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FORMATION AND RATES OF REPAIR OF POLYCYCLIC ADDUCTS AT THE NUCLEOTIDE LEVEL IN NORMAL HUMAN FIBROBLASTS

By

Dong Wei

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ABSTRACT

FORMATION AND RATES OF REPAIR OF POLYCYCLIC ADDUCTS AT THE NUCLEOTIDE LEVEL IN NORMAL HUMAN FIBROBLASTS

By

Dong Wei

Previous studies in our laboratory showed two cell-cycle-dependent mutation hot spots in exon 3 of the hypoxanthine phosphoribosyltransferase (*HPRT*) gene of normal human fibroblasts treated at early G1 phase with benzo(a)pyrene diol epoxide (BPDE) but not in the cells treated in S phase. They were not present in cells treated at S phase but appeared in cells that were treated at early G1 phase, so that cells could have at least 12 hr for repair before DNA replication. To investigate whether these two mutation hot spots are caused by inefficient DNA repair at these two sites, I adapted ligation mediated polymerase chain reaction (LMPCR), in conjunction with *Escherichia coli* UvrABC excinuclease, to study rates of repair of BPDE adducts at the nucleotide level on the nontranscribed strand of exon 3 of the *HPRT* gene in normal human fibroblasts. The rates of repair of BPDE adducts at various nucleotide positions varied significantly. The rates of repair at these two sites where mutation hot spots were located were among the

slowest, strongly suggesting that inefficient repair plays an important role in the formation of such cell-cycle-dependent mutation hot spots. To investigate whether this site-specific repair depends solely on the local DNA secondary structure, I determined the rates of repair of 1-nitrosopyrene (1-NOP)- induced adducts in the region of interest and compared the result to that found with BPDE adducts in the same region. Both chemicals form mainly guanine adducts. The average rate of repair of 1-NOP induced adducts was 2- to 3- fold faster than that of BPDE adducts. However, at a particular nucleotide position, 1-NOP-induced adducts were removed at a rate faster or slower than, or equal to that of BPDE adducts, suggesting that the rate of repair at a specific nucleotide position depends on the adduct conformation and the secondary structure of the local DNA. To investigate the distribution of BPDE adducts in human cells, I synchronized and treated them with BPDE at different stages of the cell cycle, determined the distribution of BPDE adducts, and found that the BPDE adduct distribution was essentially the same across the cell cycle, indicating that the changing physiological conditions in the nucleus did not affect the BPDE adduct formation. Comparison of BPDE adduct formation in the region of interest in DNA treated *in vivo* and *in vitro* indicated that the relative distribution of BPDE adducts at most sites *in vivo* was very similar to that *in vitro*, however, the relative level of BPDE adducts at a region of multiple guanines *in vivo* was much higher than that *in vitro*. No nucleosomal structures or other protein-DNA interactions were identified to account for this enhancement *in vivo*. Therefore, I conclude that the difference between BPDE adduct formation at the region of multiple guanines *in vivo* and *in vitro* is due to the physiological condition in the nucleus.

This work is dedicated to:

my wife, Jing Qing

my sister, Yun Wei

my mother, Junlu Yang

my father, Zhilong Wei

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Finally, I would like to thank Scott Boley for his help in English proficiency in Chapter I. I would also like to acknowledge all the past and present members of the Carcinogenesis Laboratory for their assistance, encouragement and generous friendship.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Mutations can occur when DNA is replicated in the presence of various DNA adducts, as evidenced by the fact that most of the base substitutions found in cells treated with a particular carcinogen are located where DNA adducts form (Strauss et al., 1982; Miller, 1985). However, the relative distribution of DNA adducts is usually different from the relative distribution of mutation frequencies found (Brash et al, 1987; Yang et al., 1988). One reason that may account for this is that DNA polymerases have different potentials to insert an incorrect nucleotide opposite a particular DNA adduct at a certain sequence context (Kunkel, 1992; Wang, 1991). Another major factor is DNA repair. DNA repair systems in a cell can remove DNA adducts and replace them with correct nucleotides (Sancar and Sancar, 1988), so when DNA replication starts, DNA polymerases actually encounter the changed adduct spectrum instead of the original adduct spectrum. If DNA adducts at different nucleotide positions are repaired with different efficiencies, the changed adduct spectrum may be totally different from the original adduct spectrum, resulting in a different mutation spectrum.

