adducts formed by light-activated psoralen, and by metabolites of 4-nitroquinoline oxide, cisplatin, mitomycin C, benzo[a]pyrene, and many other multi-ringed chemicals (Sancar and Sancar, 1988; van Houten, 1990). This broad substrate specificity indicates that alterations in the local conformation of the DNA resulting from the damage are recognized by the excinuclease, not the individual adduct or photoproduct.

UvrABC proteins have been purified individually and characterized in some detail (van Houten, 1990). UvrA is an ATPase and a DNA-binding protein with two DNA recognition "zinc fingers" (Doolittle et al., 1886). UvrA has higher affinity to damaged DNA than nondamged DNA and this discrimination is further enhanced by the presence of ATP. Thus, UvrA has been considered the damage recognition subunit of the excinuclease (Doolittle et al., 1986). UvrB does not hydrolyze ATP, nor does it bind to DNA by itself. However, when UvrA, UvrB, and ATP are mixed with damaged DNA, this leads to formation of a DNA-protein complex. Studies reveal that under physiological conditions, the formation of UvrB-DNA complexes is favored over UvrAB-DNA complex (Orren and Sancar, 1989, Visse et al., 1991). The stoichiometry of the UvrA and UvrB subunits in the preincision complex is: UvrA dimerizes in an ATP dependent manner, and associates with UvrB to form a $(UvrA)_2(UvrB)_1$ complex in the absence of DNA. This UvrAB complex delivers UvrB onto damaged DNA under the guidance of the UvrA dimer. The UvrA dimer then dissociates from the complex and leaves an UvrB-DNA complex (Orren and Sancar, 1989). Further evidence

favoring UvrB-DNA complex formation is that the DNase I footprint of complex formed in the presence of both UvrA and UvrB is smaller than that observed with UvrA alone, i.e., 19 base pairs (bp) compared to 33 bp (van Houten et al., 1987). In addition, high UvrA concentrations will result in nonspecific binding and an extra incision at the 15th phosphodiester bond 5' to the damaged site in the presence of UvrC. Fortunately, an uninduced *E. coli* cell only contains ~20 UvrA and 200 UvrB molecules (Sancar and Sancar, 1988). UvrC has no ATPase activity and no specific affinity for damaged DNA. However, addition of UvrC to a mixture containing UvrA, UvrB, ATP, and a damaged DNA substrate results in the dual incisions of DNA mentioned above. UvrC is absolutely required for this dual incision event since incisions do not occur in $UvrC^-$ mutants (Tang and Ross, 1985). UvrC may function to either activate nuclease center in the UvrB protein, or UvrC may contain a nuclease center that is only activated when bound to the UvrB-DNA complex (van Houten, 1990).

1.2 Model in Mammalian Cells. (A) Human: The use of cells isolated from patients with deficiencies in nucleotide excision repair advanced the understanding of the nature of nucleotide excision repair in mammalian cells. Cells from the classic XP patients tested have been proven deficient in the incision step of nucleotide excision process (Zell and Lohman, 1979). Genetic analysis based upon fusions between XP cells isolated from different patients has shown the existence of at least seven complementation groups, designated A to G (Cleaver and Kraemer, 1989), clearly demonstrating the complexity of nucleotide excision repair. Each complementation group has unique characteristics, including the degree of repair-deficiency (Zelle and Lohman, 1979; Mitchell and Nairn, 1989), and response to the cytotoxic and mutagenic effects of UV (Patton et al.,

1984). There is evidence of some heterogeneity within certain complementation groups (Fujiwara et al., 1987). In general, XP complementation group A cells have very little ability to remove CPD and 6-4's. Most XP group A cells are highly sensitive to the cytotoxic and mutagenic effect of UV. However, some XP group A cell lines, e.g., XP8LO, show higher activity of repair synthesis than others (DeWeerd-Kastelein et al., 1976). Cells from group C patients repair transcribed strand of actively transcribed genes normally while most of the rest of the genome is poorly repaired (see Section B2.). XP group D cells are reported not to repair CPD and to repair only 40% of 6-4's, compared to normal cells (Paterson, M., Cross Cancer Center, Edmonton, personal communication). Group E has a higher survival than other XP groups. It repairs CPD at the normal rate, but repairs 6-4's at 75% of the rate seen in normal cells in the first 6 hr. (Mitchell and Nairn, 1989).

Such intergroup and intragroup heterogeneity clearly implicates an involvement of multiple gene loci in the excision repair pathway. It has been reported that the XP groups A, B, and D carry mutations at different loci (see next section). It is possible that the products of different loci may act as a complex to achieve one single step of excision repair (Hanawalt and Sarasin, 1986). Alternatively, different mutations in the same locus may affect various domains of the same protein (Fujiwara et al., 1987).

(B) Rodent: Twelve complementation groups have been defined for UV-sensitive rodent cells from a large population of mutagenized cells in culture (Thompson et al., 1981, Busch et al., 1989). They differ quantitatively in their degree of deficiency. For five or these groups,

the human gene capable of correcting the deficiency has been cloned (ERCC-1,2,3,5, and 6). Intragroup heterogeneity has also been found in the rodent mutants. For example, V-H1 and UV5 belong to the same complementation ERCC-2 group. They differ in the efficiency of 6-4's repair. V-H1 cells exhibit intermediate 6-4's repair; UV5 cells are completely deficient in 6-4's repair (Mitchell et al., 1989).

The different groups of rodent mutants with various deficiencies in repair of CPD and/or 6-4's may provide insights to the relationship between excision repair pathways and different photoproducts. Rodent mutant UV61 cells were reported to have an intermediate level of UV sensitivity, measured by colony-forming ability and mutant frequency in the *HPRT* gene. Thompson and associates (1989) found that UV61 cells repair 6-4's at the normal rate. They concluded that repair of 6-4's makes a substantial contribution of increased survival and decreased mutant frequency observed in UV61 cells. However, this mutant was later found to be capable of moderate selective repair of CPD in an active transcribed gene (Lommel and Hanawalt, 1991). The partial UV resistance of this mutant was, therefore, assigned to the removal of CPD from transcriptionally active DNA. Nevertheless, 6-4's still play an important role in achieving the biological effect. This is supported by the study in a partial revertant, RH1-26, of the UV-sensitive mutant V-H1 (Zdzienicka et al., 1992). Both V-H1 and RH1-26 cells do not repair CPD as measured in the genome overall as well as in an active gene. The rate of repair of 6-4's from the genome overall was slower in V-H1 cells than in repair-proficient cells, but was restored to normal in RH1-26 cells. RH1-26 cells, despite the absence of CPD repair, have an almost normal mutagenic response compared with repair-proficient cells. These

investigators concluded that 6-4's are the main UV-induced mutagenic lesions. The relative contribution of CPD and 6-4's to UV-induced survival and mutagenicity remain controversial.

1.3 Genes Involved. Cell fusion and DNA-mediated gene transfer have demonstrated that five of the 12 rodent complementation groups can be corrected by genes from normal human cells. These cloned human DNA fragments have been designated excision repair cross-complementing rodent repair deficiency (ERCC) genes, i.e., ERCC-1, ERCC-2, ERCC-3, ERCC-5, and ERCC-6.

The first human excision repair gene, designated ERCC-1, was cloned by its ability to correct the excision repair defect in CHO mutant 43-3B which belongs to complementation group 2 (Van Duin et al, 1986). ERCC-1 was found to be located on human chromosome 19 and to be homologous with yeast repair gene RAD10. There is an α helix-turn-helix motif. This suggests that the role of ERCC-1 and RAD10 in the removal of DNA damage is mediated through a DNA-protein interaction. At the present time, ERCC-1 does not appear to correct the excision defect in any of the XP cells.

The protein encoded by the ERCC-2 gene, cloned by Weber et al. (1988) possesses a high level of homology with the yeast RAD3 protein which encodes a 5'->3' DNA helicase (Weber et al., 1990). In April 1991, Weber et al. reported in a conference abstract that ERCC-2 corrects the excision repair defect in XP group D cells and in the cells miscategorized as being in XP group H (Verneulen et al., 1992). This finding were later confirmed by Flejter et al. (1992).

The finding that the ERCC-3 gene specifically corrects the excision defect in XP group B cells certainly makes an exciting study of human excision repair (Weeda, et al., 1990). The XP group B patient's DNA

contains a C --> A mutation in the splice acceptor sequence of the last intron of the only ERCC-3 allele that is detectably expressed. Like the other ERCC genes discussed above, the predicted ERCC-3 protein harbors helix-turn-helix domains and a conserved region with DNA helicase super families. Recently, amino acid sequence analysis of ERCC-3 protein indicated that ERCC-3 protein corresponded to the human BTF2 basic transcription factor (also called TFIIH) (Schaeffer et al., 1993). It suggests that transcription and nucleotide excision repair may share common factors and hence may be considered to be functionally related.

The ERCC-5 gene, which corrects the excision repair deficiency of Chinese hamster ovary cell line of complementation group 5, was constructed by homologous intercosmid recombinants by Mudgett and MacInnes (1990). It was found to be located on human chromosome 13.

The largest repair gene cloned by using the genomic transfection approach is ERCC-6 gene (Troelstra et al., 1990). It is about 100 kb in size. The fact that the mutant that this gene corrected is specifically defective in the repair of CPD, but normal repair of 6-4 (Thompson et al., 1989), implies that the gene may be involved in the repair of CPD, but not in the repair of 6-4's, or alternatively, the mutation in the ERCC-6 gene alters the affinity of the repair complex for CPD and 6-4's. Troelstra et al., (1992) reported that introduction of a functional ERCC6 cDNA molecule into fibroblasts derived from a CS group B patient can correct their defect in preferential repair of lesions from the transcribed strand of active genes. In addition, sequence analysis of the ERCC6 gene in cells derived from a CS group B patient revealed deleterious mutations in both alleles. It provides evidence for a specific role for ERCC6 in preferential repair of active gene.

Mouse excision repair genes that are able to correct the defect of XP

cells in complementation group A (Tanaka et al., 1989) or group D cells (Arrand et al., 1989) have also been found. These two mouse repair genes have been designated XPAC (XP-A complementing gene) and XPDC, respectively. Later, Tanaka et al. (1990) isolated a human homolog of the XPAC gene. This cDNA encodes a hydrophilic protein which contains a distinct zinc finger motif indicating that it interacts directly with DNA. It was also found to be homologous to a yeast repair gene RAD14 (Bankmann, 1992). However, for the XPDC gene, sequence comparison has revealed no obvious similarities to two human excision repair genes (ERCC-1 and ERCC-2).

Another possible human excision repair gene was identified by Chu and Chang (1988) in a gel electrophoresis binding assay with a UV-irradiated DNA probe. This factor (XPE binding factor) was missing in cells isolated from one of XP group E patients (GM2415). It was found later by the same group (Patterson and Chu, 1989) that it shares many of the similarities with yeast photolyase, including DNA binding activity and structural homology. These investigators proposed that photolyase may target DNA lesions for other excision repair proteins.

Lastly, a gene able to correct XP cells from complementation group C has also been isolated using a different strategy (Peterson and Legerski, 1991).

2. Evidence of Heterogeneity of Nucleotide Excision Repair in Mammalian Cells

In 1974 Wilkins and Hart suggested heterogeneity in DNA repair as an explanation for the finding that only about one half of the CPD in the DNA of human fibroblasts were accessible to an exogenously supplied

endonuclease from *Micrococcus luteus*. Subsequently, an interesting discovery with respect to this heterogenous repair was the finding by Mayne and Lehmann that RNA synthesis blocked after UV irradiation is resumed before the total removal of UV-induced CPD has occurred (Mayne and Lehmann, 1982). Several years later work from Hanawalt's laboratory using the T4 endonuclease V to cleave DNA specifically at CPD sites, followed by quantitative Southern hybridization using probes for a specific sequence of interest, was used to determined the number of damaged sites in specific restriction fragments and the rate of repair (Bohr et al., 1985). More recently, this methodology has been modified by use of the ABC excinuclease to measure damage induced by agents other than UV (Thomas et al., 1988; 1989). Repair analysis at the level of the gene has since added new perceptions with respect to DNA repair processes in relation to chromatin structure. The term, "preferential repair", was introduced to describe the phenomenon that actively transcribed genes are repaired faster than non-coding sequences in the genome. By using strand-specific RNA probes, the preferential repair in the active transcribed gene is selective for the transcribed DNA strand (Mellon et al., 1987). This strand selectivity of DNA repair is further named "strand-specific repair". This section presents evidence of heterogeneity of excision repair from the view of the gene-specific level or the strand-specific level.

2.1 At the Gene-Specific Level (A) Rodent: Despite the dramatically slow CPD repair in the genome overall found in rodent cells compared to human cells, rodent and human cells exhibit similar levels of survival after UV irradiation (Ganesan et al., 1983). This implies some special pathways may be present in rodent cells to account for the higher than expected

survival. In 1985, the first demonstration of preferential repair of specific active genes was found in the dihydrofolate reductase (dhfr) gene of Chinese hamster ovary (CHO) cells by Hanawalt, Bohr and associates. They found more than two-thirds of the CPD had been removed from a fragment of the dhfr gene by 24 hr after irradiation (20 J/m^2) while little removal was detected in fragments upstream of the gene, and only 15% of the CPD had been removed from the genome overall (Bohr et al., 1985). This active repair of a transcribed gene was soon thereafter demonstrated for the active c-abl proto-oncogene in mouse cells (Madhani et al., 1986). Subsequently, preferential repair was studied in more detail. A study compared the rate of repair and the pattern of methylation between the 5' end and 3' end of the dhfr gene (Bohr et al., 1986a). A region of preferential repair centered around the 5' end of the gene, and a decrease in repair efficiency was found in the 3' region. Interestingly, only one major region of hypomethylation was found in the 5' end of the dhfr gene, located in the promoter region. This implies that hypomethylation plays a role in the accessibility of chromatin to repair enzymes. Ho et al. (1989) later found that *in vitro* demethylation of this 3' end of the gene significantly increased its rate of repair. It is likely that more open structure is more readily accessible to repair.

(B) Human: To determine if preferential repair is a general characteristic of the mammalian repair process or is peculiar to rodent cells, Hanawlat and associates (Mellon et al., 1986) examined the rate of CPD repair in a fragment of the DHFR gene and compared it to a nontranscribed repetitive alpha DNA sequence of normal human cells. They found that within 4 hr after irradiation (10 J/m^2), more than 60% of the CPD had been removed from DHFR sequence, whereas only 25% were removed

from the alpha repetitive sequence. These results strongly suggested that selective CPD removal from active genes is a general characteristic of mammalian DNA repair.

There have also been studies on preferential repair after UV damage in cells from individuals with repair-deficient diseases. For example, cells isolated from patients with Cockayne's syndrome, which are UV sensitive but exhibit a normal repair of the overall genome, appear to lack preferential repair of active genes (Mullenders et al., 1988; Venema et al., 1990). This illustrates that deficiencies in preferential repair may be implicated in human hereditary disease. Such an idea was strengthened by a recent finding that selective repair of the transcribed strand of DHFR gene in an *simian virus* 40 (SV40)-immortalized UV-resistant revertant of XP group A cell line (Lommel and Hanawalt, 1993). This preferential repair of CPD found in the revertant may contribute to its increased UV resistance compared to the parental XP group A cells which have virtually no repair. The studies in other XP cells, i.e., XP group C cells also support the hypothesis that preferential removal of CPD from the active genes is essential for UV resistance (Bohr et al., 1986b; Kantor et al., 1990; Venema et al., 1991). XP group C cells exhibit a relatively high survival despite a low repair capacity of 15 to 20% compared with normal cells (Cleaver and Kraemer, 1989). Studies in the rate of repair at the gene-specific level showed that XP group C cells repair damage induced by UV in the DHFR, β-actin, and adenosine deaminase (ADA) genes much faster than that in the genome overall.

2.2 At the Strand-Specific Level. (A) Cyclobutane Pyrimidine Dimers: Soon after the discovery of preferential repair of CPD in active genes, selective removal of CPD from the transcribed strand of active genes was

discovered. It was first demonstrated in DHFR genes of both human and rodent cells (Mellon et al., 1987). Strand-specific repair of CPD was subsequently found in the active human metallothionein genes (Leadon and Lawrence, 1991) and human ADA genes (Venema et al., 1991). It suggests a general importance of strand-specific repair in mammalian cells. The possible role of transcription in directing repair of CPD in the transcribed strand was further examined in detail. Leadon and Lawrence (1991) showed that inducing higher levels of transcription with a transcription activator selectively increased the rate of repair *only* on the transcribed strand of the induced gene. Treatment of cells with an inhibitor of RNA polymerase II eliminated the enhanced rate of repair on the transcribed strand without affecting the repair on the nontranscribed strand or the genome overall. Similar results were obtained by Christians and Hanawalt (1992). They proposed a hypothesis that repair on the transcribed strand of a gene is independent of repair on the nontranscribed strand and may directly involve the transcriptional apparatus. However, questions still remain about whether the transcription complex *itself* sends a signal at the stalled site to the repair machinery or whether there is a *factor* that coordinates the repair process with transcription. The latter hypothesis was supported by the isolation of a transcription-repair coupling factor (TRCF) from wild-type *E. coli* (Selby and Sancar, 1991). This TRCF can restore preferential repair in an *E. coli* mutation frequency decline mutant that is otherwise incapable of such repair, i.e., is a bacterial homologue of CS (Selby et al., 1991). Recently, the TRCF has been cloned and sequenced by Selby and Sancar (1993). It is a protein with a helicase motif and regions of sequence similarity to UvrB and RecG proteins. It stimulate the repair of

the transcribed strand only when transcription is taking place. When a lesion in the template blocks RNA polymerase, the stalled RNA polymerase can be recognized by TRCF and TRCF may recruits the $UvrA_2B_1$ complex by its affinity for UvrA and aids in the formation of UvrB-DNA complex. The TRCF and UvrA dissociate from DNA simultaneously. UvrC then binds to the UvrB-DNA complex and the dual incisions are made.

(B) 6-4 Photoproducts: Preferential repair of 6-4's was first demonstrated in the dhfr gene of CHO cells (Thomas et al., 1989). It was further supported by another study using the photochemical method in the hamster dhfr gene (Link et al., 1992). The latter study also examined the rate of repair in either strand of the dhfr gene. They found the strand bias is much *less* distinct. There are several possibilities that might explain the lack of strand-specific repair of 6-4's. First, the result of preferential repair obtained is only a reflection of preferential induction of 6-4's in a more open structure, such as an actively transcribed gene in chromatin. Mitchell and associates (1990) have shown that 6-4's are induced in significantly greater frequency in linker regions than in the core regions. The more open structure may render 6-4's more accessible to the repair enzymes, resulting in the observation of rapid repair in the active gene. Second, CPD and 6-4's may be involved in different repair pathways. These two photoproducts have been shown to differ in the degree of helix distortion they produce (Mitchell and Nairn, 1989), as well as in their cytotoxic and mutagenic effects (Thompson et al., 1989; Mitchell et al., 1989; Zdziencka et al., 1992). In addition, there are cell lines from human and rodent that are only defective in repair of either CPD or 6-4's. It is likely that CPD and 6-4's exhibit different effects on transcription and repair. Finally, the assays used

to detect the strand-specific repair of 6-4's have not been sensitive enough to detect the strand difference. The rates of repair of 6-4's in the dhfr gene measured by photoreactivation in conjuction with UvrABC treatment or by a photochemical method in these two studies are both slower than that of the genome overall measured by radioimmunoassays, i.e., 45% in the dhfr gene compared to 80% in genome overall in 4 hr after irradiation. Further detailed analyses are needed to understand these processes more clearly.

2.3 Role of Heterogeneous Repair in Mutagenesis. Obviously, preferential repair of active genes and strand-specific repair of the transcribed strand should have important implications for the process of induced mutagenesis. One of the consequences of such processes will be the accumulation of DNA lesions in non-active regions of the chromosome and in the nontranscribed strand of active genes. Mutagenic bypass of these lesions should, therefore, lead to accumulation of mutations in non-active regions of the DNA and a bias for mutations induced by lesions in the nontranscribed strand. It has been observed that after a period of time for repair after UV irradiation, there are more mutations which could be attributed to UV photoproducts in the nontranscribed strand than in the transcribed strand. McGregor and associates (1991a) found that in synchronized populations of normal cells which are allowed little or no time for repair before nucleotide excision repair, 29% of the photoproducts that result in the observed base substitutions were located in the nontranscribed strand of the Hypoxanthine (guanine) phosphoribosyl trasnferase (HPRT) gene . In populations allowed at least 6 hr of repair before nucleotide excision repair, 80% of the substitutions could be attributed to photoproducts located in the nontranscribed strand. This

switch in the strand distribution could be attributed to strand-specific repair during this 6 hr repair period because there was no such switch in the repair deficient XP group A cells. Similarly, Vrieling et al. (1989) working with asynchronized growing repair proficient CHO cells, found 11 out of 17 base substitutions were caused by photoproducts in the nontranscribed strand of the hprt gene. This bias towards the nontranscribed strand was even more dramatic when they irradiated the CHO cells with a lower dose, i.e., 85% of the mutations occurred at dipyrimidines located in the nontranscribed strand (Vrieling et al., 1991). They also found a dramatic bias in strand distribution of photoproducts in different wild-type CHO cells at a low dose (Menichini et al., 1991). These investigators gave several explanations for the differences observed in the strand distribution between treatments of high and low doses. The most favorable one is that at high doses, cells cycle progression is considerably delayed, more time for repair is available, and 6-4's could be removed from both strands in significant amounts. If 6-4's contribute to most of the mutagenicity, this will result in a much less pronounced strand bias for mutation induction at high doses.

Accumulation of mutations could cause ultimately the activation of a gene, e.g., oncogene activation. A recent experiment used B-cells from spleen in mice that are either resistant or susceptible to plasmacytomas and studied the repair efficiency of c-*myc* locus after UV irradiation. They found a deficient repair in the 5'c-*myc* region in the sensitive mouse strain compared to the resistant strain before the development of tumors (Beecham et al., 1991). Since rearrangements occur in this region 5' to c-*myc*, some relationship may exist between the local genomic DNA repair phenotype and c-*myc* activation.

3. Evidence that Nucleotide Excision Repair in Mammalian Cells is Error-Free.

There is evidence that in mammalian cells nucleotide excision repair can eliminate potentially cytotoxic and mutagenic lesions induced by various carcinogens and UV irradiation in an error-free manner. Maher and associates have performed a series of experiments to examine this hypothesis. For example, when cells with normal rates of excision repair were compared with XP cells with various capacities of excision repair for the mutagenic effect of UV radiation (Maher et al., 1979; 1982) and various carcinogens (Yang et al., 1980; Maher et al., 1977; 1988), the mutagenic effect in each strain correlated its rate of excision repair. For example, the repair-deficient XP cells are significantly more sensitive than normal cells to the mutagenic action of UV and various carcinogens. When normal cells were irradiated or treated at lower doses, there was no significant increase in mutant frequency above background. This dose response "threshold" in normal cells may reflect the result of rapid excision of DNA damage by the excision repair coupled with a brief UV-damage-induced delay in DNA replication.

The conclusion that the excision repair eliminates potentially mutagenic lesions induced by various DNA damaged agents and the process itself is virtually "error-free" is further supported by the investigations of the mutant frequency in cells treated with carcinogen at different times prior to S-phase to allow various lengths for excision repair to take place before DNA replication (Maher et al., 1977; 1979). In the repair-proficient cells, the mutant frequency decreases as a function of time for repair, until it reaches background levels. Given low doses of UV radiation, repair-deficient cells with intermediate levels

of excision repair also exhibited a gradual decrease in the mutant frequency in which the rate of recovery was similar to the rate of normal cells given a much higher dose. In contrast, the repair-deficient cells, XP group A cells that have virtually no excision repair, showed no change in the mutant frequency with the time for repair.

Studies using synchronized normal human cells and then irradiating these cells at different times in the cell cycle also supports the conclusion that excision repair is an "error-free" process (Konze-Thomas et al., 1982; Watanabe et al., 1985). If normal cells which are irradiated at the onset of S-phase just before the target gene for mutations is being replicated, the mutant frequency reaches the highest level. In contrast, the mutant frequency in cells treated in G_1-phase 6 to 15 hr before DNA replication was significantly lower than that treated in S-phase. Similar results were also obtained with human cells exposed to bulky chemical agents that form adducts that are removed by excision repair. Cells were exposed to tritiated (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) and measured the radioactivity as the number of DNA adducts either immediately or 15 hr after treatment (Yang et al., 1980). The normal cells exhibited a gradual loss of radioactivity from their DNA and a gradual decrease in the frequency of induced mutations. In contrast, there was no loss of radioactivity labeled DNA adducts from the DNA of excision repair deficient cells and their frequency of induced mutations did not change. These data strongly suggest that excision repair can eliminate the potential mutagenic lesions after UV irradiation and the process itself is "error-free".

C. The Influence of DNA Replication in Mutagenesis.

1. Replication on a Normal Template.

Organisms must replicate and repair their DNA with high accuracy so as to maintain their genetic identity. DNA is subject to damage by chemical and physical agents and also suffers errors during its replication. These lesions and errors may remain in DNA, leading to mutations and cell death. Therefore, an understanding of the fidelity of DNA synthesis is necessary for elucidating the molecular basis of mutagenesis.

Measurements of spontaneous mutation frequencies *in vitro* suggest that the average frequency of base substitutions is in the range of 10^{-5} to 10^{-7} misincorporations per base pair replicated. These frequency is even lower *in vivo* which is estimated to be in the order of 10^{-7} to 10^{-11} (Hübscher, 1983). How can such high accuracy can be achieved? Divergent approaches indicate that highly accurate DNA replication results from a multicomponent process. In this section, each of these processes will be discussed.

Processes Involved to Achieve the High Fidelity. 1.1 Nucleotide-Polynucleotide Interaction. The first step occurs as a base is actually being inserted at the growing point of the DNA chain. In the kinetic model of DNA polymerase accuracy, this discrimination step appears to result from a difference in free energy (ΔG) between correct and incorrect base pairings (Kornberg, 1969; Bessmann et al., 1974; Clayton et al., 1979). The difference in free energy between correct and incorrect Watson-Crick base pairings at equilibrium is not precisely known. However, the ΔG could be estimated by the measurements of nucleotide interactions (Pitha et al., 1968), and the stability of polynucleotide helices (Fink

and Crothers, 1972; Fresco et al., 1980).

In the passive polymerase model, the weak base pairs are rejected before incorporation (Clayton et al., 1979). In the active polymerase model, the difference in free energy between correct and incorrect base pairs is greater in the presence of polymerase than that suggested from the analyses of base pairings in the absence of polymerase. It has been suggested that the allosteric sites on the DNA polymerase that interact with the nucleotides in connection with base pairing also partipate in replication accuracy (Mildvan, 1974).

Other mechanisms have also been proposed to explain enhanced discrimination between correct and incorrect basepairs. For example, the base pairing conformation in the 3' end of a growing strand can only allow the polymerase complex to accommodate the correct nucleotide to be inserted into the 3'OH group of the previous nucleotide (Loeb, 1974; Gillin and Nossal, 1976). This conformational discrimination involves a slower rate of incorporation of an incorrect nucleotide than a correct one.

1.2 Proofreading Process. The second discrimination step during replication occurs at the growing point, removing incorrect nucleotides at the time of or immediately after incorporation. Such a proofreading process has been detected in DNA polymerases from bacteria (El-Deiry et al., 1988), bacteriophage (Marians, 1984), lower eukaryotes, and mammalian cells (Kunkel, 1988). The 3'-->5' exonuclease activity is generally believed to be responsible for this proofreading process (Loeb and kunkel, 1982). Studies on *E.coli* polymerase I show that if a mis-basepair is formed, the 3'-->5' exonuclease can hydrolyze the noncomplementary deoxynucleoside triphosphate to a free deoxynucleotide monophosphate

before the phosphodiester bond can be formed between the noncomplementary nucleotide and 3'OH terminal (El-Deiry et al., 1988). It has been suggested that the polymerase subunit can also lead to an increased affinity of the exonuclease subunit for the 3'OH terminal and result in a stimulation of proofreading activity (Maki and Kornberg, 1987). This is because of the observation that the proofreading ability of *E.coli* polymerase III depends on a synergistic interaction between the polymerase subunit and the exonuclease subunit. When the two subunits were complexed, polymerase activity increased 2-fold, and 3'-->5' exonuclease activity increased 10- to 80-fold.

Some *in vitro* replication experiments with prokaryotic and eukaryotic DNA polymerases demonstrate the existence of proofreading and its importance to fidelity. In these experiments, a biologically active DNA template, which contains a defined single base substitution mutation, is copied *in vitro* and then analyzed *in vivo*. Errors are detected by determining the frequency of reversion of the mutation to wild type (Kunkel et al., 1986; Hauser et al., 1988). When Kunkel and associates (1981) added an inhibitor of the proofreading process, e.g., nucleotide substrate pool imbalances or deoxynucleotide monophosphates into the reversion assay, they found that the accuracy of polymerase I decreases 20-fold and the accuracy of T4 DNA polymerase decreases > 500-fold. No decrease in accuracy of AMV DNA polymerase or DNA polymerase β was observed, consistent with the fact that these enzymes lack associated exonuclease activities. The reversion assay has also been designed with two vectors having the origin of replication on opposite sides of the defined base substitution to study the fidelity of replication of leading and lagging strands (Robert et al., 1991). This study showed that

exonuclease proofreading occurs on both strands and base substitution error rates are similar for both strands.

1.3 Mismatch Repair. There is evidence for a third opportunity to reduce errors. It involves incision by mismatch-specific endonucleases of noncomplementary base pairs that had been formed postsynthetically during DNA replication. In bacteria, evidence indicates that this pathway involves the discrimination between the parental and the newly synthesized strands. This is mediated by the *MutHLS*-dependent, methyl-directed pathway (Lahue and Modrich, 1989). Recognition of the newly synthesized strand is determined by a delay in methylation of specific DNA sequences (Glickman and Radman, 1980). Only the bases in the newly synthesized strand that lacks GATC methylation will be excised and filled by DNA polymerase III holoenzyme (Längle-Rouault et al., 1987).

In eukaryotes, *PMSI* protein in yeast had been identified to be homologous to the *E.coli MutL* protein, but no evidence for strand specificity of the yeast mismatch repair pathway has been found (Kramer et al., 1989). However, other evidence indicates that mismatch repair in eukaryotes is also directed to a particular strand in some way. Modrich and associates (Holmes et al., 1990) constructed an open circular DNA containing a mismatch with a strand specific incision located 808 bp from the mismatch, then assayed the mismatch repair by using nuclear extract derived from Drosophila and HeLa cells. They found that efficient, strand-specific mismatch correction did occur *in vitro* in the strand containing the incision. This implies that a DNA terminus served as a growing DNA chain signal to direct the mismatch repair. In addition, Brown and Jiricny (1988) showed that mismatch repair was also influenced by sequences flanking the mismatch. The correction tended to favor the

retention of the guanine and cytosine involved in a particular mismatch.

2. Replication on a Damaged Template.

Several studies have shown that shortly after UV irradiation, the size of newly synthesized DNA is smaller and it grows longer gradually until it reaches the size of parental DNA several hours after UV irradiation (Kaufmann and Cleaver, 1981; van Zeeland and Filon, 1982). In this section, the inhibition of DNA replication by DNA lesions and possible mechanisms involved in replication of a damaged template will be discussed. If the mutation occurs during DNA replication following carcinogen treatment, the specificity of mutation induced by various carcinogens will also be discussed.

2.1 Inhibition of Replication. (A) Effect on the Rate of Replication: Shortly after treating cells with low doses of UV or BPDE that do not produce measurable loss of cell colony-forming ability, the inhibition of initiation is observed. This is reflected in the production of abnormally small nascent DNA equivalent to the size of an average replicon, i.e., 2 x 10^7 daltons of single-stranded DNA (Kaufmann and Cleaver, 1981; Cordeiro-Stone et al., 1986). Higher doses of UV and BPDE, in addition to their effect on initiation, also inhibit the elongation of daughter strands in active replicons (Kaufmann and Cleaver, 1981; van Zeeland and Filon, 1982; Boyer et al., 1990). This effect is manifested by a dose-dependent reduction in incorporation of radiolabeled precursor into replication intermediates of > 4 x 10^7 daltons, with the concurrent production of abnormally small nascent DNA. However, at late times normal cells are able to synthesize high-molecular-weight DNA even though the parental strand still contains DNA lesions (van Zeeland and Filon, 1982).

Several different mechanisms have been proposed to explain the

formation of small molecular weight nascent DNA after UV irradiation. Lehmann (1972) first proposed that CPD blocks replication, but there may be reinitiation of DNA synthesis beyond the CPD, resulting in a gap opposite the CPD. Such gap conformation indeed is observed by others using electron microscopic analysis of irradiated-SV40 virus molecules (White and Dixon, 1984; Mezzina et al., 1988). Later, the more widely accepted model proposes that lesions located in the DNA template for the "leading" strand block or temporarily halt fork progression, whereas lesions located in the template for the "lagging" strand would not impede fork movement, but rather interfere with the completion of Okazaki fragments, giving rise to a gap (Kaufmann, 1989).

(B) Possible Mechanism Involved in Recovery of Replication after Carcinogen Treatment: Although immediately after carcinogen treatment there is inhibition of replication, the lesion-containing DNA can still be fully replicated eventually under some conditions. This implies that a cellular capacity rapidly develops after carcinogen treatment that enables cells to replicate, despite the presence of damage in DNA. The elements which determine the use of one pathway over the other may depend on the precise structure of the lesion in the template and the sequence context of the lesion (Kaufmann, 1989). However, the mechanisms involved in the recovery of DNA replication after carcinogen treatment is still a matter for speculation. In this section, different models proposed will be discussed.

The first mechanism is bypass synthesis which involves insertion of a chosen base opposite the template lesion so as to circumvent the lesion (Kaufmann, 1989). The base inserted can be randomly chosen or selectively chosen depending on the type of lesions, e.g., non-instructive or mis-

instructive lesions (see next section). This mechanism can be mutagenic leading to base substitutions.

The second mechanism involves *de novo* DNA synthesis to fill the gap in the daughter strand opposite the template lesion (Lehmann, 1972). Because the size of the gap is in agreement with the size between adjacent Okazaki fragments, this mechanism has been mainly proposed for the completion of replication when the lesion is encountered by the lagging strand (Meneghini and Hanawalt, 1976). Such a gap-filling process can possibly introduce mutations because of the presence of unexcised lesions on the template. However, the polymerase involved is unclear.

Another mechanism has been proposed for those lesions encountered by the leading strand. Such lesions will temporarily block the DNA elongation, but elongation will eventually be completed by extension of lagging strand elongation from the adjacent replicon (Painter, 1985). Such a process will allow lesions that had been encountered by the "leading" strand to now be encountered by the "lagging" strand and to be handled by the gap-filling pathway (Fujiwara et al., 1991).

Lastly, a process involving replicational bypass following strand displacement is proposed by Fujiwara and Tatsumi (1976). This process uses the sister strand as an alternative template to circumvent the lesion in the parental strand, and normal DNA synthesis is resumed past the lesion. It appears to be error-free because the sister strand contains no lesion. However, this pathway does not represent the principal one used in damaged human cells (Fornace, 1983).

In addition, the operation of DNA repair during replication will also have an indirect effect on the replication after carcinogen treatment. When repair removes the lesions in the template, this will reduce the

frequency of a replication complex encountering lesions during replication.

2.2 Sequence Specificity of Mutations. (A) Spontaneous mutation: Mutants occurring during normal cellular growth in culture contain a variety of mutations. Among these mutations, base pair substitutions have been well studied in an effort to understand the mechanism used by cells to control the level of base substitution error. Kunkel and Alexander (1986) used a forward mutational assay capable of detecting a wide variety of single base substitutions at many sites in M13mp2 DNA and showed that the mutations induced by DNA Polymerase α, β, and r exhibit distinct differences in template site preferences, as well as substrate insertion preferences of mutations. This implies that the DNA polymerase *itself* apparently plays a major role in determining the frequency of formation of various mispairs. However, in general, mispairs are formed in the following ratios: purine·pyrimidine,100 : purine·purine,10 : pyrimidine·pyrimidine,<1. Because purine·pyrimidine mispairs are the most frequenct form of mispairs, transitions, i.e., a purine substituted by another purine or a pyrimidine substituted by another pyrimidine, are favored over transversions i.e., purines substituted by pyrimidines or *vice versa*. In considering the misinsertion specificity, purines are preferred over pyrimidines for all the enzymes examined. In most cases, the preference is in the following order: adenine(A) > guanine(G) > cytosine(G) > thymine(T). These investigators suggested that this preference for purine insertion results from the stronger stacking interaction between the base at the 3'OH terminus and an incoming purine rather than an incoming pyrimidine. But this does not explain the several-fold preference for A over G. Such general features were also

seen in the spontaneous mutational spectrum of aprt gene of CHO cells (Nalbantogln et al., 1983; Grosovsky et al., 1986). In the spectrum of spontaneous mutations in the coding regions of the HPRT gene of human cells, all types of base substitutions were found, except A•T-->T•A (McGregor et al., 1991).

Several mechanisms have been suggested as the cause of spontaneous base substitutions. They are deamination of 5'-methycytosine or cytosine, formation of apurination/apyrimidine (AP) sites, oxidative damage, and errors introduced by DNA polymerase during replication. Deamination of cytosine to uracil, following by DNA replication before the uracil can be removed by mismatch repair or uracil glycosylase will generate a C-->T transition because of a mispair of adenine with uracil (Lindahl, 1982). Depurination and depyrimidination occur spontaneously at high frequency (Lindle and Nyberg, 1973). This is the clearest case of a non-instructive lesion in DNA. Using a method of transfection of SOS-induced and uninduced *E.coli* with phage containing an AP site lesion, several groups of investigators showed that in SOS-induced *E.coli*, more than 90% of the phage replicated result from the insertion of a nucleotide opposite AP sites, with a strong preference for incorporation of adenosine monophosphate (Sagher and Strauss, 1983; Kunkel, 1984; Lawrence et al., 1990).

Superoxide free radicals are produced by a variety of cellular metabolic processes (Ames, 1983). 8-oxoguanine is a primary product of oxygen-free radical reaction with DNA, and it has been shown that mammalian DNA polymerases selectively insert adenosine nucleotides across from such lesions to result in G•A mispairs (Shibutani, et al., 1991). This might explain the formation of G•C-->T•A transervsions. Mutational

spectra can also be profoundly influenced by the accuracy of DNA replication. For example, a CHO cell line with a 10-fold increase in the pool of deoxycytosine triphosphate has a corresponding increased mutation frequency of aprt gene (Meuth, 1981). The majority of mutations are the result of the misincorporation of the nucleotide in excess.

(B) Mutations Induced By DNA-Damaging Agents: DNA lesions that are induced by DNA-damaging agents and cause mutations have been classified into two major groups: mis-instructive lesions and non-instructive lesions (Boiteux et al., 1978). Mis-instructive lesions can lead to transition mutations. For example, O^6-methylguanine and O^4-ethylthymine with altered hydrogen-binding specificities appear as adenine and cytosine to DNA polymerase, respectively. They can result in G•C --> A•T and A•T --> G•C transitions, respectively. DNA alkylating agents, such as ethyl methanesulfonate (EMS) induce G•C-->A•T transitions because the O^6-alkylguanine residues in DNA produced by this agent appear as adenine to DNA polymerase (Lebkowski et al., 1986; Ashman and Davidson, 1987 . Misinstructive lesions can also lead to transversion mutations. For example, ethyl nitrosourea (ENU) induces A•T-->T•A and A•T-->C•G transversions in 30% of the mutations, which corresponds to a low but significant level of O^2-ethylthymine residues in the DNA of treated cells (Eckert et al., 1988).

In addition to the AP aites, the most widely studied mutagen that induces non-instructive lesions is UV light. It induces non-instructive lesions such as CPD and 6-4's, although TT cyclobutane dimers have been considered as a mis-instructive lesion recently (Lawrence, et al., 1990). In most of the model systems, including the *lacI* gene in *E.coli* (Miller, 1985), the shuttle vector in mammalian cells (Hauser, et al., 1986;

Protic-Sabljic et al., 1986; Bredberg et al., 1986), and endogenous HPRT gene in human cells, including excision repair deficient XP group A cells (McGregor et al., 1991a), the majority of base substitutions induced by UV are G•C-->A•T transitions. However, the pattern of mutations induced at the hamster hprt locus are a mixture of transitions and transversions (Vrieling et al., 1989).

The role for CPD and 6-4's in mutation induction, especially their relative mutagenic effect and in the cause of G•C-->A•T transitions, has been controversial for years. To clear up this contradiction, Lawrence and associates (Banerjee et al., 1988) constructed a single stranded vector that contains a unique *cis-syn* TT CPD. In the absence of SOS induction, there are only a few progeny plaques. This implies that a single dimer blocks replication in at least 99.5% of the molecules. In the SOS-induced *E.coli*, the CPD-containing vectors produced 27% of the plaques of that seen in the CPD-free vectors. Sequence analysis showed that the normal sequence was formed in 93% of them. This implies a preferential insertion of adenine. Among the mutations observed, about 90% of these were targeted at the 3' thymine of the CPD, the first nucleotide encountered by polymerase. The types of mutations were not random: T-->A transversion were more common than T-->C transition, and no T-->G transversions were observed. A similar study was performed for normal and Dewar isomer forms of 6-4's between adjacent thymine dimers (LeClerc, et al., 1991). In the absence of SOS induction, vectors carrying both types of 6-4's were rarely replicated. This suggests 6-4's are strong replication blocking lesions. Interestingly, in the SOS-induced cells, the error frequency of replication past the normal isomer form is more than 90%. Equally striking, 93% of the mutations were T-->C transitions at the 3' thymine of the 6-4's. In the mutations induced by

Dewar isomer form, these values were 53% and 46%, respectively. These investigators suggested that the high error frequency and specificity arise from the formation of a stable T·G mispair. Preferential incorporation of deoxy-adeninosin monophosphate is unlikely to be a significant mechanism with the mutagenic structure of 6-4's in this case. Taking these studies together, 6-4's are more likely to be responsible for most mutations of T-->C at TT sites, wherase most T-->A transversion are likely to be the result of CPD.

The effect of agents which add bulky adducts to the DNA has also been examined. For example, BPDE exhibits a strong mutational specificity with G residues which accounts for 90% of the mutations in the *supF* t-RNA gene replicated in *E.coli* (Mazur and Glickman, 1988), the *supF* gene replicated in human cells (Yang et al., 1987), and in the endogenous *HPRT* gene in human cells (Chen et al., 1990). These data are consistent with the formation of N^2-substituted guanine adducts. However, the types of mutations induced are different, being predominantly G·C-->T·A transversion for both *supF* and *HPRT* gene studies in human cells, but being a mix of single base-pair deletions, transversions, or transitions at G·C base-pairs in the *supF* gene study in *E.coli*. It has been suggested that the majority of BPDE-induced mutations in eukaryotic DNA arise as a result of the N^2-position of guanine, while mutations induced in *E.coli* are a result of the N^7-substituted guanine. This suggestion is supported by the observation that the (-)BPDE isomer, which produces a high proportion of N^7-substituted guanines, is a more effective mutagen in bacteria. In contrast, the (+)BPDE isomer, which produces N^2-substituted guanines, is more mutagenic in mammalian cells (Mazur and Glickman, 1988). This observation raises the possibility that the biologically relevant BPDE-DNA adducts differ between *E.coli* and human cells.

D. Insights into Sunlight-Induced Skin Cancer in XP Variant Patients.

1. Clinical Characteristics of XP variant patients.

Xeroderma pigmentosum (XP) is a rare autosomal recessive disease in which patients develop solar damage, pigmentation abnormalities, and malignancies in the area of skin exposed to sunlight (Robbins et al., 1974). Patients have been found world-wide and in all races (Fujiwara et al., 1987). It occurs in approximately 1 person in 250,000 in the United States and Europe but is considerably higher in Japan (1 in 40,000) and Egypt (Cleaver et al., 1989). The frequency of patients in the variant group is about 25% of all XP patients found world wide (Cleaver et al., 1989). Many but not all XP variant patients have an acute sun sensitivity in early infancy. The tendency for this acute sun sensitivity subsides later in life compared with classic XP patients,i.e., those with defective nucleotide excision repair (Cleaver et al., 1989). Freckles develop on sun-exposed areas early in life and skin on the exposed areas becomes dry and scaly. Later some areas of the skin become atrophic and develop a brown to red rough appearance and an increasing numbers of wart-like skin lesions. Basal and squamous cell carcinomas usually first appear in childhood or adolescence and occur predominantly in those areas receiving the most sun exposure (Robbins et al., 1974). There is also a remarkably increased prevalence of malignant melanomas in these patients (Lynch et al., 1967). Other tumors that occur include basi-squamous cell carcinomas, keratoacanthomas (Stevanovic, 1961), capillary hemangioma, and epitheliomas. Ophthalomalogical symptoms such as dyspigmentation and telangiectases of the eyelids are often observed in such patients (Thielmann et al., 1991). Only a few patients have been described with

neurological abnormalities (Kraemer et al., 1987).