The first evidence of site-specific repair came from studies with *Escherichia coli* UvrABC excinuclease, the major components of nucleotide excision repair in *E. coli*, which excises various DNA adducts (Sancar and Sancar, 1988). Measurement of the excision efficiencies of various DNA adducts by UvrABC excinuclease *in vitro* indicated

that UvrABC excises the same DNA adducts at different nucleotide positions with different efficiencies (Thomas et al, 1985; Seeberg and Fuchs, 1990) , suggesting that the local DNA sequence context can affect the excision efficiency of UvrABC *in vitro*. What happens *in vivo* remained unknown until Kunala and Brash (1992) determined rates of excision repair of UV induced cyclobutane pyrimidine dimers (CPDs) in the *lacI* gene of *E. coli* at single nucleotide resolution, by labeling the desired fragments and mapping the relative frequencies of CPDs formed at each site, using T4 endonuclease V (T4EV), which specifically cuts CPDs (Brash, 1988). They found that rates of repair of CPDs at various nucleotide positions varied significantly, and most of the UV induced mutation hot spots found in the *lacI* gene were the sites where the rates of repair were slow (Schaaper et al, 1987), suggesting that site-specific DNA repair plays an important role in formation of mutation hot spots in *E.coli.*

In human cells, nucleotide excision repair (NER) is believed to be carried out by at least 11 different proteins (Hoeijmakers and Bootsma, 1994). The wide range of substrates recognized by NER suggests that NER recognizes the structural changes in DNA induced by formation of DNA adducts instead of the adducts themselves (Sancar and Sancar, 1988). Thus, NER in human cells should also be site-specific. However, rates of repair at the nucleotide level in human cells are very difficult to study because of the complexity of the human genome. Tornaletti and Pfeifer (1994) overcame this difficulty by using ligation mediated polymerase chain reaction (LMPCR). They cut the DNA isolated from UV-irradiated cells with T4 endonuclease V, and mapped the positions and frequencies of cuts with LMPCR. They found that CPDs in the *p53* gene

are repaired site-specifically in normal human fibroblasts. They also found that 7 out of 8 mutation hot spots in the *p53* gene found by Brash et al. (1991) in human squamous cell and by Ziegler et al. (1993) in human basal cell carcinomas were sites where rates of repair were slow, further confirming that inefficient repair contributes to the formation of mutation hot spots. Gao et al. (1994) also demonstrated site-specific repair of CPDs at the promoter region of the *PGK1* gene.

All these studies of rates of repair *in vivo* at the nucleotide level made use of T4EV. However, T4EV can not recognize DNA adducts induced by chemical carcinogens, such as bulky polycyclic compounds. At the time I undertook my investigation, there were no reports about the distribution of any bulky adducts at the nucleotide level in human cells, nor were there reports about rates of repair of bulky adducts at the nucleotide level. Highly purified *E.coli* UvrABC excinuclease, which can excise essentially all bulky DNA adducts leaving a 5' phosphate group at the site of the cut, had just become available. Thus it seemed theoretically possible to combine UvrABC and LMPCR to investigate at the nucleotide level the formation and repair of bulky DNA adducts in endogenous DNA of human cells.

The objectives of this present work were (1) to investigate the formation and repair of BPDE adducts on the nontranscribed strand of exon 3 of the *HPRT* gene in normal human fibroblasts; (2) to investigate the rates of repair of 1-nitrosopyrene-induced adducts at the nucleotide level in the region of interest and compare them with those of BPDE adducts in the same region to determine the contribution of the adduct conformation and the local DNA secondary structure to the rate of repair at a particular nucleotide position; (3) to

investigate the distribution of BPDE adducts at the nucleotide level in exon 3 of the *HPRT* gene in human cells across the cell cycle, compare this to that found in purified DNA treated with BPDE *in vitro*, and assess the effect of physiological conditions to the formation of BPDE adducts.

Chapter I reviews the literature dealing with nucleotide excision repair, methods available to study nucleotide excision repair at various level, and the effects of protein-DNA interactions on DNA adduct formation and DNA repair; Chapter II consists of a manuscript published in the March 1995 issue of **Proceedings of the National Academy of Sciences U.S.A., 92,** pp.2204-2208. It describes the research I carried out to determine rates of repair of BPDE adducts at the nucleotide level on the nontranscribed strand of exon 3 of the *HPRT* gene in normal human fibroblasts and correlates the result with mutagenesis data obtained by previous studies in our laboratory; Chapter III consists of a manuscript written in the format of **Molecular and Cellular Biology**. It describes my work to compare the rates of repair of 1-NOP induced adducts and BPDE adducts to determine the contribution of the adduct conformation and the local DNA secondary structure to the rate of repair at a particular nucleotide position; Chapter IV consists of a manuscript written in the format of **Carcinogenesis**. It describes my work to determine the distribution of BPDE adducts at the nucleotide level in human cells treated *in vivo* and in purified DNA treated *in vitro*, and compare them to assess the effect of physiological conditions on BPDE adduct formation.

LIST OF REFERENCES

LIST OF REFERENCES

Brash, D.E. 1988. in *DNA repair : A Laboratory Manual of Research Procedures,* eds. Friedberg, E.C. & Hanawalt, P.C. (Dekker, New York), 327-345.

Brash, D.E., Seetharam, S., Kraemer, K.H., Seidman, M.M.& Bredberg, A. 1987. Photoproduct frequency is not the major determinant of ultraviolet light base substitution hot spots or cold spots in human cells. *Proc. Natl. Acad. Sci. USA*, **84**:3782-3786.

Brash, D.E., Rudolph, J.A., Simon, J.A., Lin, A., McKenna, G.J., Baden, H.P., Halperin, A.J. & Ponten, J. 1991. A role for sunlight in skin cancer:UV-induced *p53* mutations in squamous cell carcinoma. *Proc. Natl. Acad. Sci. USA*, **88**:10124-10128.

Gao, S., Drouin, R. & Holmquist, G.P. 1994. DNA repair rates mapped along the human *PGK1* gene at nucleotide resolution. *Science*, **263**:1438-1440.

Hoeijmakers, J.H.J. & Bootsma, D. 1994. Incisions for excisions. *Nature*, **371**:654-655.

Kunala, S. & Brash, D.E. 1992. Excision repair at individual bases of the *Escherichia coli* lacI gene: relation to mutation spots and transcription activity. *Proc. Natl. Acad. Sci. USA*, **89**:11031-11035.

Kunkel, T.A. 1992. DNA replication fidelity. *J. Bio. Chem.*, **267**:18251-18254.

Miller, J.H. 1985. Mutagenic specificity of ultraviolet light. *J. Mol. Biol.*, **182**:45-68.

Sancar, A. & Sancar, G.R. 1988. DNA repair enzymes. *Annu. Rev. Biochem.*, **57**:29-67.

Seeberg, E. & Fuchs, R.P.R. 1990. Acetylaminofluorene bound to different guanines of the sequence-GGCGCC-is excised with different efficiencies by the UvrABC excision nuclease in a pattern not correlated to the potency of mutation induction. *Proc. Natl. Acad. Sci. USA,* **87**:191-194.

Schaaper, R.M., Dunn, R.L. & Glickman, B.W. 1987. Mechanisms of ultraviolet-induced mutation. Mutation spectra in the *Escherichia coli lacI* gene for a wild type and an excision-repair-deficient strain. *J. Mol. Biol.*, **198**:187-202.

Strauss, B., Rabkin, S., Sagher, D. & Moore, P. 1982. The role of DNA polymerase in base substitution mutagenesis on noninstructional templates. *Biochimie*, **64**:45-68.

Tornaletti, S. & Pfeifer, G.P. 1994. Slow repair of pyrimidine dimers at *p53* mutation hot spots in skin cancer. *Science*, **263**:1436-1438.

Wang, T.S.-F. 1991. Eukaryotic DNA polymerases. *Annu. Rev. Biochem.*, 60:513-552.

Yang, J.-L., Maher, V.M. & McCormick, J.J. 1988. Kinds and spectrum of mutations induced by 1-nitrosopyrene adducts during plasmid replication in human cells. *Mol. Cell. Biol.*, **8**:3364-3372.