2. Characteristics of XP Variant Cells in Culture.

2.1 Cytotoxicity. (A) UV: Sensitivity to the killing effect of UV_{254nm} radiation has been shown in asynchronously growing fibroblasts from skin biopsies of at least four of XP variant patients including XP4BE (Maher et al., 1975; 1976a; Myhr et al., 1979), XP13BE (Maher et al., 1975; Maher and McCormick, 1980), XP7TA and XP30RO (Maher et al., 1975; Arlett et al., 1975). XP4BE cells have also been shown to sensitive to the killing effect of broad spectrum simulated sunlight (Patton et al., 1984). This cytotoxicity was determined from the decrease in the cells' colony-forming ability after UV irradiation. In general, the slope of survival curve of XP variant cells averaged 1.5-fold steeper than that of normal cells. UV doses corresponding to 37% survival of colony-forming ability were about 3.75 J/m^2 for XP variant cells and 5 J/m^2 for normal cells.

When cells are plated in the various concentrations of caffeine containing medium after UV irradiation for 7-10 days, caffeine causes a dose-dependent increase in the cytotoxicity of UV in XP variant cells (XP4BE and XP13BE) even at non-toxic or only sightly toxic concentrations. This synergistic effect of caffeine on the cytotoxicity of UV irradiation is not seen in normal human cells or in classical XP cells (Maher et al., 1975). Similar results were found with XP7TA and XP30RO cells (Arlett et al., 1975).

A study measuring the cytotoxicity of UV when irradiation was performed at certain points in the cell cycle of synchronized XP4BE cells showed that XP4BE cells irradiated in early G_1-phase gave the same cytotoxicity as cells irradiated just before S-phase (Watanabe et al., 1985). However,

if cells were irradiated in the G_0 state to allow at least 16 hr prior to S-phase, cells exhibited a higher survival than those irradiated in G_1- or S-phase. The increased in survival found in XP4BE cells was quite comparable to normal cells similarly irradiated. No change in survival occurred in excision repair-deficient XP group A cells (Konze-Thomas, et al., 1982). Thus, excision repair before proceeding through the cell cycle decreases the potentially lethal effect of UV in XP4BE and normal cells.

(B) Other Carcinogens: The cytotoxicity following exposure to four aromatic amide carcinogens (Maher and McCormick, 1976a) or K-region epoxide derivatives of four carcinogenic hydrocarbons (Maher et al., 1977) was also determined in XP4BE variant cells along with normal cells and several XP cell lines with various levels of deficiency in the capacity of excision repair. For each carcinogen derivative, the ratio between the slope of the survival curve for the particular XP cells and that for the normal cells was approximately equal to the ratio of the slopes for the cells irradiated with UV. In general, XP variant cells show only a slightly enhanced cytotoxicity to these chemical carcinogens compared with normal cells, but are not as sensitive as classic nucleotide excision repair defective XP cells. A similar enhanced cytotoxicity was found in cells following exposure to ENU (Simon et al., 1981) and 4-nitroquinoline-1-oxide (4NQO) (Dollery et al., 1983). In addition, the synergistic effect of caffeine on the survival of XP variant cells after exposure to hydrocarbon epoxides resembles that found for UV irradiation (Maher et al., 1975).

2.2 Mutagenicity of UV Irradiation. Although XP variant cells show only a slightly enhanced cytotoxicity to UV, they are extremely sensitive to

the mutagenic action of UV_{254nm} (Maher et al., 1976b; Myhr et al., 1979; Patton et al., 1984) and of simulated sunlight (Patton et al., 1984). In fact, they are far more sensitive than most classical XP cells and are almost as sensitive as excision repair deficient XP group A cells (Patton et al., 1984) when they are compared under conditions of maximum expression of the 6-thioguanine (TG) or azaguanine resistance phenotype. Asychronously growing diploid fibroblasts from XP variant patients show a higher frequency of TG resistant colonies than asychronously growing diploid fibroblast from normal donors when compared at equal UV doses or at equally cytotoxic doses. The slope of the curve plotting mutant frequency as a function of dose in an asynchronous population of XP variant cells is at least 5 times steeper than that of normal cells. At a low dose, causing little or no cell killing in either cell type, UV_{254nm} induces HPRT mutants at a frequency of 200×10^{-6} in XP variant cells compared to only 20×10^{-6} in normal cells, and at still lower doses, the frequency is 25- to 100-fold higher in XP variant cells than that in normal cells (Patton et al., 1984). What is more, if caffeine at nontoxic doses, is present in XP variant cells during the 7 to 9 days between UV irradiation and the beginning of mutant selection, it causes a significant synergistic increase in the mutagenicity of UV, similar to that found for the cytotoxicity of UV (Maher et al., 1976b).

Such UV hypermutability found in the XP variant cells may account for development of skin cancer on sunlight-exposed parts of the body of XP variant patients. The possible cause of this UV hypermutability will be discussed in Section 3 and 4. It should be noted that a comparison of the HPRT mutant frequency in synchronized XP variant and normal cells following exposure to BPDE showed that for every dose and time point

similarly treated at a specific phase in the cell cycle, the frequencies induced in the XP variant and normal cells are almost identical (Watanabe, et al., 1985). Thus, the hypermutability of XP variant cells seems to be specific to UV irradiation.

2.3 Transformation to Anchorage Independence. XP variant cells have also been shown to be much more sensitive than normal cells to transformation to anchorage independence by UV_{254nm} radiation (McCormick et al., 1986; Boyer et al., 1991). The anchorage independence is determined from the ability of cells to grow in semi-solid agar containing serum which gives a comparable background frequency between different cell lines tested. The sensitivity of XP variant cells to induction of anchorage independence by UV paralleled their sensitivity to UV-induced mutant frequency. This supports the hypothesis that transformation to anchorage independence results from the mutations induced by UV.

2.4 Reactivation of Damaged Viral Template. Another method to reveal the difference between XP variant and normal cells is host cell reactivation (HCR). This technique assays the ability of UV-irradiated virus to form plaques on monolayers of human fibroblasts (Day, 1974). The ability of UV-damaged viruses to undergo DNA repair and replication in infected cells often parallels the cells' ability to survive UV damage. In general, classical XP cells have a reduced HCR of UV-irradiated virus compared to normal cells (Abrahams and van der Eb, 1976). Although XP variant cells show a normal rate of nucleotide excision repair in other assays (See Section 3), they show a reduced HCR for UV-irradiated SV40 virus (Abrahams and van der Eb, 1976) as well as adenovirus (Day, 1975). XP variant cells were shown to have approximately 60% of the HCR of normal cells. However, there are conflicting results concerning the HCR of UV-irradiated Herpes

simplex virus (HSV) (Coppy and Menezes, 1981; Ryan and Rainbow, 1986).

When the infected host cells are also irradiated, the surviving fraction of UV-irradiated virus is increased (Abrahams et al., 1984; Ryan and Rainbow, 1986). However, the increased levels of the surviving fraction in XP variant cells appear to be markedly dependent on the virus tested and experimental protocol employed. For example, XP variant cells were shown to have normal levels for HSV-1 reactivation (Abrahams et al., 1984), elevated levels for HSV-2 reactivation (Ryan and Rainbow, 1986), and reduced levels for adenovirus reactivation (Jeeves and Rainbow, 1983) compared to normal cells. These differences may imply that virally coded enzymes contribute to the reactivation of damaged viruses in XP variant cells.

When cells are UV-irradiated before infection of unirradiated virus, there is also an increase of virus mutation frequency among the surviving virus in irradiated cells compared to that in unirradiated cells, implying a transient loss of fidelity in DNA replication. Such enhanced mutagenesis (EM) has been seen with many viruses (Abrahams et al., 1984). Abrahams and colleagues (1984) have examined the time course of appearance of enhanced survival and enhanced mutagenesis of unirradiated virus in both normal and XP cells including XP variant cells. They found that in all cell types examined except the XP variant cells, EM followed a similar time course of appearance as did enhanced survival. However, maximal expression of the EM function was delayed by two days with respect to enhanced survival in the XP variant cells. The mechanism involved in the abnormal kinetics of EM found in XP variant cells is still unknown. It may be relative to their defect in DNA replication after UV irradiation (See Section 4).

3. Evidence that Defective Excision Repair Is Not the Mechanism Involved in the XP Variant Cells' Predisposition to Sunlight-Induced Skin Cancer.

3.1 Normal Rate of Nucleotide Excision Repair in Genome Overall. Cells from the classic XP patients tested have proved to be deficient in nucleotide excision repair of UV-induced DNA damage, and it has been suggested that the defective nucleotide excision repair is responsible for the development of skin cancers in these patients (see Section B). However, cells isolated from the variant type of XP patients appear to have a normal or nearly normal rate of nucleotide excision repair although these patients have a high incidence of severe skin cancer. XP variant patients have a normal rate of UV-induced tritiated thymidine (^{3}H-TdR) incorporation into their peripheral blood lymphocytes (Burk et al., 1971), skin fibroblasts (Cleaver, 1972), and epidermal cells (Robbins et al., 1972; Kondo et al., 1987). The level of repair ability of XP variant cells in such assays is in the range of 70 to 100 percent of normal cells. This level is higher than that of classic XP cells. XP variant cells have also been shown to have a normal rate of excision repair of CPD in the genome overall by measuring the rate of loss of CPD-specific endonuclease sensitive sites (Zelle and Lohman, 1979) and of CPD-specific monoclonal antibody sites (Roth et al., 1987). The rate of repair of another major UV photoproduct, i.e., 6-4's, was also shown to be normal in a radioimmunoassay (Mitchell, et al., 1987).

Additional evidence of a normal rate of nucleotide excision repair can be found in several other assays. For example, XP variant cells have a similar rate of repair of closely opposed CPD dimers (Lam and Reynolds, 1986) and a similar sensitivity of aphidicolin, a possible inhibitor of a

repair polymerase, as normal cells (Tyrrell and Amaudruz, 1987). Although the rate of repair at the gene-specific level in the XP variant cells has not been examined yet, a study measuring the rate of recovery of RNA synthesis after UV irradiation in XP variant and normal cells showed that both types of cells have a similar recovery rate (Mayne and Lehmann, 1982). This implies that preferential repair of actively transcribed genes also occurs in XP variant cells.

3.2 Excision Repair in XP Variant Cells Is an Error-Free Process. When replication occurs on a template containing unexcised lesions, mutations can result (Konze-Thomas et al., 1982). Maher and colleagues designed an experiment to determined whether excision repair is an error-prone process and results in mutations in XP variant cells (Watanabe et al., 1985). They irradiated both normal and XP variant cells under conditions that allowed the cells various lengths of time for excision repair before DNA replication and measured the frequency of mutants induced. They found that the relative ratios of the decrease in mutant frequency with time for excision repair before DNA replication in XP variant and normal cells are equal. The mutant frequency in both types of cells decreases as a function of time for repair, until it reaches background levels. This implies that the excision repair is responsible for removing the lesions and the process itself is error-free in XP variant cells as that in normal cells.

3.3 Possible Defects in Repair. Although XP variant cells excise UV photoproducts from the genome overall normally, there are observations that indicate an existence of a possible defect in excision repair. For example, UV_{313nm} radiation at 37°C induced excessive fragmentation of DNA in XP variant skin fibroblasts. No such fragmentation was seen in normal

and XP group A cells (Netrawali and Cerutti, 1979). This suggests that a late step in excision repair of lesions induced by UV_{313nm} such as the ligation of parental DNA fragments may be defective in XP variant cells. Another important observation is that some preparations of whole cell extract isolated from XP variant cells are unable to carry out excision repair on purified UV-irradiated plasmids, whereas some extracts are as efficient as that of normal cells (Wood et al., 1988; Hansson et al., 1991). This suggests an unusually labile excision repair protein in XP variant cells which easily loses activity during preparation of cell extracts. Whether this reflects an altered repair protein is still unknown. It has been suggested that the lack of repair of purified DNA by XP variant cell extracts reflects inability of the repair complex to repair regions of DNA that are relatively free of chromatin, such as the replication fork. This raises the possibility that XP variant cells are defective in repair at the replication fork (Cleaver et al., 1990).

It is also possible that the deficiency in the repair ability of XP variant cells, if any, resides in a repair system other than the nucleotide excision repair pathway. One study shows that XP variant cells contain less photoreactivating enzyme than do normal cells. The activity is about 10% that of the normal cells in three XP variant cell lines, i.e., XP4BE, XP13BE, and XP7TA (Sutherland and Oliver, 1975). Attempts by several other groups to demonstrate this photoreactivation have been unsuccessful or the results were ambiguous (Cleaver and Kraemer, 1989). Recently, Sancar and colleagues found that at least in Hela cells and human white blood cells, photolyase activity is lower than the detectable level (Li et al., 1993). Therefore, the contribution of this low level of photoreactivating enzyme in XP variant cells to the development of

sunlight-induced skin cancer in these patients seems to be less important.

4. Evidence that Defective Replication May Be Involved in the XP Variant's Inherited Predisposition to Sunlight-Induced Skin Cancer.

4.1 Defective Replication After UV. (A) Effect on Daughter Strand Elongation: The defect in DNA replication after UV irradiation of XP variant cells was first demonstrated by Lehmann et al. (1975). When they measured the size of newly synthesized DNA after UV irradiation in three XP variant cell lines, i.e., XP4BE, XP7TA and XP3ORO, and compared it to that in normal and XP group A cells, they found after pulse-labeling the XP variant cells for 1 hr with tritiated thimine (^{3}H-TdR), the size of newly synthesized DNA of XP variant cells is much smaller than that of normal cells, and it is even smaller than that of XP group A cells. When cells are incubated with fresh medium containing unlabled TdR for various time after the pulse-labeling period and examined for the molecular weight of DNA, the time taken to synthesize DNA of molecular weight equal to parental DNA is much longer in XP variant cells than in normal and XP group A cells. However, Lehmann and his colleagues could not distinguish the difference among all cell lines specifically in the rate of elongation because the daughter DNA that is already present at the time of UV irradiation will be labeled and indistinguishable from the daughter DNA that is synthesized after UV irradiation.

van Zeeland and Filon (1982) later developed a technique to nick UV-irradiated DNA *in situ* by introducing CPD-specific endonuclease into permeablized cells following UV irradiation. Since the stretches of daughter strand DNA already present at the time of UV irradiation will contain CPD, the CPD-specific endonuclease will be able to cut off these

stretches, leaving the DNA synthesized after UV irradiation intact. They showed that within 15 min after irradiation, the size of newly synthesized DNA in normal cells is already greater than the interdimer distance and continues to increase in size at the same rate during the next few hours, so that within 4hr it is 14 times the interdimer distance. In XP group A cells, the newly synthesized DNA also elongates rapidly, although more slowly than in normal cells. However, in two XP variant cell lines, i.e., XP30RO and XP6DU, no increase in length occurs during the first 15 min, and they are much slower in daughter strand growth following UV irradiation, especially during the first 2 hr.

Similarly, Boyer et al. (1990) reported that for a given dose of UV, normal and XP variant cells receive the same number of UV lesions, but the variant cells are three to four times more sensitive to inhibition of daughter strand growth. An average of 5.1 CPD per replicon was needed to inhibit DNA strand growth in normal cells, but only about 1.4 were needed for XP4BE and XP115LO variant cells. These investigators proposed that normal cells express a mechanism for rapid bypass of UV-induced lesions which is not expressed in XP variant cells. This mechanism facilitates bypass of 67% of UV lesions with little difficulty in normal cells, but XP variant cells lack such a mechanism.

(B) Effect on Initiation: The inhibition of replication by UV irradiation has also been characterized by velocity sedimentation of pulse-labeled newly synthesized DNA in alkaline sucrose gradients to see the effect specifically on initiation of DNA replication from origin. This effect is reflected by a selective inhibition of synthesis of daughter DNA with the same length as an average replicon, i.e., 2×10^7 daltons of single-stranded DNA , within 30-60 min after UV irradiation (Kaufmann and

Cleaver, 1981). Such studies show that low doses of UV (< 1 J/m^2) specifically inhibit replicon initiation in both normal and XP group A cells. For example, 30 min after irradiation with 0.8 J/m^2, incorporation of ^{3}H-TdR into molecular weight of 2 x 10^7 daltons was 50% of that in unirradiated cells. Shortly after, the molecular weight of these replicon-size DNA gradually increased and parental-size DNA reappeared in 2 hr. In contrast, XP variant cells showed a 60% inhibition of replicon initiation after 30 min even at 0.3 J/m^2 of UV. A dose of 0.8 J/m^2, which caused only the inhibition of replicon initiation in normal and XP group A cells, but did not interfere with the elongation of daughter strand growth, caused an exaggerated inhibition of replicon initiation and also an inhibition of elongation of daughter strand growth in XP variant cells. Little or no recovery of replicon initiation was observed even after 2 hr (Kaufmann and Cleaver, 1981; Cordeiro-Stone et al., 1986).

It was proposed that the stronger effect of UV on initiation and slower recovery rate seen in the XP variant cells are due to either a lack of a mechanism of very rapid lesion bypass (Kauffman and Cleaver, 1981) or an inability to activate alternative sites of replicon initiation by XP variant cells (Fujiwara et al., 1991). The latter possibility has been eliminated by Griffiths and Ling (1991) who showed that XP variant cells are capable of activating alternative sites of replicon initiation. When cells were pulse labeled for 25 min with high specific activity ^{3}H-TdR followed by a 25 min incubation in low specific activity ^{3}H-TdR, there were several distinct replicon figures seen by autoradiographic observation. These replicon figures have a center area of high grain density which trails off to a lower grain density on both sides. The heavily labeled region represents the center of an replicon origin. After UV irradiation,

the distance between two such adjacent distinct replicon figures decreases. This suggests an activation of alternative sites of replicon initiation after UV irradiation. Such a decrease in distance between adjacent replicon figures has been seen in both normal and XP variant cells.

(C) Effect of Caffeine: When the irradiated cells are incubated with caffeine-containing medium throughout the whole period after irradiation before cells are measured for the molecular weight of DNA, caffeine almost completely prevents the increase of the molecular weight of newly synthesized DNA in irradiated XP variant cells, whereas no such effect is seen in normal and classic XP cells. This synergistic effect of caffeine on inhibition of DNA replication by UV has been seen in many XP variant cell lines, e.g., XP4BE, XP7TA, XP30RO (Lehmann et al., 1975), XP3KO, XP5TO (Fujiwara and Tatsumi, 1976), XP5KA (Yagi and Takebe, 1989), and XP16KO (Fujiwara et al., 1991).

The mechanism involved in the synergistic effect of caffeine on inhibition of DNA replication by UV found in XP variant cells is still unclear. It is unlikely that XP variant cells metabolize the caffeine differently from other cell lines because the catabolic pathway of caffeine in XP variant cells seems to be qualitatively similar to that in normal cells (Goth and Cleaver, 1976). It has been proposed that caffeine, which binds reversibly to single-stranded DNA and to UV-irradiated DNA, competes with repair/or replication enzymes for the damage sites. In the XP variant cells, one of these repair/or replication enzymes is defective and has a lower binding affinity to the DNA (Lehmann et al., 1975). This still remains an open question because caffeine is extensively metabolized (Goth and Cleaver, 1976). Whether there is enough

caffeine inside cells to bind to DNA or to damaged sites is questionable.

Another more likely explanation for the caffeine potentiation seen in the variant cells is that caffeine increases the number of replication origins (Painter, 1980) and this results in more unrepaired damage being located just ahead of replication forks. If this is true, XP variant cells would face more problems in replicating pass these unexcised lesions during replication. Evidence comes from the observation that caffeine produces a large increase in sensitivity in rodent cell lines and in several SV40-transformed human cells lines that proliferate rapidly (Cleaver, 1989). In addition, the effect of caffeine on DNA replication after UV irradiation is similar in XP variant cells and mouse cell lines (Fujiwara and Tatsumi, 1976; Yagi and Takebe, 1989).

4.2 Replication after Other Carcinogen Treatments. Since XP variant cells show difficulty in replicational bypass of UV photoproducts, studies which examine the effect on DNA replication of several other carcinogens that are also repaired by the excision repair pathway were performed in XP variant cells. One of such carcinogen is BPDE. However, XP variant cells (XP4BE and XP115LO) displayed a pattern of response to the inhibition of DNA strand growth by BPDE that was almost identical to that observed in normal cells (Cordeiro-Stone et al., 1986; Boyer et al., 1990). It was proposed that XP variant cells are deficient in some gene products which enable normal cells to bypass certain UV photoproducts, but that this gene product does not affect the sensitivity of XP variant cells to BPDE adducts (Cordeiro-Stone et al., 1986).

Two other chemical carcinogens, i.e., 4NQO and N-acetoxy-2-acetyl-aminoflurene (AAAF) which are involved in short-patch repair and excision repair precesses, respectively, were also examined for their effect on DNA

replication in XP variant cells. The rate of increase in size of newly synthesized DNA in XP variant cells (XP7TA) was as rapid as normal cells after treatment with 4NQO (Dollery et al., 1983). In contrast, in cells treated with AAAF, the XP variant cells (XP4BE and XP13BE) are slower in increasing the size of new DNA than normal cells or even than classic XP cells (D'Ambrosio and Setlow, 1978).

A recent study shows that XP variant cells also have a defect in replicational bypass of the psoralen adducts that are structually similar to CPD (Misra et al., 1993). Although repair of psoralen adducts is similar to that of normal cells, XP variant cells can not bypass the interstrand cross-links and can only bypass half of the monoadducts compared to normal cells during replication. These studies provide indirect evidence that gene products which are involved in replicational bypass of DNA lesions may be different depending on the structure of lesion formed in DNA.

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CHAPTER II

XP variant cells are less likely than normal cells to incorporate dAMP opposite photoproducts during replication of UV-irradiated plasmids

(UV mutagenesis/ *supF* gene/ error-prone replication)

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ABBREVIATIONS USED

bp, base pair(s); *supF*, tyrosine amber suppressor tRNA structural gene; SV40, simian virus 40; XP-V, SV40-transformed xeroderma pigmentosum variant cell line; UV, ultraviolet radiation of 254nm wavelength

SUMMARY

Xeroderma pigmentosum (XP) variant patients show the clinical characteristics of the disease, with increased frequencies of skin cancer, but their cells have a normal, or nearly normal, rate of nucleotide excision repair of UV-induced DNA damage and are only slightly more sensitive than normal cells to the cytotoxic effect of UV radiation. However, they are significantly more sensitive to its mutagenic effect. To examine the mechanisms responsible for this hypermutability, we transfected an XP variant cell line with a UV_{254nm}-irradiated shuttle vector carrying the *supF* gene as a target for mutations, allowed replication of the plasmid, determined the frequency and spectrum of mutations induced, and compared the results with those obtained previously when irradiated plasmids carrying the same target gene replicated in a normal cell line (Bredberg *et al.* Proc. Natl. Acad. USA, **83**:8273, 1986). The frequency of mutants increased linearly with dose, but with a slope 5 times steeper than that seen with normal cells. Sequence analysis of the *supF* gene showed that 52 out of 53 independent mutants generated in the XP variant cells contained base substitutions, with 62/64 of the substitutions involving a dipyrimidine. 28% of the mutations involved A·T base pairs, with the majority found at position 136, the middle of a run of three A·T base pairs. (In the normal cells, this value was only 11%.) If the rate of excision of lesions from *supF* in the two cell lines is equal, our data suggest that XP variant cells are less likely than normal cells to incorporate dAMP opposite bases involved in photoproducts. If such incorporation also occurs during replication of chromosomal DNA, this could account for the hypermutability of XP variant cells with UV irradiation.

INTRODUCTION

It is now widely recognized that the transformation of normal cells into tumorigenic cells is a multi-step process, and substantial evidence indicates that mutations play a fundamental role in cellular transformation and carcinogenesis, as well as in many inheritable diseases and developmental anomalies (1, 2). However, our understanding of the factors and influences governing the formation of these changes in gene structure is considerably less advanced. Cells isolated from patients with the rare, autosomal recessive disorder xeroderma pigmentosum (XP) present a unique model system for investigating of DNA repair and mutagenesis in human cells. In the present study we made use of a shuttle vector assay to investigate the kinds of mutations induced when a UV-irradiated plasmid replicated in cells derived from the class of XP patients called XP variants to provide clues to the mechanisms responsible for the hypermutagenic effect of UV radiation on these cells.

XP variants inherit the characteristic predisposition to sunlight-induced skin cancer, but unlike the majority of XP patients, their cells do not exhibit a significant deficiency in the rate of nucleotide excision repair of endogenous UV-induced DNA damage, including both cyclobutane pyrimidine dimers (3-8) and pyrimidine (6-4) pyrimidone photoproducts (7,8). Cells from XP variant patients have an abnormality in the manner in which DNA replicates on a template containing UV lesions (9-11) and an inability to convert a very minor UV photoproduct to an excisable lesion (12). They are only slightly more sensitive than cells from normal donors to the cytotoxic effect of UV, but significantly more sensitive to its mutagenic action (13-15). However, the molecular mechanism(s) responsible for the abnormal sensitivity of the XP-V cells to UV-induced mutations has

not been explained.

To examine this question, we UV-irradiated a shuttle vector, pS189 (16), carrying the *supF* gene as the target for mutations, and transfected the plasmids into a simian virus 40 (SV40)-transformed XP variant cell line (XP-V) where they could be replicated by the human cell polymerase(s). The progeny plasmids were analyzed for the frequency of *supF* mutants and the kinds of mutations and their location in the gene was determined. The results were compared with those reported by Bredberg *et al.* (17) who used the same assay in a repair-proficient cell line from a normal donor. We found a dose-dependent decrease in yield of replicated plasmids and a corresponding increase in the frequency of *supF* mutants. The slope of the mutant frequency curve was 5 times steeper than that seen with the normal cells. Sequence analysis of the *supF* gene from 53 independent mutant plasmids indicated that an abnormally high proportion of the base substitutions involved A·T base pairs (bp), with many at a unique "hot spot", position 136, in the middle of a run of three A·T bp.

MATERIALS AND METHODS

Cells and Plasmid. The XP-V cell line, an SV40-immortalized derivative of XP cell line GM2359, was kindly provided by Dr. Roger Schultz (University of Maryland, Baltimore, MD). The cells were grown in modified MCDB-110 medium (18) prepared with Earle's salts and supplemented with 10% fetal calf serum (GIBCO) and antibiotics. The ampicillin-sensitive indicator bacterial host was *Escherichia coli* SY204, carrying an amber mutation in the ß-galactosidase gene and in the tryptophan gene (19). The 5337 bp shuttle vector, pS189 (16), a deletion derivative of pZ189 (20), contains the tyrosine amber suppressor tRNA gene (*supF*) flanked by the gene for ampicillin resistance and the bacterial origin of replication (16). It also contains an origin of replication that facilitates its replication in mammalian cells.

UV Irradiation. The plasmid DNA was diluted with Tris-EDTA buffer (10mM Tris-HCl, pH 7.5, with 1 mM EDTA) to 50 μg/ml immediately before irradiation and 1 ml was pipetted into a sterile plastic 60 mm tissue culture dish that had been placed on ice. The plasmids were irradiated with the indicated doses of UV from an unfiltered germicidal lamp at a dose rate of 2.5 $J/m^2/sec$, then precipitated with ice cold ethanol and redissolved in Tris-EDTA buffer at a concentration of 500 μg/ml and stored at -20°C until used.

Transfection and Rescue of Replicated Plasmids. The procedures used for $CaPO_4$ coprecipitation transfection were essentially as described (21), but with the density of the cells increased from 1 X 10^4 cells/cm^2 to 3 X 10^4 cells/cm^2 (1.5 X 10^6 cells per 100 mm diam. dish), and with the amount of plasmid per dish increased from 6 μg to 40 μg. The cells were harvested after the transfection, and progeny plasmids were extracted as described

(22). To distinguish between independent mutants with identical mutations and putative siblings derived from a single event, progeny plasmids obtained from each dish of cells were maintained and assayed separately. Prior to bacterial transformation, the plasmids were treated with *DpnI* to digest any DNA that still had the bacterial methylation pattern to ensure that the purified DNA was derived from plasmids that had replicated in the human cells.

Bacterial Transformation and Mutant Characterization. The techniques used were essentially as described (21). Briefly, progeny plasmids were assayed for mutant *supF* genes by transforming SY204 bacterial cells to ampicillin resistance and selecting on agar plates containing ampicillin, an indicator dye, and isopropyl β-D-thiogalactoside. On this medium, bacterial transformants containing plasmids with a mutant *supF* gene form light blue or white colonies; those with a wild type *supF* gene form blue colonies. Mutant colonies were restreaked on these agar plates and on plates lacking tryptophan to confirm the phenotype, and then the plasmids were amplified and purified using a small scale alkaline lysis procedure (23), and analyzed by electrophoresis on 0.8% agarose gels for altered DNA mobility. Plasmids without evidence of gross alterations were sequenced as described (21).

RESULTS

Yield of Plasmids and Mutation Frequency. Before beginning the study, we found we could increase the yield of plasmid DNA obtained from the XP-V cells 60-fold by increasing the amount of plasmid DNA per transfection from 6 μg to 40 μg, and increasing the cell density to 3 X $10^4/cm^2$. As shown in Fig. 1A, UV-irradiation caused by a dose-dependent decrease in yield of replicated plasmids from XP-V cells, a decrease that was not found by Bredberg *et al.* (17), using GM637 cells from a normal donor as their host cells. The yield after a dose of 200 J/m^2 was 33% of the unirradiated control.

There was a corresponding dose-dependent increase in the frequency of *supF* mutants (Fig. 1B), reaching 330 X 10^{-4} with a background of 3 X 10^{-4} at a dose of 500 J/m^2. The slope of the mutant frequency curve was 5 times steeper than that found previously (17) using the GM637 cell line as host. Table 1 gives the number of plasmids analyzed and their characterization.

Spectrum of Mutations Produced in the *supF* Gene of UV-Irradiated Plasmids that Replicated in XP-V Cells. DNA sequence analysis (Fig. 2) of 53 equivocally independent mutants from passage of the UV-irradiated plasmids through the XP-V cells revealed 64 base substitutions at 27 sites and showed that 52/53 mutants contained base substitutions. The one plasmid with a rearrangement in the *supF* gene came from a plasmid preparation that received 200 J/m^2. As noted in Table 2, the majority of mutants (41/53) contained only a single base substitution. 3/53 had tandem substitutions, 6/53 had two base substitutions, but not located in tandem, one had three separate base substitutions, and one contained a complex mutation. All except two base substitutions (site 101 and 114) were found at sites of adjacent pyrimidines. The A·T --> T·A transversion

Figure 1. (A) Yield of plasmids following replication in XP-V cells (circles), as estimated from the relative frequency of transformation of bacteria to ampicillin resistance, as a function of the UV dose to the plasmids. (B) Frequency of *supF* mutants induced in plasmids replicated in XP-V cells (circles) as a function of UV dose to the plasmid.. Comparable data for plasmids that replicated in GM637 cells (triangles), from a normal donor. These latter data, taken from Bredberg *et al.* (17), are reproduced for purpose of comparison.

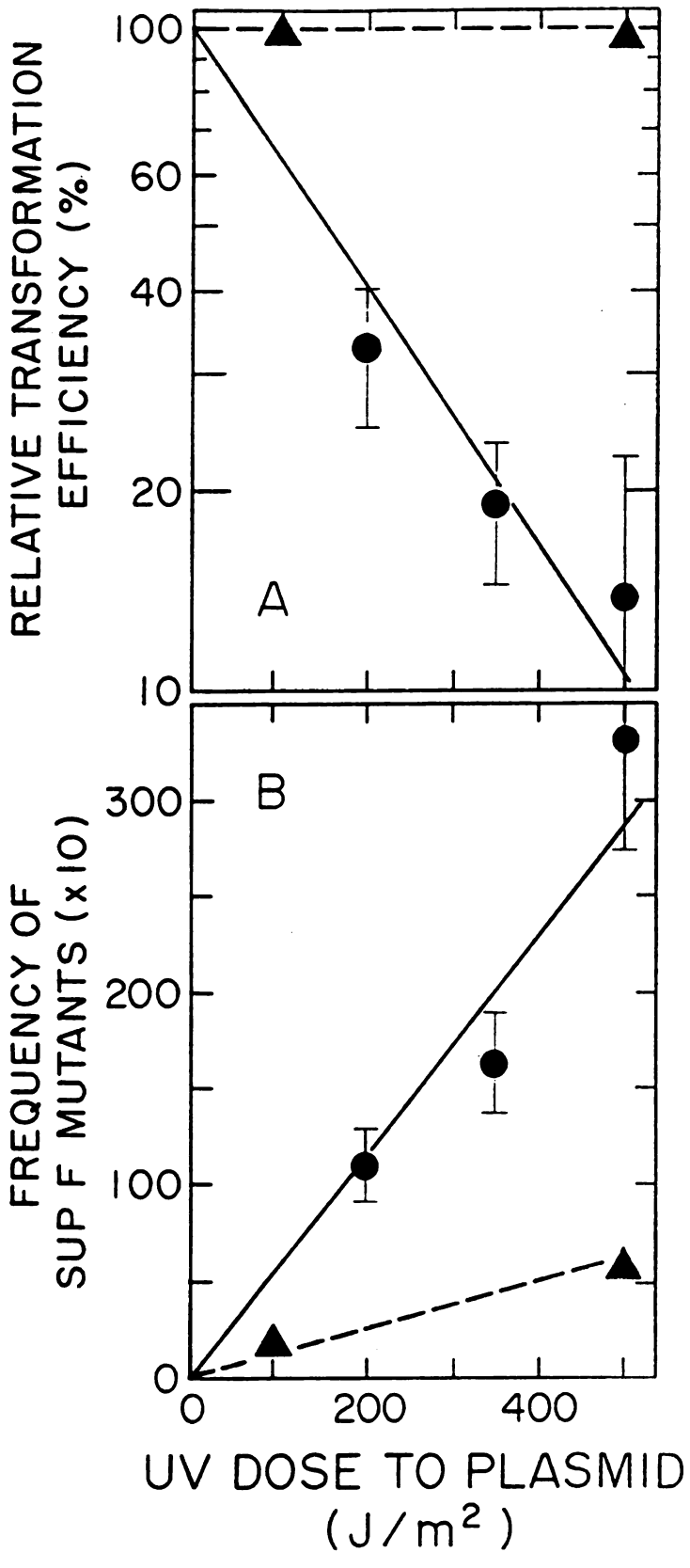
RELATIVE TRANSFORMATION EFFICIENCY (%)
100
60
40
20
10
A
B
FREQUENCY OF SUP F MUTANTS (x10)
300
200
100
0
0
200
400
UV DOSE TO PLASMID
(J/m²)

Figure 1

Table 1. Analysis of mutants obtained by transformation of *E. coli* with progeny of UV-irradiated pS189 generated during replication in XP-V cells

UV dose to plasmid J/m^2	No. of human cell trans-fection expts.	*supF* mutants per trans-formant*	Frequency of *supF* mutants (10^4)	Plasmids with altered gel mobility per no. examined†	Characterization of sequenced mutants: Total *supF* genes sequenced	No. with rearrangements	No. with point mutations‡	Mutants with point mutations per 10^4§
0	32	9/29,566	3	0/9	0	ND¶	ND	ND
200	5	35/3,072	114	0/35	18	1	17	109
350	5	26/1,566	166	0/26	14	0	14	166
500	5	34/1,017	334	0/34	21	0	21	334

*White or light blue colonies were restreaked on plates lacking tryptophan to ensure that the inability of the cell to metabolize X-Gal resulted from inactivation of the *supF* gene.

†Alteration visible on agarose gel (> 150 bp).

‡Substitution, deletion, or insertion of 1 or 2 bp.

§Calculated from fraction of mutants with point mutations times the observed frequency (column 4). The fraction of mutants with point mutations is the number in column 8 divided by that in column 6 plus those mutants showing altered gel mobility (numerator, column 5).

¶ND, not determined.

Figure 2. Location of independent mutations in the structural region of the *supF* tRNA gene. Every tenth base and the anticodon is underlined. The mutations observed in the progeny of the irradiated plasmids are placed below the sequence. The mutations underlined represent tandem mutations. In the class of two base substitutions and three base substitutions, the mutations connected by the dashed line represent the individual mutants with multiple base substitutions. The caret shows the location of an inserted cytosine. The asterisks indicate the prominent "hot spots".

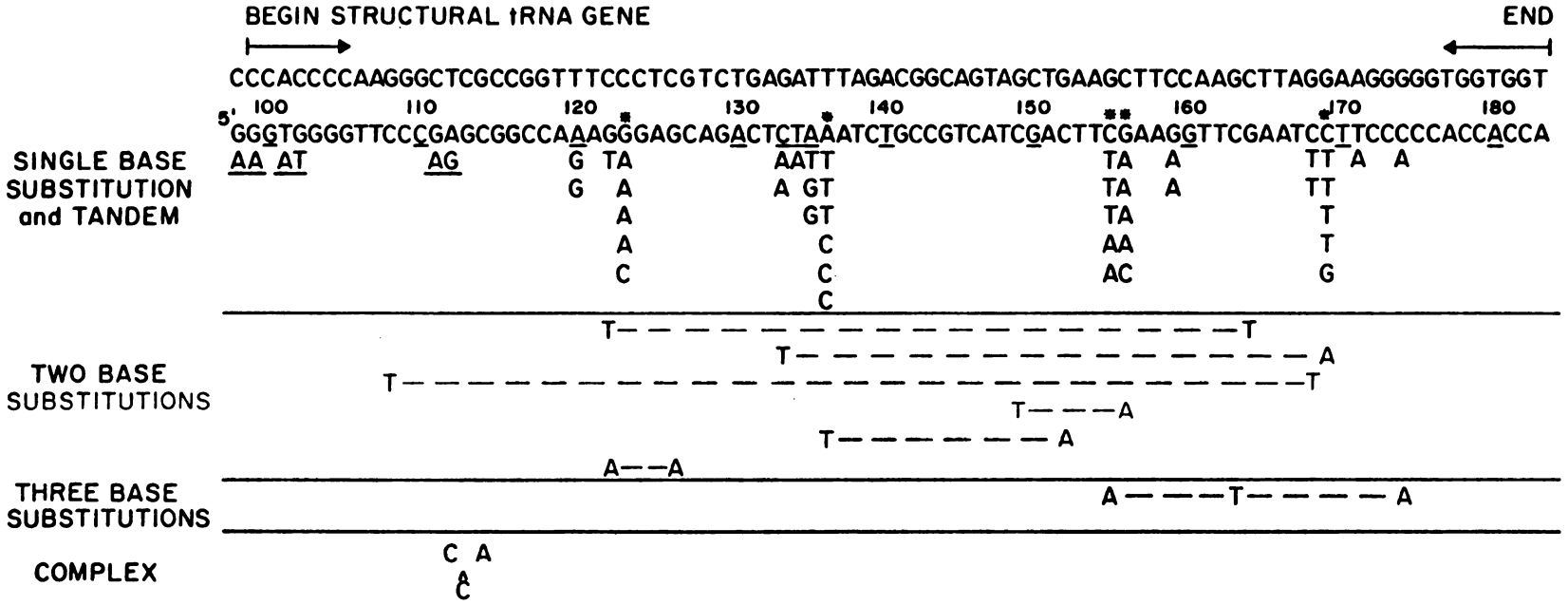
BEGIN STRUCTURAL tRNA GENE
END
CCCACCCCAAGGGCTCGCCGGTTTCCCTCGTCTGAGATTTAGACGGCAGTAGCTGAAGCTTCCAAGCTTAGGAAGGGGGTGGTGGT
5' 100 110 120 130 140 150 160 170 180
GGGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAAGGTTCGAATCCTTCCCCCACCACCA
SINGLE BASE SUBSTITUTION and TANDEM
AA AT
AG
G TA
G A
A
A
C
AATT
A GT
GT
C
C
C
TA A
TA A
TA
AA
AC
TT A A
TT
T
T
G
TWO BASE SUBSTITUTIONS
THREE BASE SUBSTITUTIONS
COMPLEX
C A
C

Figure 2

Table 2. Analysis of sequence alterations generated in the *supF* gene by replication of UV-irradiated plasmids in XP-V and GM637 cells

Sequence alterations	Number of plasmids with base changes	
	XP-V	GM637*
Single base substitutions	41 (77%)	44 (49%)
Tandem base substitutions	3 (6%)	16 (18%)
Multiple base substitutions	8 (15%)	28 (31%)
Two base substitutions		
≤ 15 bases apart	3	--§
35-59 bases apart	3	--§
Three base substitutions	1	--§
Complex†	1	4
Insertions and deletions‡	0	1 (1%)
Gross rearrangement	1 (2%)	0
Total	53	89

*Data obtained in cell GM637 are from Bredberg *et al.* (17) and are shown here to allow easy comparison.

†Plasmids with insertion or deletion accompanied by one or two base substitutions.

‡Plasmids contain insertion or deletion only.

§Data not available.

at site 101 was part of a tandem substitution, and the G•C --> T•A transversion at site 114 was part of the complex triple mutant. All 41 single base substitutions could have occurred at the 3' side of the photoproduct, but 14/41 definitely occurred there, i.e., those located at sites 134, 135, 155, and 156. In the mutant with base substitutions at position 122 and 126, the mutated site at 126 was found at the 5' side of a dipyrimidine. Two other mutants had two base substitutions (i.e., at sites 136 and 152; and at sites 149 and 156) which could not be explained by the presence of two photoproducts in one plasmid, since the dipyrimidines at those positions are located on opposite strands.

Table 3 compares the types of base pair substitutions observed in the *supF* gene of UV-irradiated plasmids passaged through XP-V cells with those found using GM637 cells. In both cases, the major class of base pair substitution was the G•C --> A•T transition, but the frequency of this change with plasmids from XP-V cells was much lower than from GM637 cells. In plasmids from XP-V cells, transversions made up 47% of the substitutions in the *supF* gene, a frequency twice as high as that observed with GM637 cells. In addition, the types of transversions differed, i.e., plasmids from XP-V cells had twice the frequency of G•C --> T•A transversions, and base pair substitutions involving A•T base pairs occurred at a frequency of 28% with XP-V cells compared to 11% with GM637 cells. These differences are statistically significant ($P<0.01$). 67% of these A•T substitutions occurred at TTT sites (at positions 120, 135, and 136).

Mutational "Hot Spots" for UV-Induced Mutations. As shown in Fig. 2, there were five prominent "hot spots" i.e., position 123, 136, 155, 156, and 169. It should be noted that 12 bases pairs intervene between

position 123 and 136, and between position 156 and 169. Except for site 136, each "hot spot" involved a G·C base pair. Site 136, which is located in the middle of a run of three A·T base pairs was the strongest "hot spot", with 11% of the total base substitutions found there. This "hot spot" is unique to plasmids replicated in XP-V cells, i.e., it has not previously been found with UV irradiated *supF* genes that have been replicated in mammalian cells (17,24,25). All the mutations at this site were transversions, whereas those at the other four hot spots were mainly transitions.

DISCUSSION

Because the frequency of *supF* mutants in our study was so much higher than background, we are quite certain that the mutations resulted from UV radiation. The increase in frequency was linearly related to dose as predicted for mutations resulting from single UV-induced photoproducts, and in all except two of the 64 base substitutions observed, the pyrimidine involved was adjacent to another pyrimidine. The exception at position 101, which involved a tandem mutation 101-102, may have been caused by a rare UV lesion consisting of a purine flanked by pyrimidines, i.e., CAC (26). The other exception, at position 114, was part of a complex triple mutant with two base substitutions and one insertion. It is not easy to imagine the origin of this complex set of changes. However, if they were triggered by photoproducts at positions 111-112 and/or 112-113, it should be noted that in no case was dAMP incorporated.

Even the mutations we designate as "multiple base substitutions" (Table 2) appear to be targeted to UV photoproducts: (a) the frequency of plasmids that contained multiple base substitutions was directly related to the dose; (b) the proportion of mutant plasmids containing multiple base substitutions increased as a function of dose, i.e., 11% (2/18) at 200 J/m^2; 14% (2/14) at 350 J/m^2; 19% (4/21) at 500 J/m^2; (c) the distribution of types of base substitutions in the mutants with multiple base substitutions was almost the same as that in mutants with single or tandem base substitutions, e.g., 44% vs 45% for G·C --> A·T; 7% vs 8% for A·T --> G·C, etc; (The latter results differ significantly from those of Seidman *et al.* (27), who found that more G·C --> T·A transversions and fewer G·C --> A·T transitions with the plasmids with multiple mutations than with the plasmids with single or tandem base substitutions.) and (d)

in three out of our six mutants involving two base pair substitutions, the distance between the substitutions was at least 35 bp, suggesting that two photoproducts were involved. Two photoproducts in a single plasmid is clearly possible since Hauser *et al.* (24) estimates that, under the experimental conditions used for the present study, the mean number of photoproducts per *supF* gene induced by 500 J/m^2 is one, and a Poisson distribution predicts that approximately 35% of the *supF* genes will receive more than one photoproduct. Even the two mutations located close to each other (site 122 and 126) could have resulted from two independent photoproducts. In the other two mutants with two base substitutions, non-tandem, the mutations cannot be explained by two photoproducts in a single plasmid since the dipyrimidines that would have been involved are located in opposite strands, (i.e., sites 136 and 154; and sites 149 and 156). Such mutations could arise if a second base change occurred spontaneously during replication of a plasmid containing a targeted base substitution. Another possible explanation is that recombination between two plasmids carrying *supF* mutations occurred during replication in the host cells. A third possibility , and one proposed by Seidman *et al.* (27) is that the multiple base substitutions are the result of an error-prone polymerase that gains access to the DNA by excision repair single strand breaks.