Ziegler, A., Leffell, D.J., Kunala, S., Sharma, H.W., Gailani, M., Simon, J.A., Halperin, A.J., Baden, H.P., Shapiro, P.E., Bale, A.E. & Brash, D.E. 1993. Mutation hot spots due to sun light in the *p53* gene of nonmelanoma skin cancer. *Proc. Natl. Acad. Sci. USA*, **90**:4216-4220.

CHAPTER I

LITERATURE REVIEW

A. Cancer and DNA Repair

In vitro and *in vivo* studies indicate that human cancer is the result of a series of mutations that permanently change the genetic information (Cairns, 1981; Ames, 1989). In human cells, most of the genetic information is stored in the genomic DNA and stably transferred to the progeny cells through a semi-conservative replication mechanism. Mutations can occur when DNA is damaged and a DNA polymerase tries to replicate DNA in the presence of DNA damage (Strauss et al., 1982). Although human cells are constantly subjected to various kinds of internal and external damage, mutations rarely happen in normal human cells. This is because the integrity of DNA is constantly protected by various DNA repair systems. DNA repair systems can detect and remove abnormally modified nucleotides (e.g., bulky DNA adducts) (Sancar and Sancar, 1988) or normal but mismatched nucleotides (Modrich, 1994), so no mutations will occur and the genetic information will be kept unchanged when these repair systems are operating effectively.

The importance of DNA repair in preventing cells from becoming tumorigenic is evidenced in hereditary diseases that affect DNA excision repair, such as xeroderma

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pigmentosium (XP) (Cleaver, 1968; Kraemer, 1980), Cockayne Syndrome (CS) (Guzzetta, 1972) and trichothiodystrophy (Lehmann et al., 1988). XP arises when the main pathway for nucleotide excision repair (NER) contains an inherited defect (Kraemer, 1980). Due to this defect, the cells of XP patients are more sensitive to the lethal and mutagenic effects of most mutagens, such as ultraviolet (UV) light from the sun, and the cells are therefore more prone to acquire the genetic changes necessary to become tumorigenic within a much shorter period than normal cells when exposed to comparable levels of UV radiation. These patients usually suffer an age-specific incidence of skin cancer that is several thousands times higher than normal individuals. They typically develop multiple skin tumors which lead to death from metastatic squamous or basal cell carcinoma or malignant melanoma (Kraemer, 1980).

Eight complementation groups of XP have been identified so far (Cleaver, 1990), and the defective genes responsible for the aberrant excision repair function have been cloned for all groups except XP variants (Hoeijmakers and Bootsma, 1994). Studies on the cloned genes have shown that their products are required at critical steps of NER, such as recognition of DNA damage or excision of DNA damage (Hoeijmakers and Bootsma, 1994). In addition, some excision repair components, which are defective in some XP patients, are found involved in other important cellular functions such as RNA transcription (Buratowski, 1993). For example, the ERCC 3 (excision repair cross complementation 3) protein was originally identified as a protein complementing the defective excision repair in Complementation Group 3 rodent cells (Weeda et al., 1990a). Later it was shown that the deficiency in ERCC 3 resulted in XP complementary group

B, in which not only photosensitivity (a symptom typical for XP patients) is found, but also many other non-skin associated abnormalities such as the neural disorders typical in patients with Cockayne's Syndrome (Weeda et al., 1990b). Recent studies have shown that the ERCC 3 protein is a major component of the transcription factor II H, which is required for RNA polymerase II transcription (Schaeffer, 1993). The defect in its dual functions during the development of affected individuals may underlie the non-skin abnormalities of these XP group B patients (Hanawalt, 1994).

Defective nucleotide excision repair is not the only DNA repair process implicated in human cancer. Recently it was found that deficiency in DNA mismatch repair was implicated in an important familial cancer-prone disease, hereditary nonpolyposis colorectal cancer (HNPCC) (Modrich, 1994). Cell lines derived from tumors of patients with HNPCC accrue mutations at rates that can be more than a hundred times that of normal human cells (Parsons et al., 1993). Biochemical analysis of a number of these cell lines has consistently revealed an associated deficiency in strand-specific mismatch repair. The majority of HNPCC cases are attributed to a defect at any one of the multiple loci responsible for the mismatch repair system, e.g., the *hMSH2* (Fishel et al., *1993), hMLH1* (Papadopoulos et al., 1994), *hPMS1* (Bronner et al., 1994) and *hPMS2* (Nicolaides et al., 1994) genes. It has been hypothesized that the initial event in development of HNPCC tumors is the functional loss of a critical mismatch repair activity (Modrich, 1994). This loss will result in genetic destabilization, which can lead to mutations. Mutations in some important genes may circumvent the growth regulatory system in the cell and result in cancer. Recently, the tumor growth factor-β receptor genes, which are important for