Since we and Bredberg *et al.* (17) used very similar protocols for irradiating our plasmid DNA, it is unlikely that our plasmids received a higher dose of UV and carried more lesions unless the dosimetries were not comparable. Another possible explanation for the higher frequency of mutants in plasmids from XP-V cells than in those from GM637 cells is that the rate of excision repair of the photoproducts in the plasmids was significantly slower in XP-V cells than in GM637. However, XP variant

cells are reported to excise UV-induced lesions from their endogenous DNA at virtually the same rate as normal cells (3-8). Wood *et al.* (28) recently found that, in contrast to their earlier observations (29), cell-free extracts from XP variant cell lines can exhibit a normal rate of excision of UV photoproducts from DNA plasmids. Nevertheless, if the higher frequency merely reflected a higher number of unex- cised lesions in the *supF* gene, the *kinds* of mutations observed in plasmids from XP-V and GM637 cells should have been very similar. They were not. It is not likely that the excision process itself introduces mutations, since Watanabe *et al.* (30) showed that if XP variant cells are synchronized and irradiated at various times prior to DNA replication of the HPRT gene during S-phase, the frequency of 6-thioguanine resistant mutants decreases with time post-irradiation before S-phase. If the cells are prevented from replicating for 24 hr after UV, the mutant frequency is decreased to background levels. No such decrease occurs if the cells are incapable or virtually incapable of excision repair (31).

To explain the hypermutability of the XP variant cells, Watanabe *et al.* (30) suggested that the process the XP variant cells use to replicate past unexcised UV photoproducts differs from that of normal cells, i.e., either the XP variant cells uses a more "error prone" process or the normal cells use a more "error-free" process. These investigators could not distinguish between these two possibilities, but in view of the findings of Cleaver and his associates (32-34) on the increased blocking effect UV photoproducts have on DNA initiation and chain elongation in the variant compared to normal cells, Watanabe *et al.* (30) suggested that "some process unavailable to the XP variant cells is operating in the normal cells."

The results of the present study comparing the types of base pair substitutions observed in the *supF* gene of plasmids derived from XP-V and GM637 cells support this hypothesis. The data suggest that the polymerase(s) of the XP-V cells is less likely than that of GM637 cells to incorporate dAMP opposite bases involved in UV photoproducts during DNA replication. This is because there was a significantly lower frequency of G•C --> A•T transitions, 45% compared to the 73% observed with the GM637 cells. Many investigators (35-37) suggest that the preference for the G•C --> A•T transitions among UV-induced mutations results from dAMP being preferentially incorporated by the DNA polymerase opposite a non-instructive lesion. In addition there was a significantly higher frequency of A•T base pair substitutions, 28% compared to 11% (Table 3) an occurrence that would result if the polymerase failed to incorporate dAMP opposite photoproducts involving thymidine. Note that the strongest "hot spot" in the *supF* spectrum from XP-V cells occurred at position 136, which necessarily involved a T•T photoproduct. There were no mutations at site 136 in plasmids from GM637 cells, and none of the "hot spots" found in plasmids from GM637 cells involved A•T base pairs (17). If during replication of their endogenous genome, XP variant cells also are less likely than normal human cells to incorporate dAMP opposite bases involved in UV photoproducts, this would contribute to their hypermutability with UV radiation.

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CHAPTER III

Evidence from mutation spectra that the UV hypermutability of xeroderma pigmentosum variant cells reflects abnormal, error-prone replication on a template containing photoproducts

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SUMMARY

Xeroderma pigmentosum variant (XP) patients are genetically predisposed to sunlight-induced skin cancer. Fibroblasts derived from these patients are extremely sensitive to the mutagenic effect of ultraviolet radiation and are abnormally slow in replicating DNA containing UV-induced photoproducts. However, unlike cells from the majority of XP patients, XP variant cells have a normal, or nearly normal rate of nucleotide excision repair of such damage. To determine whether their UV hypermutability reflected a slower rate of excision of photoproducts specifically during early S-phase when the target gene for mutations, i.e., the hypoxanthine (guanine) phosphoribosyltransferase gene (*HPRT*), is replicated, we synchronized diploid populations of normal and XP variant fibroblasts, irradiated them in early S phase, and compared the rate of loss of cyclobutane pyrimidine dimers and 6-4 pyrimidine-pyrimidones from DNA during S-phase. There was no difference. Both removed 94% of the 6-4's within 8 hr and 40% of the dimers within 11 hr. There was also no difference between the two cell lines in their rate of repair during G_1-phase. To see if the hypermutability resulted from abnormal error-prone replication of DNA containing photoproducts, we determined the spectra of mutations induced in the coding region of the *HPRT* gene of XP variant cells irradiated in early S- and G_1-phase, and compared them with those found in normal cells. The majority of the mutations in both types of cells were base substitutions, but the two types of cells differed significantly from each other in the kinds of substitutions observed either in mutants from S-phase ($P<0.01$) or from G_1-phase ($P=0.03$). In the variant cells, the substitutions were mainly transversions (57% in S, 73% in G_1). In normal cells, transversions were much rarer (8% in S, 24% in

G_1) ($P<0.001$ for S, $P<0.01$ for G_1). In the normal cells irradiated in S, the majority of the substitutions were G·C --> A·T, and most involved CC photoproducts in the transcribed strand. In the variant cells irradiated in S, substitutions involving cytosine in the transcribed strand were G·C --> T·A transversions almost exclusively. G·C --> A·T transitions made up a much smaller fraction of the substitutions than in normal cells ($P<0.02$), and virtually all of them involved photoproducts located in the nontranscribed strand. The data strongly suggest that XP variant cells are much less likely than normal cells to incorporate dAMP or dGMP opposite the pyrimidines involved in photoproducts. This would account for their significantly higher frequency of mutants and might explain their abnormal delay in replicating a UV-damaged template.

INTRODUCTION

Xeroderma pigmentosum (XP) variant patients inherit a predisposition to sunlight-induced skin cancer and develop the same clinical characteristics of the disease as do classic nucleotide excision repair-deficient XP patients (26). However, in contrast to the cells from the classic XP patients, fibroblasts derived from XP variant patients are reported to excise UV photoproducts at a normal, or near normal rate (5, 21, 26, 35). Maher et al. (15) and Myhr et al. (22) showed that such cells are only slightly more sensitive than cells from normal donors to the cytotoxic effect of UV, but are *significantly* more sensitive to its mutagenic action ($\geq$ 5-times-steeper slope). If, as evidence suggests, mutations are causally involved in carcinogenesis, such hypermutability can help explain the genetic predisposition of XP variant patients to skin cancer on sunlight-exposed parts of the body. However, a fundamental question still remains, i.e., What mechanism(s) is responsible for the UV hypermutability of XP variant cells?

The hypermutability cannot be accounted for by error-prone excision repair because when synchronized populations of XP variant cells were irradiated at various times prior to S-phase to allow different lengths of time for excision repair before DNA replication, the mutant frequency decreased as a function of time for repair, until it reached background levels (32). One possible explanation for the UV hypermutability in the variant cells is that their replication fork encounters more photoproducts than does that of normal cells. This would be the case if the slightly lower rate of excision repair sometimes reported for XP variants reflects a significantly slower rate *specifically* during *S-phase*. A second

possible explanation for the hypermutability is that the number of unexcised photoproducts is the same for both types of cells, but the replication complex of the XP variant cells is defective and less likely than that of normal cells to incorporate the correct nucleotide opposite the photoproduct during S-phase replication.

This second hypothesis is supported by the results of several groups of investigators who showed that XP variant cells replicate DNA containing UV photoproducts with greater difficulty than do normal cells (2, 6, 11, 13, 23, 29). For example, Boyer et al. (2) reported that for a given dose of UV, normal and XP variant cells receive the same number of UV lesions, but the variant cells are 3 to 4 times more sensitive to inhibition of daughter strand growth. An average of 5.1 cyclobutane pyrimidine dimers (CPD) per replicon was needed to inhibit DNA strand growth in normal cells, but only 1.4 were needed for XP variant cells. Similarly, van Zeeland and Filon (29) showed that within 15 minutes after irradiation, the size of nascent DNA in normal human fibroblasts is greater than the interdimer distance and continues to increase in size at the same rate during the next few hours, so that within 4 hr it is 14 times the interdimer distance. However, in the XP variant cells, no increase in length occurs during the first 15 minutes, and for the next few hours the rate of increase is six times slower than normal. These data are consistent with the replication forks of XP variant cells being blocked at photoproducts longer than normal cells are, and taking much longer for transdimer synthesis. Additional data supporting the second hypothesis comes from our recent finding that the kinds of mutations induced when a UV-irradiated plasmid is allowed to replicate in XP variant cells differ significantly from those seen with normal cells (31).

To determine whether there was a difference between the two cell types in their *rate* of excision repair during S-phase, we synchronized normal and XP variant cells, irradiated them at the onset of S-phase, harvested them immediately or after various hours of time for excision repair, and analyzed them for rate of loss of photoproducts, using antibodies specific for CPD or 6-4 pyrimidine-pyrimidones (6-4's). We found no difference in the rate of excision of either photoproduct. To determine whether there was a difference between the two cell types in the *kinds* of mutations induced, we determined the spectrum of *HPRT* mutations in the XP variant and compared the results with those from normal cells. The kinds of base substitutions in the two cell types differed significantly, strongly supporting the hypothesis of abnormal error-prone replication bypass of photoproducts by the XP variant cells.

MATERIALS AND METHODS

Cells and media. Normal fibroblasts, designated NFSL89, were explanted from the foreskin of a normal newborn as described previously (17). XP variant cells, XP4BE (CRL 1162), were obtained from the American Type Culture Collection (Rockville, MD). Cells were routinely cultured in Eagle's minimal essential medium containing 10% fetal bovine serum. For selection of thioguanine (TG) resistant cells, this medium was supplemented with 40 μM TG. For thymidine incorporation experiments, it was supplemented with [^{3}H]-TdR (New England Nuclear, Dupont, Wilmington, Del.) (5 μCi/ml of medium, 78.5 Ci/mmol).

Determining onset of S-phase in synchronized cells. Cells were driven into the G_0 state by density inhibition as described previously (12) and stimulated to enter the cell cycle by being 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, as well as the length of the S-phase, was determined by measuring the incorporation of 15 minutes pulses of [^{3}H]-TdR into acid-insoluble material as described previously (9).

Exposure to UV light and determination of cytotoxicity and mutant frequency. Cells were released from the G_0 state and plated at 10^4 cells/cm^2 for mutagenicity studies and at cloning densities for cytotoxicity determination. One hour after the cells began S-phase (17 hr after release from G_0) or in early G_1-phase (6 hr after release), the culture medium was aspirated, and the cells were washed with phosphate-buffered saline and irradiated with UV_{254nm} as described previously (24), and refed with culture medium. The cytotoxicity was determined from the decrease in the cells' colony-forming ability. The target cells for

mutation analysis were allowed an 8-day expression period and then selected for TG resistance as described previously (17). When clones developed 14 days later, they were isolated and *HPRT* cDNA was amplified directly from the original clones. The mutant frequency was determined from the number of TG resistant clones per 10^6 clonable cells as described previously (17).

Amplification of *HPRT* cDNA and DNA sequencing. First-strand cDNA was synthesized directly from mRNA in lysates of 100 to 500 cells, using polymerase chain reaction as described previously (34). The amplified *HPRT* coding region was sequenced, using the conditions recommended by the manufacturer (see protocols for DNA sequencing with Sequenase version 2.0, U.S. Biochemical, Cleveland, OH). The three primers used were those described in reference 34. This reference also gives the concentration of three dideoxy-chain termination mixes. For the fourth, i.e., tube T, the concentration was 150 µM of each dNTP/3 µM ddTTP.

Measurement of the rate of repair of specific photoproducts. XP variant and normal cells in exponential growth were plated at a density of ~7 X $10^3/cm^2$, i.e., 1/8 of their density at confluence, and allowed to undergo three population doublings in medium containing [^{14}C]-TdR (0.02 µCi/ml, 59 mCi/mmol; New England Nuclear, Dupont) to label the DNA. When they reached confluence, the cells were refed with fresh unlabeled medium each day for 3 days and then held at confluence in the absence of mitogens for 3 more days to achieve the G_0 state. The cells were released from G_0 and irradiated with 6 J/m^2 in early G_1- or S-phase. Following irradiation, the cells were either harvested immediately or incubated for various periods of time up to 22 hr. Any DNA replication occurring during this

period did not influence the measurement of repair rates since the DNA samples were normalized to equal amounts of parental (^{14}C-labeled) DNA. The DNA was isolated and assayed as described previously (20) for the presence of CPD and 6-4's using polyclonal antibodies that specifically recognized either of these photoproducts. The radioimmunoassay consisted of 2 μg of heat-denatured parental (prelabeled) DNA from the human cells and 10 pg of ^{32}P-labeled UV-irradiated pBR322 plasmid DNA competing for CPD-specific antibody-binding sites or 6-4-specific antibody-binding sites. To determine the amount of excision repair that had occurred, the extent of inhibition of ^{32}P-bound antibodies by the human DNA was converted to percent of antibody sites remaining, using a standard curve.

RESULTS

Rate of loss of photoproducts in synchronized populations of normal and XP variant cells. To test the hypothesis that during S-phase XP variant cells excise UV photoproducts at a slower rate than do normal cells, we synchronized large populations of both types of cells by release from the density-inhibited G_0 state, and irradiated them with 6 J/m^2 17 hr after release at the onset of S-phase. (The time of onset of S in the two types of cells plated at 10^4 cells/cm^2 was verified.) Irradiated cells were harvested immediately or after various hours of incubation, and the DNA was assayed for the rate of removal of CPD and 6-4's, using antibodies specific for these photoproducts. (For purposes of comparison we also measured the rate of removal of photoproducts during G_1-phase.) There was no difference between the XP variant and the normal cells in the rate of repair of either photoproducts during either S-phase (Fig. 1A) or G_1-phase (Fig. 1B). Both types of cells exhibited very rapid repair of 6-4's during S-phase, i.e., >90% were removed within 6 hr. The rate of CPD during S-or G_1-phase was significantly slower than that of 6-4's, i.e., only 40% removed within 11 hr, but there was no significant difference between the two types of cells. For both cell lines the extent of removal of CPD after 20 hr was somewhat greater during G_1- than S-phase.

Comparative study of the spectrum of mutations induced by UV. To test the hypothesis that the UV-hypermutability of XP variant cells reflects an abnormally error-prone replication complex, we determined the spectrum of mutations induced by 4 J/m^2 in synchronized populations of XP variant cells irradiated in early S-phase (17 hr after release from G_0) or in early G_1-phase (6 hr after release), and compared the results with what we had

Figure. 1 Rate of repair of 6-4 photoproducts and cyclobutane pyrimidine dimers by XP variant cells and normal cells during S-phase (A) or G_1-phase (B). XP variant cells (XP-V; open symbols) and normal cells (NF; closed symbols) were irradiated with UV light at 6 J/m^2, harvested immediately or incubated for the indicated time before their DNA was assayed for the number of binding sites of antibodies (Ab) against 6-4's (circles) or CPD (triangles). The data were obtained from two separate experiments; each point is the average of at least two separate assays. The error bars are calculated as the square root of the sample variance in separate experiments.

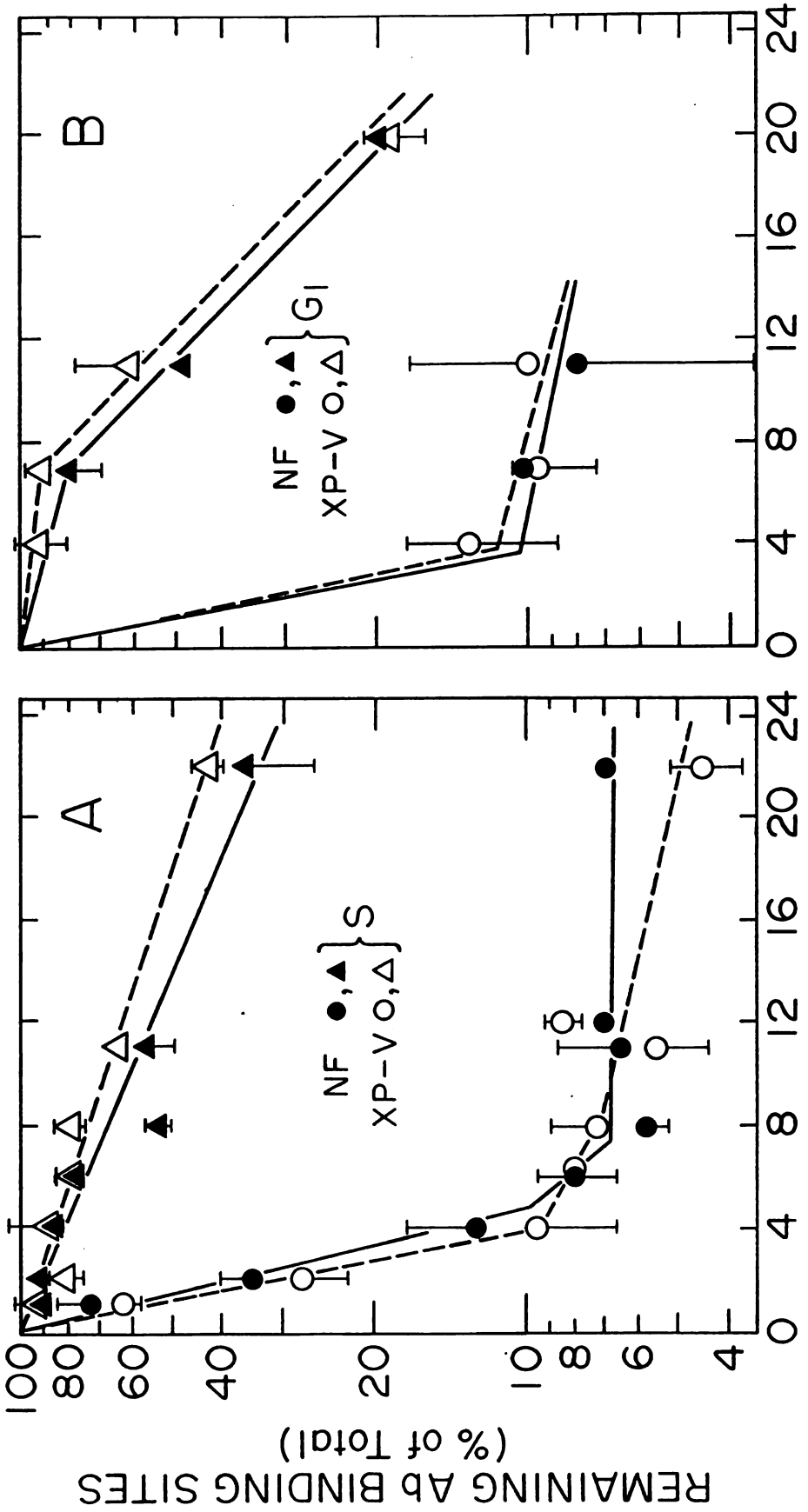
REMAINING Ab BINDING SITES
(% of Total)
100
80
60
40
20
10
8
6
4
A
B
NF ●,▲
XP-V ○,△
S
G1
0
4
8
12
16
20
24
REPAIR TIME (Hours)

Figure 1

obtained previously (18) with normal cells under similar conditions. To facilitate analysis of unequivocally independent mutants, we plated the synchronized XP variant cells into a series of individual dishes (eight populations for cells to be irradiated in early S-phase, and 11 populations for those to be irradiated in early G_1). In addition, there was a set of unirradiated control cells. The survival of the cells irradiated in early S was 19% of the unirradiated control; that of the cells irradiated in G_1 was 23%. The frequency of TG resistant mutants averaged 680 X 10^{-6} $\pm$ 260 X 10^{-6} for the S-phase cells and 220 X 10^{-6} $\pm$ 150 X 10^{-6} for the G_1-phase cells; the background frequency averaged 18 X 10^{-6} $\pm$ 14 X 10^{-6}. The large variance in these values reflects the fact that, rather than using very large populations of pooled cells as we do when our purpose is to determine frequencies, we used a series of smaller populations that were deliberately kept separate from each other to avoid analyzing siblings.

(i) Mutations found in cells irradiated in S-phase. The results of our analysis of 37 unequivocally independent mutants from XP variant cells irradiated in early S-phase are shown in Tables 1 and 2. Eleven (30%) appeared to have a splice site mutation. One of these (VS11) also had a base substitution. Twenty-one of the other 26 mutants contained only a single base substitution; two contained tandem base substitutions; and three had two substitutions, non-tandem. For 28 of the substitutions, the "premutagenic lesion" could be assigned to a dipyrimidine. In three cases, the substitution involved a cytosine flanked by two adenines. Such ACA sites represent rare UV photoproducts (1).

McGregor et al (18) reported data obtained by sequencing 22 independent mutants from normal cells irradiated in early S-phase. They found eight

(36%) with putative splice site mutations and 14 with base substitutions. The total number of base substitutions they analyzed was 19. To increase that number before trying to compare the mutation spectra of XP variants and normal cells, we analyzed 8 additional independent mutants derived from those earlier experiments, and obtained eight more base substitutions, along with a putative splice site mutation. These additional data, along with those reported by McGregor et al. (18), are shown in Tables 2 and 3.

The *kinds* of base substitutions induced in the two types of cells differed significantly ($p=0.001$ using the Chi square test). In the XP variant cells, 43% (13 of 30) of the base substitutions involved A·T, and 12 of these 13 (92%) were targeted to TT dipyrimidines, with 10/12 located in runs of T's. In the normal cells, only 28% of the base substitutions (7/25) involved A·T, with no more than 5 targeted to TT dipyrimidines, and only 2 of these TT dipyrimidines located in runs of T's. The most significant difference between the variant cells and the normal cells was the distribution of G·C --> A·T transitions. Such transitions made up the majority (64%) of the substitutions in the normal cells, and virtually all of them were targeted to photoproducts located in the transcribed strand. In contrast, G·C --> A·T transitions made up a much smaller fraction of the substitutions in the variant cells ($P<0.02$), and all of them were targeted to photoproducts in the nontranscribed strand. Substitutions involving cytosine in the transcribed strand were exclusively G·C --> T·A transversions. In the variant cells, 57% (17 of 30) of the substitutions were transversions compared with only 8% (2 of 25) in the normal cells ($P<0.001$). In addition, the types of transversions differed significantly.

Table 1.

[a]In addition to the independent mutants listed, another mutant, VS4, had an A·T --> G·C base substitution at position 314 causing a Tyr to Cys amino acid change. However, there was no discernible photoproduct at that site.