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negatively regulating the cell growth, were found mutated in many HNPCC tumors with mismatch repair deficiency (Markowitz et al., 1995). The mutations found were either insertions or deletions, which is consistent with deficient mismatch repair.

DNA repair systems do not act alone in human cells. They constantly interact with other cellular processes that are important in the regulation of DNA repair. A typical example is *p53*, an important tumor suppressor gene that has been found mutated in half of human tumors tested (Vogelstein and Kinzler, 1992). One of the functions of the p53 protein is to serve as a messenger between DNA repair systems and the cell cycle control process (Vogelstein and Kinzler, 1992). When cellular DNA suffers certain kinds of damage, the p53 protein can halt the cell cycle in the G1 phase to allow the DNA repair system time to repair the DNA before replication. It has been hypothesized that one consequence of *p53* mutations is that the subsequent defect in the G1 arrest mechanism does not allow enough time for repair of the damaged DNA and so the cell will be mutation-prone and therefore cancer-prone.

A normal cell is not tumorigenic, and if the integrity of its DNA can be protected by DNA repair systems, a tumor will never occur. In order to understand how mutations are generated and to better prevent mutations from occurring, it is necessary to understand what the DNA repair is and how the DNA repair systems work.

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B. DNA Repair systems

1. Basic DNA repair systems

Cells use DNA repair systems to keep their DNA "damage-free" from either internal events such as spontaneous generation of apurine/apyrimidine sites, oxidative damage, etc., or external agents such as ultraviolet light (UV), X-rays and chemical carcinogens. DNA repair systems can be basically divided into two categories:

(1). Direct repair systems. These systems employ specific processes to reverse the chemical reaction causing a modified base. Examples of direct repair systems are the photoreactivation of cyclobutane pyrimidine dimers (CPDs) (Sancar, 1990) or 6-4 pyrimidine pyrimidones ((6-4)s) (Todo et al., 1993) by various photolyases, which cut the extra bond formed in CPDs or (6-4)s reverting them back to normal pyrimidines; the removal of the methyl group from O^6-methylguanine by O^6-DNA alkyltransferase (Lindahl et al., 1988), which transfers the methyl group to itself reverting the O^6-methylguanine to a normal guanine.

(2). Indirect repair systems. The basic repair strategies used by these systems involve the removal and replacement of the incorrect base (mismatch repair) or the modified base (excision repair). In mismatch repair, the substrate is a pair of normal nucleotides which have paired incorrectly (Modrich, 1994). In *E. coli*, this mismatched base pair is recognized by MutS, which then recruits MutL (Grilley et al., 1989). This complex activates a latent d(GATC) endonuclease assocciated with MutH (Au et al., 1992). Since the adenine in the d(GATC) sequence is always methylated except in newly synthesized DNA (Meselson, 1988), and this d(GATC) endonuclease incises the unmethylated strand

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at a hemimethylated d(GATC) sequence (Au et al., 1992), the incision by the d(GATC) endonuclease will be on the same strand with the incorrect nucleotide that has been inserted mistakenly during DNA replication. This incision is followed by an excision reaction, in which MutS, MutL, MutU and an appropriate exonuclease remove the portion of the unmethylated strand spanning the GATC site and the mismatch (Grilley et al., 1993). The gap is then filled by DNA polymerase III and DNA ligase. Excision repair, the other type of indirect repair, can be divided into base excision repair and nucleotide excision repair (Sancar and Sancar, 1988) In base excision repair, the modified base is removed by a DNA glycosylase, the abasic nucleotide is excised, and the resulting gap is filled by DNA polymerases and DNA ligases (Sancar and Sancar, 1988). In nucleotide excision repair, an oligonucleotide containing the nucleotide with the modified base or bases is removed, and the gap generated is then filled by DNA polymerases, using the complementary strand as the template. The final nick is sealed by DNA ligases (Sancar and Sancar, 1988).

Among these repair systems, nucleotide excision repair (NER) is the major DNA repair mechanism in most species. NER is a general repair system that can repair essentially all DNA lesions, although at different efficiencies (Sancar and Sancar, 1988). Most importantly, NER is the only mechanism found to repair bulky adducts such as those induced by benzo(a)pyrene and 1-nitrosopyrene (Sancar and Sancar, 1988). Detailed discussion of the nucleotide excision repair in *Escherichia coli* (procaryotes), *Saccharomyces cerevisiae* (eucaryotes) and human cells (eucaryotes) is essential to the subject of this dissertation.

2. Nucleotide Excision Repair in *E. coli*

E.coli is the classical model for nucleotide excision repair. The basic stepwise process of NER comes from studies in *E. coli,* and this process was later found to be universal (Sancar and Sancar, 1988; Huang et al., 1992). DNA adducts are first recognized, and DNA endonucleases are then recruited to generate dual incisions, located 5' and 3' of the DNA lesion. The oligonucleotide containing the DNA adduct is then displaced and the gap is filled by a DNA polymerase using the complementary strand as the template. Finally, a DNA ligase closes the nick completing the NER process. In *E.coli*, three proteins, namely UvrA, UvrB and UvrC, are employed to recognize the DNA lesion and excise the oligonucleotide containing the lesion. UvrD and DNA polymerase I then fill the gap, and the final nick is sealed by DNA ligase I (Sancar and Sancar, 1988; van Houten, 1990).

The first step of NER in *E. coli* is carried out by UvrA (Seeberg and Steinum, 1982), a protein which has an energy-favored tendency to dimerize and form the UvrA dimer, the reactive form of UvrA. The UvrA dimer can interact with both undamaged and damaged DNA molecules (Mazur and Grossman, 1991). It has been suggested that after the UvrA dimer binds, it can move progressively along the DNA to the damage site because UvrA has ATPase and DNA helicase activity and thus can move along the DNA (Grossman and Thiagalingan, 1993). The DNA/$(UvrA)_2$ complex then undergoes a conformational change and UvrB, which also has a cryptic ATPase activity, is directed to this complex to form $(UvrA)_2$UvrB/DNA. However, it is also possible that the UvrA dimer first interacts with UvrB, and that this $(UvrA)_2$UvrB complex binds and progresses

along the DNA and locates the DNA lesion, because strong evidence shows that $(UvrA)_2$ can form a complex with UvrB in vitro, and this $(UvrA)_2UvrB$ is capable of binding DNA and locating the site of the damage (Orren and Sancar, 1989).

After the $(UvrA)_2UvrB$ complex reaches the site of the DNA lesion, the conformation of this complex changes in an endothermic manner (Grossman and Thiagalingan, 1993) possibly due to the abnormal structure induced by the DNA adduct or photoproducts. DNA footprinting assays have shown the footprint of this $(UvrA)_2UvrB$ complex changing from 33 bp (which is also the footprint of $(UvrA)_2$ on DNA) to 19 bp (which is the footprint of UvrB), suggesting that the protein that directly contacts DNA changes from UvrA to UvrB (Orren and Sancar, 1989; Visse et al., 1992). This conformational change eventually leads to release of the UvrA dimer. UvrB will then unwind and kink the DNA at the damage site, resulting in a much tighter interaction between UvrB and DNA.

The stable complex between DNA and UvrB after release of UvrA is able to attract the UvrC protein (Orren and Sancar, 1989). It is highly likely that the special conformation of the UvrB/DNA complex serves as the binding substrate of UvrC, because *in vitro* UvrC can not bind either UvrB or DNA containing an adduct (Orren and Sancar, 1989). Both UvrB and UvrC have endonuclease activity, and in the UvrBUvrC/DNA complex, UvrB makes the first incision at the 4th or 5th phosphodiester bond 3' to the site of the damage, possibly independent of the action of UvrC (Lin and Sancar, 1992). This is followed by the incision made by UvrC at 8th phosphodiester bond 5' to the site of the damage (Sancar and Tang, 1993). This dual incision generates an oligonucleotide 12-13 nucleotides in length containing the DNA lesion.