[b]Sequence of the nontranscribed strand. The sequence is shown in a 5' to 3'orientation. The lowercase letters represent the sequence in an intron. The sites of substitution are underlined.

[c]The sequence shown is from the appropriate strand and is listed in a 5' to 3' orientation. The site of substitution is underlined.

[d]T, transcribed; NT, nontranscribed.

[e]Contained more than one mutation, nontandem.

[f]Mutant VS68 also had an A·T --> T·A base substitution at position 196 where an ACA photoproduct was located. This resulted in the Cys to Ile amino acid change.

TABLE 1. Kinds and locations of mutations induced in the coding region of the *HPRT* gene in XP variant cells (XP4BE) irradiated in early S-phase

Mutant[a]	Position	Exon	Type of mutation	Surrounding sequence[b]	Amino acid change	Premutagenic-photoproduct[c]	Strand containing photoproduct[d]
Base substitutions involving cytosine:							
VS16[e]	145	3	G•C --> A•T	CGT CTT GCT	Leu --> Phe	TC or CT	NT
VS66	151	3	G•C --> A•T	GCT CGA GAT	Arg --> STOP	TC	NT
VS58	464	6	G•C --> A•T	AAT CCA AAG	Pro --> Leu	CC	NT
VS70	464	6	G•C --> A•T	AAT CCA AAG	Pro --> Leu	CC	NT
VS77	464	6	G•C --> A•T	AAT CCA AAG	Pro --> Leu	CC	NT
VS78	506	7	G•C --> A•T	ACC CCA CGA	Pro --> Leu	CC	NT
VS11[e]	601	8	G•C --> A•T	AGG GAT TTG	Asp --> Asn	TC or CC	T
VS1	115	2	G•C --> C•G	CCT CAT GGA	His --> Asp	TC	NT
VS73	40	2	G•C --> T•A	GAT GAA CCA	Glu --> STOP	TC	T
VS62[e]	118	2	G•C --> T•A	CAT GGA CTA	Gly --> STOP	CC	T
VS23	134	2	G•C --> T•A	GAC AGgtaa	Arg --> Met	CC or CT	T
VS68	197	3	G•C --> T•A	CTC TGT GTG	Cys --> Ile[f]	ACA[f]	T
VS59	509	7	G•C --> T•A	CCA CGA AGT	Arg --> Leu	TC	T
VS33	562	8	G•C --> T•A	TTT GTT GTA	Val --> Phe	ACA	T
VS17	606	8	G•C --> T•A	GAT TTG AAT	Leu --> Phe	TC	T
VS42	606	8	G•C --> T•A	GAT TTG AAT	Leu --> Phe	TC	T
VS9	617	9	G•C --> T•A	GTT TGT GTC	Cys --> Phe	ACA	T
Base substitutions involving thymine:							
VS31	205	3	A•T --> G•C	CTC AAG GGG	Lys --> Glu	TT	T
VS16[e]	295	3	A•T --> G•C	GAT TTT ATC	Phe --> Leu	TTTT	NT
VS6	296	3	A•T --> G•C	GAT TTT ATC	Phe --> Ser	TTTT	NT
VS61	296	3	A•T --> G•C	GAT TTT ATC	Phe --> Ser	TTTT	NT
VS76[e]	374	4	A•T --> G•C	ACT TTA ACT	Leu --> Ser	TTT	NT
VS48	392	5	A•T --> G•C	GTC TTG ATT	Leu --> Ser	TT	NT
VS75	294 295	tandem 3	A•T --> C•G A•T --> C•G	GTA GAT TTT GAT TTT ATC	Asp --> Glu Phe --> Val	TTTT	NT
VS62[e]	417	6	A•T --> C•G	GAC ACT GGC	No change	CT	NT
VS64	436	6	A•T --> C•G	ACT TTG CTT	Leu --> Val	TTT	NT
VS26	604	8	A•T --> T•A	GAT TTG AAT	Leu --> Met	TTT	NT
VS76[e]	605	8	A•T --> T•A	GAT TTG AAT	Leu --> STOP	TTT	NT
VS30	643	9	A•T --> T•A	GCA AAA TAC	Lys --> STOP	TTTT	T

Table 2. Mutants with putative splice site mutations

Mutations induced in coding region of *HPRT* gene in:	Mutant	Missing exon
XP4BE cells in early S-phase	VS34	2
	VS14	4
	VS53	5
	VS7	8
	VS11[a]	8
	VS20	8
	VS32	8
	VS56	8
	VS79	8
	VS60	8
	VS37	18 bp missing from 1st part of exon 9, 610-627
Normal cells in early S-phase	NUS2[b]	4
	NUS30	4
	NUS18[b]	5
	NUS17[b]	5
	NUS22[b]	7
	NUS11[b]	7
	NUS10[b]	8
	NUS12[b]	8
	NUS20[b]	10 bp deletion, 536-545 very near 5' end of exon 8
XP4BE cells in early G_1-phase	VG92	4
	VG98	4
	VG106	7
	VG61	8
	VG96	8
	VG107	8
	VG24	21 bp missing from 1st part of exon 8, 533-553
	VG91	21 bp missing from 1st part of exon 8, 533-553

[a]Contained more than one mutation, nontandem.

[b]Reported previously by McGregor et al. (18).

TABLE 3. Kinds and locations of mutations induced in the coding region of the *HPRT* gene of normal human cells irradiated in early S-phase

Mutant[a]	Position		Exon	Type of mutation	Surrounding sequence[b]	Amino acid change	Premutagenic photoproduct[c]	Strand containing photoproduct[d]
Base substitutions involving cytosine:								
NUS23	103		2	G·C --> A·T	AGG GTG TTT	Val --> Met	CC	T
NUS24	173		3	G·C --> A·T	ATG GGA GGC	Gly --> Glu	TC or CC	T
NUS21	208 209	tandem	3	G·C --> A·T G·C --> A·T	AAG GGG GGC	Gly --> Lys	CCCCCC	T
NUS27[e]	390		5	G·C --> A·T	AAT GTC TTG	No change		NT
NUS16	463 464	tandem	6	G·C --> A·T G·C --> A·T	AAT CCA AAG	Pro --> Leu	TCC	NT
NUS14	471		6	G·C --> A·T	AAG ATG GTC	Met --> Ile	CC	T
NUS7	500 501	tandem	7	G·C --> A·T G·C --> A·T	AAA AGG ACC	Arg --> Lys	TCCT	T
NUS4	505		7	G·C --> A·T	ACC CCA CGA	Pro --> Ser	CCCC	NT
NUS28	509		7	G·C --> A·T	CCA CGA AGT	Arg --> Gln	TC	T
NUS1[f]	568		8	G·C --> A·T	GTA GGA TAT	Gly --> Arg	CC or CT	T
NUS29	569		8	G·C --> A·T	GTA GGA TAT	Gly --> Glu	TC or CC	T
NUS5	599 600	tandem	8	G·C G·C --> A·T	TTC AGG GAT	Frameshift	CCCT	T
NUS3	601		8	G·C --> A·T	AGG GAT TTG	Asp --> Asn	TC or CC	T
NUS9	447		6	G·C --> C·G	TCC TTG GTC	Leu --> Phe	CC	T
NUS6	600		8	G·C --> C·G	TTC AGG GAT	Arg --> Ser	CCC	T
Base substitutions involving thymine:								
NUS8	122		2	A·T --> G·C	GGA CTA ATT	Leu --> Pro	CT	NT
NUS1[f]	123		2	A·T --> G·C	GGA CTA ATT	No change	TT	T
NUS26	294		3	A·T --> G·C	GTA GAT TTT	No change	TTTT	NT
NUS27[e]	392		5	A·T --> G·C	GTC TTG ATT	Leu --> Ser	TT	NT
NUS19	498 499	tandem	7	A·T --> G·C A·T --> G·C	GTG AAA AGG GTG AAA AGG	No change Arg --> Gly	CCTTTT	T
NUS15	596		8	A·T --> G·C	TAC TTC AGG	Phe --> Ser	TT or TC	NT

[a]The mutants with identifying numbers lower than 23 were reported previously by McGregor et al. (18). They are included along with information from additional independent mutants from the present study. In addition to the independent mutants listed, two other mutants, NUS13 and NUS25, had an A·T --> C·G or A·T --> T·A base substitution at position 84 or 200, respectively. These substitutions caused a Tyr to STOP codon change or a Val to Glu amino acid change, respectively. However, there was no discernable photoproduct at either site.

[b-d]See Table 1, footnotes *b* to *d*.

[e]This mutant also contained an A·T --> G·C base substitution at position 392. The change at position 390 may have resulted from the TT photoproduct at position 391 and 392.

[f]See Table 1, footnote e.

(ii) Mutations found in cells irradiated in early G_1 to allow time for repair. We also analyzed 24 independent mutants from the XP variant cells irradiated in early G_1-phase (Tables 2 and 4). Eight of the 24 (33%) appeared to have a splice site mutation. Fifteen of the other 16 contained single base substitutions; one contained a tandem base substitution. All but two of the pyrimidines involved in these substitutions were located adjacent to another pyrimidine. For ease of comparison, data reported by McGregor et al. (18) for 22 mutants from normal cells irradiated in G_1-phase were included in Table 5. The kinds of base substitutions we found in XP variant cells from G_1-phase (Table 4) differed significantly from what was reported for normal cells similarly irradiated (Table 5) ($p=0.03$, using the Chi square test). In the XP variant cells, only 27% (4/15) of the substitutions were transitions, compared to 76% (13/17) in the normal cells ($p<0.01$). Only 13% (2/15) of the substitutions in the variant cells were G·C --> A·T transitions, compared to 47% (8/17) in the normal cells ($p<0.02$). Table 6 shows the distribution of base substitutions found in XP variants and normal cells irradiated in G_1-phase and in S-phase.

Strand distribution of the premutagenic lesions in the two types of cells. Knowledge of the kinds of photoproducts induced by UV (1, 8) allowed us to infer from the sequence data, the strand in which the photoproducts that resulted in the observed mutations, i.e., the premutagenic lesions, were located in the gene. These are listed in the last column of Tables 1, 3, and 4, and the totals are compared in Table 7. In the mutants derived from XP variant cells irradiated in S, the 29 premutagenic lesions were distributed: 41% transcribed strand, 59% nontranscribed strand. In the mutants derived from normal cells

TABLE 4. Kinds and locations of mutations induced in the coding region of the *HPRT* gene in XP variant cells irradiated in early G_1 phase

Mutant[a]	Position	Exon	Type of mutation	Surrounding sequence[b]	Amino acid change	Premutagenic photoproduct[c]	Strand containing photoproduct[d]
Base substitutions involving cytosine:							
VG36	145	3	G·C --> A·T	CGT CTT GCT	Leu --> Phe	TC or CT	NT
VG13	151	3	G·C --> A·T	GCT CGA GAT	Arg --> STOP	TC	NT
VG80	96	2	G·C --> T·A	GAT TTG GAA	Leu --> Phe	CC	T
VG104[e]	195	3	G·C --> T·A	GCC CIC TGT	Leu --> Pro[e]	CICT	NT
VG122	209	3	G·C --> T·A	AAG GGG GGC	Gly --> Val	CCC	T
VG111	325	4	G·C --> T·A	GAC CAG TCA	Gln --> Lys	CC	NT
VG21	529	7	G·C --> T·A	CCA GAC TTT	Asp --> Tyr	TC or CT	T
VG15	580	8	G·C --> T·A	CTT GAC TAT	Asp --> Tyr	TC	T
VS56	628	9	G·C --> T·A	AGT GAA ACT	Glu --> STOP	TC	T
Base substitutions involving thymine:							
VG104[e]	194	3	A·T --> G·C	GCC CIC TGT	Leu --> Pro[e]	CICT	NT
VG84	488	7	A·T --> G·C	AGC ITG CTG	Leu --> Ser	CI or IT	NT
VG10	203	3	A·T --> C·G	GTG CIC AAG	Leu --> Arg	CI or IC	NT
VG89	344	4	A·T --> C·G	ATA AAA GTA	Lys --> Thr	TITT	T
VG53	92	2	A·T --> T·A	GAG GAT TTG	Asp --> Val	IC	T
VG79	295	3	A·T --> T·A	GAT ITT ATC	Phe --> Ile	TITT	NT

[a]In addition to the independent mutants listed, one mutant, VG4, had an A·T --> T·A base substitution at position 109, causing an Ile to Phe amino acid change, and another mutant, VG117, had an A·T --> T·A substitution at postion 407, causing a Ile to Lys amino acid change. However, there was no discernable photoproduct at either site.

[b-d]See Table 1, footnotes *b* to *d*.

[e]This mutant contains a tandem mutation.

Table 5. Kinds and locations of mutations induced in the coding region of the *HPRT* gene of repair-proficient diploid cells irradiated in G_1 phase

Mutant	Position	Exon	Type of mutation	Surrounding sequence	Amino acid change	Premutagenic photoproduct	Strand with affected photoproduct[a]
Base substitutions involving cytosine:							
NUG6	44	2	G•C --> A•T	GAA CCA GGT	Pro --> Leu	CC	NT
NUG3	73 74	tandem 2	G•C --> A•T G•C --> A•T	ATA CCT AAT	Pro --> Phe	CC	NT
NUG14	112 113	tandem 2	G•C --> A•T G•C --> A•T	ATT CCT CAT	Pro --> Phe	CC	NT
NUG12	209	3	G•C --> A•T	AAG GGG GGC	Gly --> Glu	CCCCCC	T
NUG6	371	4	G•C --> A•T	TCA ACT TTA	Thr --> Ile	CT	NT
NUG19	464	6	G•C --> A•T	AAT CCA AAG	Pro --> Leu	CC	NT
NUG5	212	3	G•C --> T•A	GGG GGC TAT	Gly --> Val	CCCCC	T
NUG2	43	2	G•C --> C•G	GAA CCA GGT	Pro --> Ala	CC	NT
Base substitutions involving thymine:							
NUG4	124	2	A•T --> G•C	CTA ATT ATG	Ile --> Val	TT	T
NUG11	241	3	A•T --> G•C	GAT TAC ATC	Tyr --> His	TT	NT
NUG18	392	5	A•T --> G•C	GTC TTG ATT	Leu --> Ser	TT	NT
NUG17	532	7	A•T --> G•C	GAC TTT GTT	Phe --> Leu	CT or TT	NT
NUG1	603 604	tandem 8	A•T A•T --> G•C	GAT TTG AAT	Frameshift	TTT	NT
NUG10	67	2	A•T --> T•A	TTT TGC ATA	Cys --> Ser	TT	NT
NUG7	108	2	A•T --> T•A	GTG TTT ATT	Phe --> Leu	TTT	NT

[a] NT, Nontranscribed; T, transcribed.

TABLE 6. Types of base substitutions induced in the coding region of the *HPRT* gene in XP variant cells and normal cells irradiated in early G_1- and early S-phase phase

Type of base substitution	Number of substitutions observed[a]			
	Cells irradiated in G_1-phase		Cells irradiated in S-phase	
	XP variant	Normal[b]	XP variant	Normal[c]
Transitions	(27)[c]	(76)	(43)	(92)
G·C --> A·T	2 (13)	8 (47)	7 (23)	16 (64)
A·T --> G·C	2 (13)	5 (29)	6 (20)	7 (28)
Transversions	(73)	(24)	(57)	(8)
G·C --> C·G	0 (0)	1 (6)	1 (3)	2 (8)
G·C --> T·A	7 (47)	1 (6)	9 (30)	0 (0)
A·T --> C·G	2 (13)	0 (0)	4 (13)	0 (0)
A·T --> T·A	2 (13)	2 (12)	3 (10)	0 (0)
	15	17	30	25

[a]Numbers in parentheses are percentages of total base substitutions.

[b]Data are from McGregor et al. (18) and are included for ease of comparison.

[c]Values include data reported by McGregor et al. (18) along with those obtained in this study.

TABLE 7. Strand distribution[a] of the premutagenic lesions responsible for the mutations observed in XP variant and normal cells irradiated in early S-phase and early G_1-phase

	Strand distribution of the premutagenic lesions[b]			
	Cells irradiated in S		Cells irradiated in G	
Strand	XP Variant	Normal	XP Variant	Normal[c]
Transcribed	12 (41%)	14 (67%)	7 (50%)	3 (20%)
Nontranscribed	17 (59%)	7 (33%)	7 (50%)	12 (80%)
	29	21	14	15

[a]Strand assignments were made on the basis of which strand contained the photoproduct that presumably resulted in the observed mutation (see Table 1-4).

[b]Numbers in parentheses are percentages.

[c]Data for normal cells are taken from McGregor et al. (18) and are included for purposes of comparison.

irradiated in S, the 21 premutagenic lesions were distributed 67% transcribed strand, 33% nontranscribed strand. The chance that the strand distributions in the two types of cells are equal is p=0.08, using the Chi square test. In the normal cells irradiated in G_1 to allow at least six hours for excision repair prior to S-phase, the strand distribution was 20%:80%. In the XP variant cells irradiated in G_1 and allowed even more time for excision repair prior to S-phase, the distribution was 50%:50%.

DISCUSSION

The data in Table 6, comparing XP variant and normal cells, especially those from cells irradiated in early S-phase so that there would be little or no time for excision repair before replication of the *HPRT* gene (9), support the hypothesis that the significantly higher frequency of mutants in the XP variant cells reflects an abnormally error-prone replication complex bypassing unexcised lesions. The hypermutability of the XP variant cells cannot be explained merely by assuming that their *HPRT* gene contains more unexcised photoproducts than remain in normal cells. If that were the explanation, the *kinds* of base substitution and the *strand distribution* of the premutagenic photoproducts in the XP variant cells should be similar to those seen in excision repair-deficient from group A (18). Instead, they differ significantly ($p=0.03$). The data suggest that the replication complex of XP variant cells is less likely than that of normal cells to incorporate dAMP and dGMP opposite photoproducts during replication.

Evidence that the normal cells exhibit preferential incorporation of *dAMP* opposite photoproducts is the high proportion of G·C --> A·T substitutions, i.e., 64% in the cells irradiated in S-phase, and the very low proportion of substitutions involving thymine (28%). The preference for G·C --> A·T transitions among UV-induced base substitutions has also been seen in excision repair-deficient XP cells (18), as well as in many other studies of UV-induced mutations, including the *hprt* locus of an excision repair-deficient hamster strain (30), the *aprt* locus of CHO cells (7), and mutations induced on a shuttle vector in SV40-transformed fibroblasts from normal donors and from classic XP patients (3, 28, 33), and has been attributed to preferential incorporation of dAMP opposite a photoproduct (10, 14, 25). The vast majority of these involved

photoproducts located in the nontranscribed strand. In the variant cells, almost none (one of nine) were. Instead, virtually all of the substitutions involving cytosine in the transcribed strand were G·C --> T·A transversions. No such transversions are seen in normal cells. The XP variant cells showed a high proportion of substitutions involving thymine, 13 of 30 (43%), and 92% of these (12/13) resulted from photoproducts that had to have consisted of a TT dipyrimidine. The vast majority of these involved photoproducts that were located in the nontranscribed strand. Less frequent incorporation of dAMP opposite UV photoproducts by the XP variant cells would result in a significantly higher frequency of mutants since thymine is the base most frequently involved in UV photoproducts (8).

The data in Table 6 for the cells irradiated in early S-phase also suggest that the XP variant cells are less likely than normal cells to incorporate *dGMP* opposite UV photoproducts. Transversions made up a much higher proportion of the substitutions in the XP variant cells than in the normal cells (57% in XP variant cells, 8% in normal cells), implying that XP variant cells are less likely than normal cells to incorporate purines opposite pyrimidines involved in photoproducts. If the XP variant cells were to incorporate dAMP less frequently, as discussed above, but incorporated dGMP at the normal frequency, G·C --> T·A transversions would be very rare instead of occurring at a rate of 29%. Moreover, substitutions involving thymine should constitute a very large proportion of the total substitutions observed. They did not. Instead, they were only 43%, suggesting less frequent incorporation of dGMP, as well as dAMP. Less frequent incorporation of dGMP opposite UV photoproducts by the XP variant cells would also contribute to their significantly higher frequency of UV-induced mutants.

The majority of the substitutions seen in normal cells irradiated in S phase

are G·C --> A·T transitions from lesions in the transcribed strand (Tavle 3). If XP variant cells are less likely than normal cells to incorporate *purines* opposite photoproducts, the strand distribution of the pyrimidines involved in the observed mutations from S-phase cells should differ from normal. Table 7 shows that this is the case. The ratio of base substitutions involving G·C and A·T base pairs was 57:43 in XP variant cells irradiated in early S-phase. Therefore, it is not surprising that the pyrimidines involved in the mutations observed in the XP variant cells irradiated in early S-phase were evenly distributed between both strands, viz., 41% transcribed, 59% nontranscribed. The corresponding ratios in the normal cells are 72:28 and 67%:33%.

The strand distribution of the premutagenic lesions in mutants derived from the normal cells irradiated with 6 J/m^2 in mid G_1-phase (6 hr prior to S) differed from that seen in normal cells irradiated in S-phase, i.e.,20:80 compared to 67:33 ($p<0.01$) (Table 6). As suggested previously (18), this difference in strand distribution can be attributed to transcription-coupled, strand-specific repair of photoproducts in the transcribed strand during the 6 hr repair period (19). In the XP variant cells irradiated with 4 J/m^2 and allowed $\geq$ 11 hr for repair before S, there was no such difference in the strand distribution (50:50 compared to 41:59). This similar strand distribution between the XP variant cells irradiated in S- and G_1-phase could reflect a lack of strand-specific repair in the *HPRT* gene of the variant cells. However, the overall rate of excision of CPD and 6-4's in XP variant cells was equal to that in the normal cells, and Mayne and Lehmann (16) showed that the rate of recovery of RNA synthesis in XP variant cells after UV irradiation is the same as in normal cells. The latter data imply that preferential repair and probably strand-specific repair also occurs in XP variant cells. A more likely explanation for the similar strand distribution in the XP variant cells irradiated in S- and G_1-

phase is that during the 11 or more hours available for repair before S-phase, the XP variant cells irradiated in G_1 removed the majority of the photoproducts from both strands. If so, among the mutants that we recovered, the distribution of premutagenic lesions (pyrimidines involved in a mutation) might well reflect the original distribution, i.e., that seen in mutants recovered from the S-phase cells that had little or no time for repair. Removing photoproducts preferentially from the transcribed strand would be obscured. In the study by McGregor et al. (18), the normal cells had less time for repair before the onset of S-phase, and therefore, the preferential removal of lesions from the transcribed strand could be detected.

In summary, our data strongly suggest that the significant difference between XP variant and normal cells in the *kinds* of base substitutions induced by UV results from XP variant cells being less likely than normal cells to incorporate dAMP and dGMP opposite unexcised photoproducts during replication. Unlike normal cells, the variant cells incorporate dTMP opposite cytosine-containing photoproducts located in the transcribed strand, and dAMP opposite such lesions mainly in the nontranscribed strand. The mechanisms responsible for this infrequent incorporation of dAMP and dGMP (nucleotide pool imbalances in the XP variant, abnormal binding affinity between nucleotides and the replication complex, etc.) can best be investigated using an in vitro replication fidelity assay, such as that employed by Roberts and Kunkel (27) or by Carty et al. (4). An error-prone replication process would account for the XP variant cells' UV hypermutability. If the replication complex was stalled because of an inability to incorporate purines opposite photoproducts, this might account for the XP variant cells' abnormal delay in producing nascent DNA of a size greater than the interdimer length.

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CHAPTER IV

Rate of repair of the two major UV photoproducts in the *HPRT* gene of normal human fibroblasts and XP variant fibroblasts

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SUMMARY

Xeroderma pigmentosum (XP) variant patients are genetically predisposed to sunlight-induced skin cancer, and cells from these patients have a significantly higher frequency of UV_{254nm}-induced mutations than do cells from normal donors. We showed recently that the spectrum of mutations in the hypoxanthanine (guanine) phosphoribosyltransferase (*HPRT*) gene induced by UV in XP variant cells differs very significantly from that found in normal cells (Wang et al., Mol. Cell. Biol. 1993, 13:4276-4283). Although that study showed that XP variant cells excise UV photoproducts from their overall genome at the same rate as normal cells, their higher frequency of UV-induced mutations in the HPRT gene might reflect a slower than normal rate of repair of photoproducts from specific active genes, such as *HPRT*. In addition, if XP variant cells differed significantly from normal cells in rate of repair of a particular type of UV photoproduct in the *HPRT* gene, this might explain the observed differences in the spectrum of mutations induced in these two types of cells. To test these hypotheses, we compared the rate of repair of the two major UV photoproducts, 6-4 pyrimidine-pyrimidone photoproducts (6-4's) and cyclobutane pyrimidine dimers (CPD) in each strand of the *HPRT* gene of synchronized XP variant and normal cells irradiated in early G_1-phase. In the normal cells, the rate of repair of 6-4's from the *HPRT* gene was faster than from the genome overall but both strands appeared to be repaired equally fast, with 72% lost from the transcribed strand and 65% from the nontranscribed strand in 1 hr. We showed previously that only 40% of 6-4's were lost from the overall genome in that period of time after a dose of 6 J/m^2. The rate of loss of CPD from the transcribed strand of the *HPRT* gene in the normal cells was 63% within 6 hr; from the nontranscribed strand it was 47%.

This was much faster than the rate of loss of CPD from the genome overall, i.e., 22% in 6 hr after a dose of 6 J/m^2. Similar comparative studies in the XP variant cells are not yet complete, but evidence to date suggests that the rates of repair of 6-4's in the two strands of the *HPRT* gene are similar to those in normal cells. However, the data we have obtained so far suggest that XP variant cells are slower than normal cells in the rate of repair of CPD from each strand of the *HPRT* gene. In fact, the rate of loss of CPD appears to be similar to the rate of loss from their genome overall. If this proves to be the case, this slow repair of CPD in XP variant cells would contribute to their UV hypermutability.

INTRODUCTION

Xeroderma pigmentosum (XP) variant patients inherit the predisposition to sunlight-induced skin cancer characteristic of classic nucleotide excision repair-deficient XP patients (1). Fibroblasts from XP variant patients have a significantly higher frequency of UV_{254nm}-induced mutations in the hypoxanthanine (guanine) phosphoribosyltransferase (*HPRT*) gene than do cells from the normal donors (2,3). However, in contrast to the cells of classic XP patients, XP variant cells are reported to excise UV photoproducts at a normal or near-normal rate, as measured by the unscheduled DNA synthesis in nonreplicating cells after UV irradiation (4,5). Recently, we showed that synchronized populations of XP variant cells excise the two major UV photoproducts, cyclobutane pyrimidine dimers (CPD) and 6-4 pyrimidine-pyrimidone photoproducts (6-4's), at a normal rate during the S-phase and G_1-phase of the cell cycle (6). However, that same study showed that the kinds and location of mutations induced in the coding region of the *HPRT* gene of UV-irradiated XP variant cells differ significantly from those in the normal cells. We concluded that if XP variant cells also excise the two photoproducts at a normal rate in specific actively-transcribed genes, such as the *HPRT* gene, then for a given dose of UV, the replication complexes of normal and XP variant cells encounter the same number of unexcised UV photoproducts, but XP variant cells use an abnormal error-prone process to bypass UV photoproducts during replication, and this results in their UV hypermutability.

In the same study, we also showed that there are significant differences between XP variant and normal cells in the distribution of photoproducts that resulted in the observed mutations, i.e., the

premutagenic lesions. In the mutants derived from cells irradiated in early S-phase to allow virtually no time for repair before the *HPRT* gene is replicated, the premutagenic lesions were distributed 67% transcribed strand: 33% nontranscribed strand in normal cells, whereas the strand distribution was 41%:59% in XP variant cells (P=0.08). In the normal cells irradiated in mid-G_1 phase to allow at least 6 hr for repair prior to S-phase, the frequency of mutants decreased by half, and the strand distribution of the premutagenic photoproducts changed to 20%:80%, suggesting a strand-specific loss of photoproducts from the transcribed strand during the 6 hr repair period (7). Allowing these cells at least 11 hr for repair prior to S-phase lowered the frequency to near-background level. However, when the XP variant cells were irradiated in early G_1-phase to allow 11 hr for repair prior to S, the frequency of mutants decreased by about 65% and yet, there was no significant change in the strand distribution of the premutagenic lesions (50%:50% versus 41%:59%). The significant difference between XP variant and normal cells in the strand distribution of the premutagenic lesions in cells irradiated in S-phase suggests that XP variant cells handle the photoproducts remaining in DNA very differently from normal cells. This might explain the higher than normal frequency of mutations in these cells. However, the lack of a change in the strand distribution between the XP variant cells irradiated in S- and in G_1-phase predicts a lack of strand-specific repair in the *HPRT* of XP variant cells. If this is true, it could also play a role in the abnormally high frequency of UV-induced mutations in the *HPRT* gene of these cells. What is more, if XP variant cells were slower than normal cells in the rate of repair of a particular type of UV photoproduct in the HPRT gene, this might help explain the differences between these

two types of cells in the spectrum of mutations induced.

The present study was designed to measure the rate of loss of the two major photoproducts from these two types of cells, to determine if they carry out preferential and strand-specific repair of photoproducts in the *HPRT* gene, and whether XP variant cells differ from normal cells in their rate of repair of photoproducts in this gene. We synchronized the cells, irradiated them in early G_1 phase, harvested the cells immediately or after various times post-irradiation to allow time for repair, and assayed the DNA for the number of photoproducts remaining in specific strands of the *HPRT* gene. To assay for CPD, digested DNA was treated with CPD-specific T4 endonuclease V (T4 endo V); to assay for 6-4's, the DNA was treated with photolyase, which specifically reverts the CPD, and then treated with UvrABC excinuclease. The samples were analyzed by Southern hybridization using strand-specific probes.

In the normal cells, the rate of repair of 6-4's from the *HPRT* gene was faster than from the genome overall, but both strands appear to be repaired equally fast. For CPD, we found evidence of preferential and strand-specific repair of CPD in the *HPRT* gene. Although the research on the rate of excision repair in the XP variant cells is still in progress, the evidence to date suggests that the rate of repair of 6-4's in the *HPRT* gene of XP variant cells is very similar to that seen in normal cells. However, the evidence to date suggests that XP variant cells are slower than normal cells in the rate of repair of CPD in each strand of the *HPRT* gene. In fact, the loss of CPD does not appear to be much faster than the rate of loss of CPD from their genome overall. If this proves to be correct, slow repair of CPD in XP variant cells should contribute to their UV hypermutability.

MATERIALS AND METHODS

Cells, Cell Culture, and Synchronization. Diploid human male fibroblasts, designated NFSL89, were explanted from the foreskin of a normal newborn as described (8). XP variant cells, XP4BE (CRL1162), were derived from skin biopsies of a male XP variant patient that were obtained from the American Type Culture Collection (Rockville, MD). Cells were routinely cultured in Eagle's minimal essential medium containing 10% fetal bovine serum. For synchronization, cells were driven into the G_0 state by density inhibition and nutrient depletion as described (9). To stimulate the cells to reenter the cell cycle, they were released from confluence and plated in serum containing medium at a density of 10^4 cells per cm^2. For thymidine incorporation experiments, serum containing medium was also supplemented with [^{3}H]thymidine (5 μCi/ml of medium, 78.5 Ci/mmol; New England Nuclear, Dupont, Wilmington, DE).

UV Irradiation and Post-UV Incubation. Cells in early G_1, six hours after release from G_0, were rinsed with phosphate-buffered saline (PBS) and irradiated with UV_{254nm} as described (10). The doses of irradiation were 9-14 J/m^2 for the assay of the rate of repair of CPD and 33-40 J/m^2 for the repair of 6-4's. Cells were lysed immediately after UV irradiation or incubated in fresh medium for various periods of time to allow time for repair before being assayed for the number of unexcised lesions. Cells were washed with ice-cold PBS before being lysed. The lysis buffer contained 50mM Tris·HCl, pH 8.0, 10mM EDTA, 100mM NaCl, 50% SDS, and proteinase K at 100μg/ml.

Isolation and Purification of DNA. The experimental conditions and protocol for DNA extraction have been described previously (11). Briefly, the purified DNA was digested with *Bam*HI (5 units/ μg of DNA) at 37°C for

4-12 hr, deproteinated by extraction with phenol, phenol/chloroform, and finally chloroform, precipitated with ethanol, redissolved in TE buffer (10mM Tris HCl, pH 7.5, 1mM EDTA) at a concentration of 0.6 mg/ml and used for further excision reaction. The completion of digestion was verified by electrophoresis of 1/20 of the samples in agarose minigels.

T4 Endonuclease V Excision Reaction. T4 endo V was kindly provided by Dr. Stephen Lloyd (U. of Texas Medical Branch, Galveston, TX). Twelve micrograms of digested DNA from each repair time point was used for the assay of CPD repair. As an internal standard, 6 pg of EcoRI-linearized plasmid was added to each DNA sample. This mixture was divided into two aliquots which were then treated in parallel, one with 6 ng of T4 endo V and the other with endonuclease buffer alone (10 mM Tris, pH 7.4, 10 mM EDTA, 100 mM NaCl, 1 μg/ml bovine serum albumin (BSA)). The final volume of each reaction mixture was 20 μl. Samples were incubated for 3 hr at 37°C. The reaction was stopped by the addition of 1:10 dilution of the alkaline loading dye of a concentration of 0.75 M NaOH, 15 mM EDTA, 37.5% Ficoll, and 0.45% Bromcresol green. The samples were then ready for electrophoresis.

Photolyase Reaction. *Escherichia coli* DNA photolyase was kindly provided by Drs. Aziz and Gwendolyn Sancar (U. of North Carolina, Chapel Hill, NC). Details concerning the preparation and properties of photolyase have been published (12) . Eighteen micrograms of digested DNA (0.6 mg/ml), along with 6 pg of linearized plasmid for an internal standard, was incubated with a buffer containing 50 mM Tris, pH7.5, 100 mM NaCl, 1mM EDTA, 10 mM β-mercaptoethanol, 100 μg/ml DNase-free BSA, and 0.25 μg of photolyase per μg of DNA in a final volume of 50 μl in a 0.5 ml Eppendorf tube. The reaction mixture was incubated for 5 min at room temperature in the dark.

The tube was then placed on ice in a petri dish and covered with a plastic dish lid to avoid formation of additional dimers in the DNA during photoreactivation. Photoreactivation was initiated by illuminating the tube for 20 min with photoreactivating light supplied by GE black lights (F15T8/BLB, λ_{max}=365$_{nm}$) at a distance of 20 cm from the sample. At the end of the reaction, the DNA was deproteinated, precipitated, and redissolved in TE buffer as described above. The photoreactivated DNA was further divided into three aliquots. One aliquot was treated with T4 endo V as described above to determine whether the photoreactivation was complete. Another was treated with UvrABC excinuclease complex. A third was incubated with excinuclease buffer.

UvrABC Excision Reaction. The UvrA, UvrB, and UvrC protein subunits were kindly provided by Dr. Pieter van de Putte (Leiden Univ., Leiden, the Netherlands). Six micrograms of photoreactivated DNA was exposed to UvrABC excinuclease. The excinuclease reaction mixture was composed of 6 μg of digested DNA plus 17 *p*mol of each of the three subunits of UvrABC in a final volume of 250 μl of buffer containing 50 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 75 mM KCl, 2 mM ATP, 1 mM dithiothreitol, and 1 μg/μl BSA. At the end of 1 hr of incubation at 37°C, the reaction was stopped by adding proteinase K at 0.1 μg/μl and 0.1% SDS to each sample and incubating for 1 hr at 37°C. The DNA was purified by ultrafiltration using Centricon-30 tubes (Amicon, Beverly, MA) and then precipitated and redisolved in TE buffer as described above. Alkaline loading dye was added to the sample as described above and the sample was ready for electrophoresis.

Gel Electrophoresis, Southern Blot Analysis, and Hybridization Probes. Immediately before being loaded and electrophoresed, the DNA samples in the alkaline loading dye were incubated at 55°C for 30 min to ensure that

they were completely denatured. The samples were loaded and electrophoresed at 27-29 volts, room temperature for 12-15 hr in a 0.6% agarose gel containing 30 mM NaOH and 1 mM EDTA. After electrophoresis, the DNA was depurinated with acid, using standard procedure (13), and transferred to a Zeta-Probe GT membrane (Bio-Rad) under the conditions recommended by the manufacturer. The hybridization probe has been described previously (11). The ^{32}P-labeled riboprobes were generated by using the method described by Melton et al. (14), with the modifications suggested by Boehringer Mannheim. Hybridization and post-hybridization washing were performed using the conditions described previously (11).

Quantitation. The intensities of bands were quantified with a Bioinage densitometer as described (11). The average number of T4 endo V- or UvrABC-sensitive sites per fragment was calculated by the Poisson distribution equation, as described by Bohr and Okumoto (15). Briefly, the fraction of fragments containing no photoproducts (the zero class) is equal to the ratio of the amount of full-length fragments in the enzyme-treated and untreated samples. The average number of photoproducts per fragment was calculated from the zero class measurements by using the Poisson expression (the number of photoproducts per fragment = -ln of the zero class). For example, if the integrated optical density of the full length fragment of treated and untreated samples were 0.55 and 1.50, respectively, the percentage of fragments containing no photoproducts (the zero class) will be 0.55/1.50 = 0.37. Therefore, the average number of photoproducts will be -ln 0.37 = 1. These calculations took into consideration the non-specific incisions produced per 20-kb fragment by the T4 endo V and by UvrABC excinuclease, which ranged from 0 to 0.19 and 0 to 0.08, respectively.

RESULTS

Determination of the Delay in Onset of DNA Replication Induced by UV_{254nm}. Before analyzing the rate of repair of UV photoproducts in the DNA of irradiated cells, we determined of the delay in onset of DNA replication induced by UV. Cells were released from the G_0 state and plated into a series of dishes at 10^4 cells per cm^2. After 6 hr, when cells had become fully flattened, they were irradiated at a dose of 10 J/m^2 and the incorporation of [^{3}H]-thymidine was measured at various times after irradiation for up to 16 hr for normal cells or up to 22 hr for XP variant cells. In the unirradiated populations, DNA replication began about 15 hr after releasing from confluence in both types of cells. In the irradiated populations, there was no incorporation of thymidine whatsoever at any of the times examined (data not shown). The total absence of any DNA replication after irradiation of the synchronized cells in early G_1 with a dose of 10 J/m^2 simplified the assay of repair because there was no interference with the repair analysis by newly synthesized DNA during the period of repair time examined.

Repair of Cyclobutane Pyrimidine Dimers in Normal Human Cells. Synchronized normal cells were irradiated in early G_1 phase at a dose of 9 J/m^2 or 14 J/m^2. In each experiment, one irradiated population was harvested immediately to measure the initial number of CPD in the individual strands of the 20-kb *Bam*HI fragment of *HPRT* gene. The other irradiated populations were harvested after various times for up to 16 hr after irradiation to allow time for repair before being assayed for the number of CPD remaining in the *HPRT* gene. The unirradiated population served as a control to be used to determine if T4 endo V introduced any

non-specific incisions in unirradiated DNA. Representative autoradiograms of such repair studies are shown in Fig. 1. The data from two separate experiments are given in Table 1 and the curve for rate of repair as a function of time is shown in Fig. 2.

The number of incisions in the DNA of cells harvested immediately after UV irradiation increased with dose and was a little higher in the transcribed strand of the *HPRT* gene fragment than in the nontranscribed strand. However, the rates of CPD removal from the transcribed and nontranscribed strand differed significantly. For the cells irradiated at a dose of 14 J/m^2, within 6 hr, 63% of the dimers had been removed from the transcribed strand, while only 47% had been removed from the nontranscribed strand; by 16 hr, almost all the dimers had been removed from the transcribed strand, whereas 16% still remained in the nontranscribed strand of the *HPRT* gene. To determine whether the rate of repair of CPD in the *HPRT* gene was significantly faster than that in the genome overall, we compared the data in Table 1 with the data we obtained previously (6) on rate of loss of CPD from the overall genome during the G_1-phase of cell cycle, using a radioimmunoassay and antibodies specific for CPD. These data from cells irradiated with 6 J/m_2 are included in Fig. 2. The radioimmunoassay showed that within 6 hr, only 22% of CPD had been removed from the overall genome, and only about 50% of CPD had been removed from the overall genome by 11 hr.

Repair of 6-4 Photoproducts in Normal Human Cells. Based on the estimation of a lower level of incidence of 6-4's than of CPD in various studies (16,17), a dose of 33 J/m^2 was used for the assay of repair of 6-4's. In addition, because we had shown that the rate of repair of 6-4's from the genome overall was much faster than that of CPD (6), we allowed

Figure 1. Autoradiograms illustrating extent of repair of CPD in the (A) transcribed and (B) nontranscribed strands of the *HPRT* gene in normal cells. DNA was isolated from unirradiated cells (first two lanes of panel A) or from cells incubated for the indicated repair period (h) after irradiation with a dose of 9 J/m^2. DNA samples were digested with *BamH*I into a 20-kb fragment and plasmids containing the sequence to be probed were included in each DNA sample as an internal loading control. The samples were then treated (+) or not treated (-) with T4 endo V and subjected to electrophoresis and Southern hybridization with ^{32}P-labeled strand-specific probes. Upper bands correspond to the 20-kb fragment of the *HPRT* gene. Lower bands correspond to the DNA fragments serving as internal loading control. In this series of samples, the plasmid was not linearized. The band intensity of the upper bands of first two lanes of panel A is low due to the insufficient amount of DNA loaded.

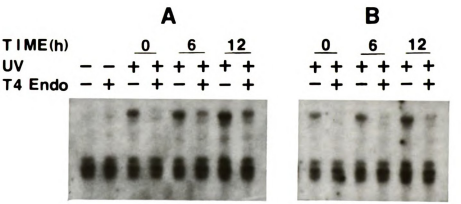

Figure 1

Table 1

Formation of cyclobutane pyrimidine dimers in the individual strands of the 20-kb *Bam*HI fragment of the *HPRT* gene and their rates of removal in normal cells

Fluence J/m^2	Repair time (hr)	Transcribed strand		Nontranscribed strand	
		Incisions/ fragment[a]	Percent repaired	Incisions/ fragment[a]	Percent repaired
14	0	2.94	0	2.79	0
	7	0.65	78	1.55	44
	16	0.07	98	0.45	84
9	0	2.23	0	2.17	0
	6	0.82	63	1.14	47
	12	0.16	93	0.37	82

[a]Calculated from the densitometric scanning as described. The nonspecific incisions, ranging from 0 to 0.08, have been subtracted.

Figure 2. Rate of removal of CPD from the transcribed (circles) and nontranscribed (squares) strands of the *HPRT* gene and from the genome overall (triangles) in normal cells. The data for the *HPRT* gene was determined by using T4 endo V. Open symbols indicate data obtained from cells irradiated with 9 J/m^2; closed symbols from cells irradiated with 14 J/m^2. The data for the overall genome were determined by a radioimmunoassay using antibodies specific for CPD and are taken from our previous study (6) for ease of comparison.

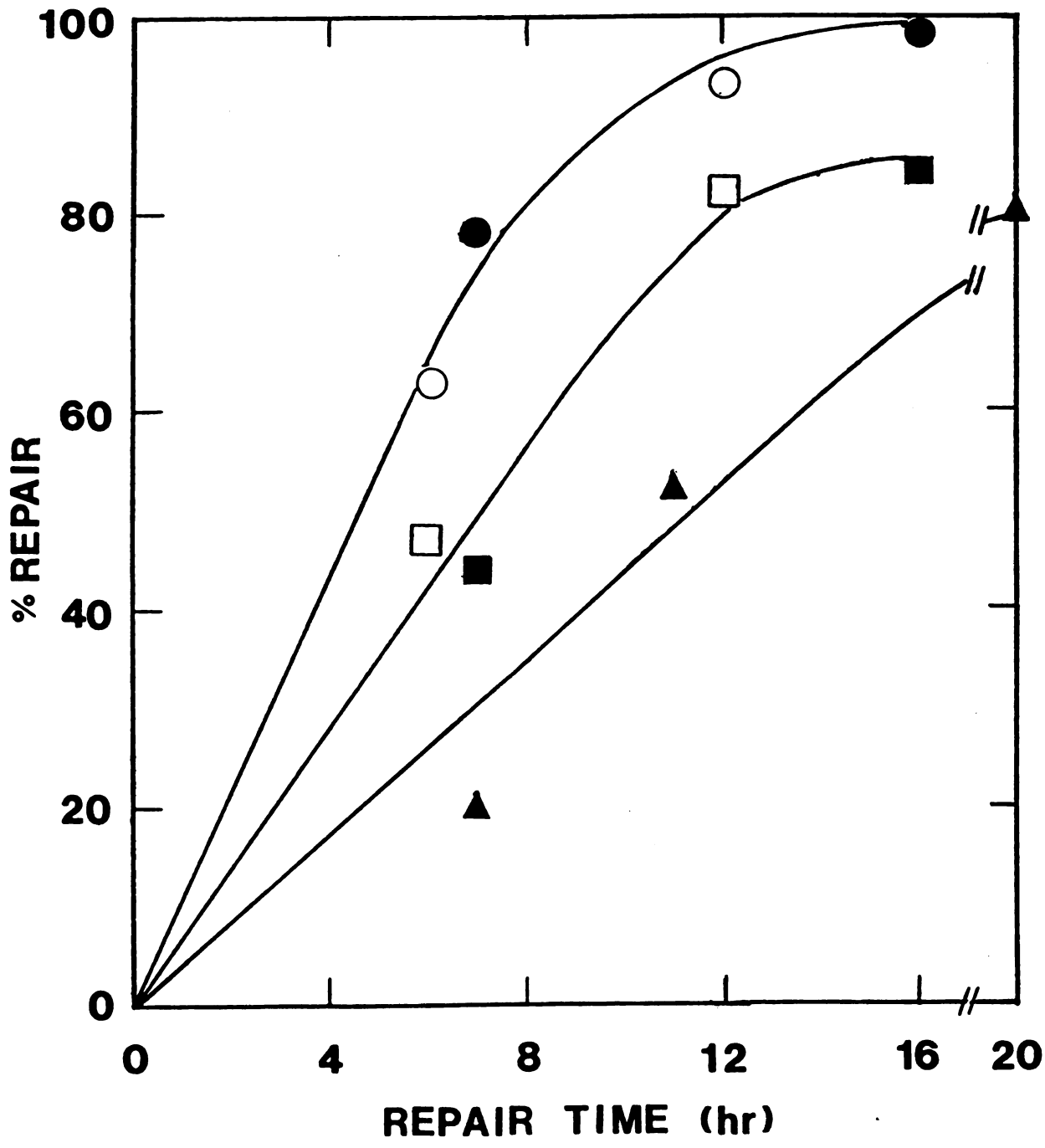

Figure 2

shorter time periods for repair of 6-4's, i.e., 0-4 hr repair periods. The data are given in Table 2 and the curve for rate of repair as a function of time is shown in Fig. 3.