UvrB and UvrC remain bound to DNA until the arrival of UvrD (DNA helicase II) and DNA polymerase I (Husain et al., 1985; Orren et al., 1992). Although it is not clear whether DNA helicase II recognizes the 5' nick of the DNA or interacts with the postincision complex in a special way, DNA helicase II binds the postincision complex, and with its 3'-5' helicase activity, removes both UvrC and the oligonucleotide containing the DNA lesion (Orren et al., 1992). UvrB is most likely dislodged by DNA polymerase I when the polymerase recognizes the 3'-OH of the 5'-incision and fills in the gap with its polymerase activity, using the complementary strand as the template (Orren et al., 1992). DNA ligase I then seals the final nick, and the NER process is complete (Sancar and Tang, 1993).

Besides the basic nucleotide excision repair, there is transcription-coupled repair in *E.coli* (Mellon and Hanawalt, 1989; Selby and Sancar, 1993), evidenced by the fact that the transcribed strand of an active gene is repaired faster than the nontranscribed strand. Since most forms of DNA damage on the transcribed strand can block the RNA polymerase, it was suggested that the stalled RNA polymerase serves as an extra signal to recruit the DNA repair machinery (Selby and Sancar, 1990; Selby and Sancar, 1991). However, studies with an *in vitro* system constructed with various purified proteins showed that the stalled RNA polymerase actually inhibited the access of UvrABC excinuclease, suggesting that other factors are involved *in vivo* (Selby and Sancar, 1990). In *E.coli*, a 120-kDa protein was discovered that might be responsible for the transcription coupled repair (Selby and Sancar, 1991). This protein was later identified as the product of the mutation frequency decline (MFD) gene, a gene defined as responsible for the

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decrease in mutations observed when UV-irradiated *E.coli* cells are incubated in starvation medium rather than in rich medium (Selby and Sancar, 1993). *In vitro* analysis of the protein shows that it is capable of removing the stalled RNA polymerase from the site of DNA damage. The UvrA protein can be retained on a column containing MFD proteins, suggesting that *in vivo* the MFD protein might help to recruit the UvrA protein (Selby and Sancar, 1993). The mechanism of transcription coupled repair has not yet been established, but it has been hypothesized that when an RNA polymerase is stalled by a DNA adduct on the transcribed strand, the MFD protein can bind and release this stalled complex. The MFD protein may also serve as a signal to attract UvrA and start the nucleotide excision repair process. In this way, DNA adducts on the transcribed strand will have more of a chance to be recognized by NER and thus be repaired faster than adducts on the nontranscribed strand.

3. Nucleotide excision repair in yeast (*Saccharomyces cerevisiae*)

Just as *E.coli* serves as a paradigm for nucleotide excision repair in procaryotes, yeast has been studied as an excellent model for NER in eucaryotes (Prakash et al., 1993). In comparison to that in *E.coli*, NER in yeast is very complex. Since the first formal search by Nakin and Matsumoto (1967) for yeast mutants abnormally sensitive to UV radiation about 30 years ago, more than 25 genes have been found to be involved in repair process (Prakash et al., 1993). These genes are categorized into three epistasis groups: RAD3, RAD6 and RAD52. The RAD3 group consists of genes required for excision repair of UV damaged DNA, including *RAD1*, *RAD2, RAD3, RAD10, RAD14* and *RAD25*. The RAD6 group contains genes required for postreplication repair of UV damaged DNA and

for UV mutagenesis, such as *RAD6* and *RAD18*. The RAD52 group contains genes that have only a marginal effect on UV sensitivity, but affect sensitivity to ionizing radiation. Due to the complexity of this system, the functions of many proteins involved in NER are still uncertain, let alone their roles in NER. Only proteins whose functions and roles in NER have been studied very thoroughly will be discussed below.