The density of the full length *HPRT* fragment of the lanes representing T4 endo V treatment after photoreactivation was about the same as of the lanes of nontreated control. Densitometric scans of these autoradiograms indicated that fewer than 0.2 of CPD remained in either strand of *Bam*HI fragment after photoreactivation (data not shown), demonstrating that the photoreactivation was complete. The scan also showed that the rate of removal of 6-4's from either strand was equally fast. Within 1 hr, 72% and 65% of the 6-4's had been removed from the transcribed and nontranscribed strands, respectively. By 4 hr, all of the 6-4's had been removed from both strands. Although there was no evidence of strand-specific repair, the rate of repair of 6-4's in either strand of the *HPRT* gene was faster than that in the overall genome measured previously (6) using a radioimmunoassay (Fig. 3). Extrapolation of the curve for the latter study shows a rate of loss of about 40% of the 6-4's in 1 hr.

Repair of UV Photoproducts in XP Variant Cells. Please note that the data presented for the XP variant cells are preliminary results taken from ongoing research. The data are preliminary because insufficient sample DNA was loaded in the gels and the band intensity of the autoradiograms corresponding to the 20-kb *HPRT* fragment was too light to be measured accurately. These experiments are being repeated. However, the preliminary data were included here because they suggest that the rate is slower than normal, and if so, this could shed light on the very significant difference between XP variant and normal cells in their spectrum of mutations induced by UV (6).

Table 2

Formation of (6-4) pyrimidine-pyrimidone photoproducts in the individual strands of the 20-kb *Bam*HI fragment of the *HPRT* gene and their rates of removal in normal cells

Fluence J/m^2	Repair time (hr)	Transcribed strand		Nontranscribed strand	
		Incisions/ fragment[a]	Percent repaired	Incisions/ fragment[a]	Percent repaired
33	0	0.54	0	0.52	0
	1	0.15	72	0.18	65
	2	0	100	0.08	85
	4	0	100	0	100

[a]Calculated from the densitometric scanning as described. The nonspecific incisions, ranging from 0 to 0.19, have been subtracted.

Figure 3. Rate of removal of 6-4's from the transcribed (circles) and nontranscribed (squares) strands of the *HPRT* gene and from the genome overall (triangle) in normal cells. The data for the *HPRT* gene was determined by photoreactivation in conjunction with UvrABC excinuclease. The data for the overall genome were determined by a radioimmunoassay using antibodies specific for 6-4's and taken from our previous study (6) for ease of comparison. The dotted line connecting time points between 0 hr and 7 hr was drawn by extrapolating the measurement obtained from the rate of removal of 6-4's during S-phase because our data in ref.6 suggest that the rate of removal of 6-4's during G_1-phase is similar to that during S-phase. The three dashed lines represent the data obtained for the rate of repair of CPD taken from Figure 2 to show the difference in the rate of repair of the two types of photoproducts.

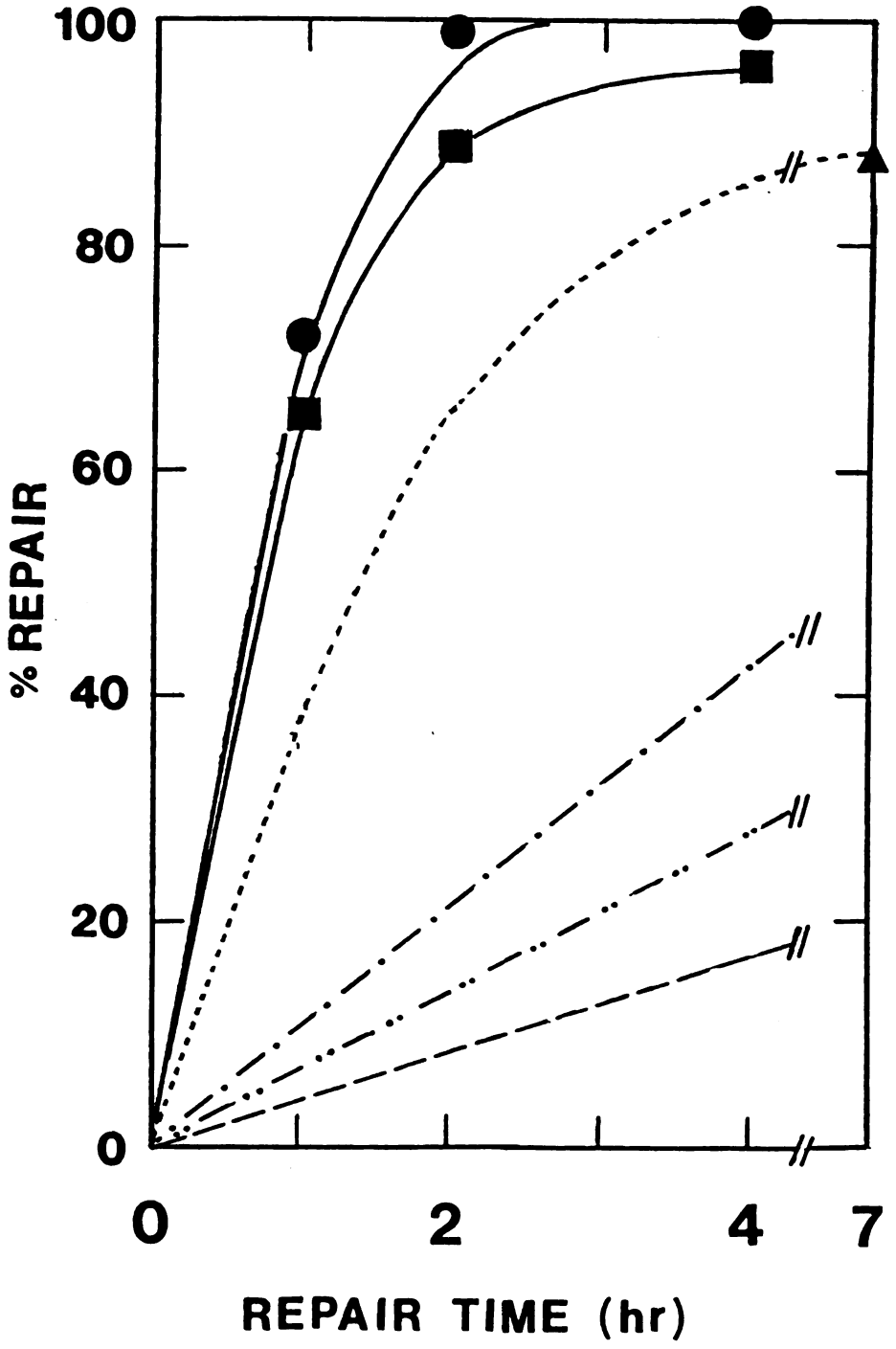

Figure 3

(i) Repair of 6-4 Photoproducts. Synchronized XP variant cells were irradiated with 33 J/m^2 or 40 J/m^2 and harvested for the assay of 6-4's repair as described above. Estimtaion of the results from these two separate irradiated populations are given in Table 3. Densitometric scans of autoradiograms suggested that the rate of 6-4's removal from either strand was equally fast and similar to that which we found with normal cells. The data suggest that within 1 hr, approximately 70% of the 6-4's had been removed from each strand; by 4 hr, it appeared that almost all of the 6-4's had been removed from each strand. If further studies prove this to be the case, then the rate of repair of 6-4's in either strand of the *HPRT* gene is faster than that which we measured previously (6) in the overall genome, using a radioimmunoassay. Extrapolation of the curve for the latter shows a rate of loss of about 40% of the 6-4's in 1 hr.

(ii) Repair of cyclobutane pyrimidine dimers. Synchronized XP variant cells were irradiated with a dose of 10 J/m^2 and harvested for the assay of CPD repair as described above. The time allowed for repair was up to 16 hr after UV irradiation. The autoradiograms used for calculating the proportion of 20-kb fragment that did not get cut by T4 endo V were almost too faint to be accurately quantified. However, estimation of the number of T4 endo V incisions and the percentage of CPD removed are given in Table 4. The data we have obtained to date, although preliminary, suggest that the rate of loss of CPD from the transcribed and the nontranscribed strand of the *HPRT* gene in variant cells is slower than from the corresponding strand in normal cells. Within 6 hr, only 56% of the dimers appeared to have been removed from the transcribed strand; while only 33% had been removed from the nontranscribed strand. By 16 hr, about 75% of the dimers appeared to have been removed from the transcribed strand,

Table 3

Formation of (6-4) photoproducts in the individual strands of the 20-kb *Bam*HI fragment of the *HPRT* gene and their rates of removal in XP variant cells

Fluence J/m^2	Repair time (hr)	Transcribed strand		Nontranscribed strand	
		Incisions/ fragment[a]	Percent repaired	Incisions/ fragment[a]	Percent repaired
33	0	0.71	0	0.90	0
	1	0.27	66	0.20	78
	2	0.08	89	0.08	91
	4	0	100	0.09	90
40[b]	0	0.88	0	1.02	0
	1	0.29	67	0.39	62
	2	0.06	93	0.29	72
	4	0	100	0	100

[a]Calculated from the densitometric scanning as described. The nonspecific incisions, ranging from 0 to 0.01, have been subtracted.
[b]The exact fluence given is not known, but is estimated to be 40 J/m^2 from the number of UvrABC cut sites obtained at time 0 hr.

Table 4

Formation of cyclobutane pyrimidine dimers in the individual strands of the 20-kb *Bam*HI fragement and their rates of removal in xeroderma pigmentsoum variant cells

Fluence J/m^2	Repair time (hr)	Transcribed strand		Nontranscribed strand	
		Incisions/ fragment[a]	Percent repaired	Incisions/ fragment[a]	Percent repaired
10	0	2.42	0	1.49	0
	6	1.06	56	0.98	33
	11	0.50	79	0.97	34
	16	0.68	72	0.93	36

[a]Calculated from the densitometric scanning as described. The nonspecific incisions, ranging from 0 to 0.07, have been subtracted.

whereas only 36% appeared to have removed from the nontranscribed strand. These experiments are being repeated. At the present time, the average rate of repair of CPD in the *HPRT* gene appears to be similar to that what we found (6) in the overall genome using a radioimmunoassay.

DISCUSSION

Our data on the rate of repair of UV photoproducts in the 5' half of the *HPRT* gene of normal cells provide direct evidence of preferential and strand-specific repair of CPD in the *HPRT* gene (Table 1, and Figure 2). This observation correlates with a strand bias for mutation induction towards photoproducts in the nontranscribed strand after at least 6 hr of repair found in normal cells (7). In addition, our results for repair of CPD in the *HPRT* gene of normal human cells are similar to what was found for the adenosine deaminase (*ADA*) and dihydrofolate reductase gene (*DHFR*) genes of normal human cells (18), and the amplified *DHFR* gene of human cells (19). However, the rate of repair of 6-4's we obtained is much faster than that obtained by other groups of investigators in the assay of the *dhfr* gene of Chinese hamster ovary (CHO) cells. Thomas and colleagues reported that 54% of 6-4's are removed from the *dhfr* gene of CHO cells in 8 hr after irradiation (20). A similar result was reported by Link et al. (21) in the *dhfr* gene of CHO cells, i.e., 62% and 43% of 6-4's are removed from the transcribed and nontranscribed strands, respectively, in 8 hr. In the present study, we found a significantly faster rate of repair of 6-4's in the *HPRT* gene of human cells, i.e., 100% and 85% repaired within 2 hr from the transcribed and nontranscribed strands, respectively (Table 2).

Possible explanations for the faster rate of repair of 6-4's we observed compared to other groups are: a) human cells may repair 6-4's faster than do CHO cells; b) although the *HPRT* and the *dhfr* gene are both actively transcribed genes, different gene fragments may have different repair efficiencies; and c) different cell culture methods were used. In

our study, the cells were synchronized and irradiated in early G_1 phase. In the studies by others, the cells were in exponential growth at the time of irradiation. It is possible that the rate of repair differs in various phases of the cell cycle.

Our data obtained with the XP variant cells, although preliminary, suggest that these cells are slower than normal cells in their rate of repair of CPD in either strand of the 5' half of the *HPRT* gene. This result is consistent with our observation that when the XP variant cells were irradiated in early G_1-phase and allowed 11 hr for repair prior to S, the frequency of mutation decreased by 65%, whereas in normal cells given the same amount of time for repair, the frequency decreased to near-background level.

The data we have obtained so far on the rate of repair of CPD in either strand of the *HPRT* gene of the XP variant cells suggest that although the rates are slower than in normal cells, the transcribed strand is repaired faster than the non-transcribed strand. However, our study of the mutation spectra in the coding region of *HPRT* gene of XP variant cells, allowed 11 hr for repair, showed no significant decrease in the proportion of mutations derived from photoproducts in the transcribed strand. This suggests a lack of strand-specific repair. Therefore, there must be other factors, in addition to the rate of repair of photoproducts, that play a role in producing the mutations in the XP variant cells.

Among the 29 mutations with assignable premutagenic lesions located in the coding region of the *HPRT* gene we observed in the XP variant cells irradiated in S-phase, there were 10 mutations with their premutagenic lesion located in the 3' half of the *HPRT* gene, i.e., from exon 4 to 9. In contrast, in the XP variant cells irradiated in G_1-phase to allow time

for repair, there were only 2 out of 14 mutations with their premutagenic lesion located in the 3' half of the gene (6). This implies a fast repair in this region. However, in the present study, the rate of repair was analyzed in a 20-kb fragment consisting of the 5' half of the 44-kb *HPRT* gene, i.e., from intron 1 to the middle of intron 3. Venema et al. (18) showed that different fragments of a gene can be repaired at different rates. The 5' half of the *ADA* gene is repaired slower than the 3' half of the gene. Both strands of the 3' half of the *ADA* gene are repaired as fast as the transcribed strand of the 5' half of the gene. Possibly, for some reason, the rate of repair in the 3' half of the *HPRT* gene in XP variant cells is also equally fast in both strands.

Our study of the spectra of mutations (6) shows that in the variant cells, the photoproducts located in the nontranscribed strand of the *HPRT* gene are much more likely to result in mutations than they do in normal cells. It is possible that the DNA polymerase involved in replication of the nontranscribed strand in XP variant cells is more error-prone when it encounters 6-4's than is that in normal cells. If so, in cells irradiated in S-phase, a high proportion of the mutations derived from photoproducts in the nontranscribed strand could be from 6-4's; the mutations from photoproducts in the transcribed strand would mainly be from CPD. After 11 hr of repair, almost all the 6-4's would be excised, and the mutations observed should be derived mainly from CPD. A faster rate of repair of CPD in the transcribed strand would bring the number of premutagenic CPD close to that found in the nontranscribed strand. This would account for our observation of an equal distribution of premutagenic lesions in the XP variant cells irradiated in G_1-phase.

More analyses of the rate of repair of CPD and 6-4's from each strand

of the *HPRT* gene in XP variant cells will be required to confirm the observations we found to date. Analysis of the rate of repair in the 3' half of the *HPRT* gene in both types of cells, as well as in other actively transcribed genes may also need to be made.

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APPENDIX I

Kinds of Mutations Found When a Shuttle Vector Containing Adducts of 1,6-dinitropyrene Replicates in Human Cells.

Nitrated polycyclic aromatic hydrocarbons (nPAHs) are common environmental contaminants produced primarily as the result of incomplete combustion processes. The predominant nPAH in diesel exhaust is 1-nitropyrene (1-NP), but 1,3-, 1,6-, and 1,8-dinitropyrene (DNP) have also been detected. Since 1,6-DNP has been shown to be one of the most mutagenic nPAHs in certain bacteria and mammalian cell assays, and strongly tumorigenic in experimental animals, it was of interest to investigate the mechanisms by which this carcinogen induces mutations. For this purpose, Boldt et al. (1991) determined whether there are specific sites in a target gene at which the carcinogen preferentially causes mutations, and if so, whether these correspond to the preferential binding sites of the compound. It was also of interest to compare the results obtained with 1,6-DNP with those obtained by Yang et al. (1988) for 1-NP in the same assay to see if there were similarities between the two structurally related carcinogens in the frequency and/or kinds of

mutations they induce. This is because both carcinogens form DNA adducts primarily with guanine, binding covalently at the C-8 position.

We measured the frequency of mutations induced when a shuttle vector plasmid carrying covalently bound residues of 1,6-DNP in the target gene, i.e., the *supF* tRNA gene, replicates in human 293 cells and compared it with what was found previously in the same assay with 1-NP. There was a linear increase in the number of adducts per plasmid as a function of applied concentration of 1,6-DNP and also in the frequency of *supF* mutants as a function of adducts per plasmid, reaching 59×10^{-4} above a background of 1×10^{-4}. The frequency of mutants induced per residue of 1,6-DNP was 1.8 times higher than that induced per 1-NP residue.

We also compared the kinds and locations (spectrum) of mutations induced by 1,6-DNP adducts in the coding region of the *supF* tRNA gene with those induced by 1-NP adducts. Both carcinogens induced mainly base substitutions, primarily G·C --> T·A transversions; but 1,6-DNP adducts produced a significant fraction of -1 frameshifts, with most of these located in a unique run of five Gs in the target gene. The fact that the spectra of mutations caused by 1,6-DNP and 1-NP were not identical indicates that both carcinogens, despite the fact that they are structurally closely related, cause their own spectrum of mutations by unique mechanisms.

The relative frequency of 1,6-DNP adducts at specific sites in each strand of the coding of the *supF* gene was measured their ability to interfere with DNA polymerase reactions. Using this assay we found that 1,6-DNP adducts were formed at every guanine, but not elsewhere in the *supF* gene. The "hot spots" for adduct formation were not perfectly correlated with "hot spots" for mutation induction. This indicates that

the ultimate biological effect of the chemical depends not only on the number of adducts originally formed, but also on such precesses as cellular DNA repair, which may remove such adducts from the plasmids before DNA replication occurs, as well as on the structure of the neighboring bases at the site of the adduct.

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APPENDIX II

Effect of Repair on Ethylnitrosourea-Induced Mutations in Human Fibroblasts.

Maher et al. (1993) have been investigating the effects of nucleotide excision repair (NER) and of O^6-alkylguanine-DNA alkyltransferase (AGT) on the kinds and locations (spectrum) of mutations induced by the alkylating agent ethylnitrosourea (ENU) in human fibroblasts. ENU reacts with 12 nucleophilic sites in DNA to induce a variety of lesions. It has been shown that the O^6-ethylguanine (O^6-EtG) and O^4-ethylthymine (O^4-EtT) are the most effective premutagenic lesions caused by ENU but O^2-ethylthymine (O^2-EtT) is also a strong candidate for mutation induction (Eckert et al., 1988). The O^6-EtG is known to be repaired by AGT, and evidence suggests that in diploid human fibroblasts it can also be repaired by NER (Simon et al., 1981; Maher et al., 1986). The repair of O^4-EtT by alkyltransferase remains uncertain in human cells, but this lesion can be repaired by NER.

To test the hypothesis that in human fibroblasts, ENU-induced premutagenic lesions are repaired by both NER and AGT, and to investigate the effect of the two kinds of repair on the spectrum of mutations induced by ENU, we (Maher et al., 1993) compared the frequency and the spectrum of

mutations induced by ENU in a series of human cells populations. These were NER/AGT-proficient normal cells and NER-deficient, AGT-proficient XP12BE cells that had or had not been pre-treated with O^6-benzylguanine (O^6-BzG). O^6-BzG has been shown to be efficient in titriating out preexisting AGT from human cells (Domaradzki et al., 1985). Use of O^6-BzG with the normal cells and the XP12BE cells gave us four populations: cells lacking both kinds of repair (NER^-/AGT^-); cells laking only NER (NER^-/AGT^+); cells laking only AGT (NER^+/AGT^-); and cells proficient in both kinds of repair (NER^+/AGT^+).

Cells lacking both kinds of repair had the highest frequency of mutants and the proportion of base substitutions involving guanine or thymine bases approximated the reported distribution of O^6-EtG, O^4-EtT, and O^2-EtT in the DNA, i.e., 7: 1.5: 7. XP12BE cells lacking NER but proficient in AGT (not pretreated with O^6-BzG) showed a lower frequency of mutants than XP12BE cells pretreated with O^6-BzG. This decrease only involved loss of premutagenic lesions involving G•C base pairs, i.e., primarily those resulting in G•C-->A•T transitions. These G•G-->A•T transitions are very probably caused by O^6-EtG (Lukash et al., 1991), indicating as expected, that AGT repairs *only* ethylated guanine bases. Normal repair-proficient cells pretreated with O^6-BzG (NER^+/AGT^-) showed a still lower frequency of mutations than cells lacking both kinds of repair. This suggested that NER repair, in the absence of AGT, also plays a role in removing premutagenic lesions induced by ENU. Sequence analysis data indicated that NER excises *both* ethylated guanine bases and ethylated thymine bases. The NER^+/AGT^+ cells showed only a slightly lower frequency of mutants than the NER^+/AGT^- cells, implying that having both kinds of repair activity gave the cells only a slight advantage over cells with NER alone.

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