NER in yeast is believed to start with recognition of the DNA damage by RAD14, a zinc metalloprotein (Bankmann et al., 1992; Guzder et al., 1993). It has been shown that RAD14 binds UV-damaged DNA with high affinity (Guzda et al., 1993), and cells with mutant *RAD14* are totally defective in the incision of UV-damaged DNA, indicating that the RAD14 protein is involved in early steps of NER (Bankmann et al., 1992). RAD14 contains a four cysteine (4C) zinc finger motif, and zinc chelation by the 4C motif could confer the damage-specific DNA binding activity upon the protein. However, RAD14 does not contain a nucleotide binding motif, has no ATPase or DNA helicase activity, and can not unwind DNA or move along DNA (Bankmann et al., 1992; Guzda et al., 1993). Thus, it is very unlikely that RAD14 can have any other functions except recognizing the damaged DNA and possibly recruiting other proteins involved in NER. RAD14 is hypothesized to recruit such proteins as DNA helicases (RAD3 and RAD25) and single strand endonucleases (RAD1/RAD10, RAD2), to carry out NER (Guzda et al., 1993).

DNA helicase activity and endonuclease activity are found in different proteins in yeast NER (Prakash et al., 1993). The endonucleases identified in yeast so far are all single-strand DNA (ssDNA)-dependent (Tomkinson et al., 1993; Habraken., 1993), leading to the suggestion that DNA helicases act first to unwind DNA, and then single-strand DNA

endonucleases make the incisions in the unwound region. RAD3 (Sung et al., 1987; Lohman, 1992) and RAD25 (Guzda et al., 1994) are examples of yeast proteins with DNA helicase activity. Mutations in these two genes can result in totally defective NER (Cox and Game, 1974; Reynolds and Friedberg, 1981; Prakash et al., 1993), strongly suggesting their involvement in early steps of yeast nucleotide excision repair.

The RAD3 protein possesses ssDNA-dependent ATPase and DNA helicase activity (Sung et al., 1987; Lohman, 1992), and translocates on the ssDNA in the 5'-3' direction. Various mutations in RAD3 were found to result in defective incision or a defective post-incision step (Naumouski and Friedberg, 1983; Higgins et al., 1983; Sung et al., 1988), indicating that RAD3 is absolutely required in incision and also functions in a step subsequent to incision. Friedberg and his colleagues reported that RAD3 is often sequestered at or near sites of base damage (Naegeli et al., 1992) resulting in loss of its helicase activity (Naegeli et al., 1993), and thus hypothesized that RAD3 may function in recognition of the DNA damage. However, Prakash and coworkers (Sung et al., 1987) found that the RAD3 helicase is not very processive and thus could not fulfill the task of unwinding the long regions of DNA packaged in chromatin *in vivo*. They suggested that the role of RAD3 in incision is to unwind the DNA in the vacinity of the damaged site to attract the single-stranded DNA endonucleases (Prakash et al., 1993). In the postincision steps, RAD3 might be necessary to displace the oligonucleotide containing the damaged base and the bound excision repair proteins, or to participate in the repair synthesis (Sung et al., 1988; Bailly et al., 1991). Another DNA helicase required for NER is RAD25 (Guzder et al., 1994b). It has been suggested that RAD 25 plays a role

in unwinding the damaged DNA to prepare it for dual incision in the damaged strand by the single-strand DNA-dependent endonucleases.

After DNA helicases bind and unwind the DNA double helix, ssDNA endonucleases can bind the DNA and make incisions. It has been shown that RAD1/RAD10 (Tomkinson et al., 1993) and RAD2 proteins (Habraken et al., 1993) in yeast have ssDNA endonuclease activity. RAD1 always interacts with RAD10 to form RAD1/RAD10 (Bailly et al., 1992), which participates in repair and recombination as a protein complex. A complex of RAD1 and RAD10 proteins with a specific interaction *in vitro* has been shown to bind ssDNA and exhibit endonuclease activity (Tomkinson et al., 1993). It has been shown that the RAD1/RAD10 enzyme makes endonucleolytic scissions in unwound regions in negatively supercoiled DNA (Sung et al., 1993a). RAD2 is also a ssDNA endonuclease but has shown no activity on unwound regions of negatively supercoiled double-stranded DNA (Habraken et al., 1993). Therefore, the involvement of these two endonucleases is most likely stepwise instead of simultaneous: the locally unwound DNA is first cut by RAD1/10, and the nicked and unwound single-stranded damaged region can then be cut by RAD2. Recent studies using specially-synthesized "spray-arm" oligonucleotides, in which partially double-stranded oligonucleotides with a spray 5' or 3' end are used to test the preference of a ssDNA endonuclease to the 3' or 5' ends of an unwound "bubble" in double-stranded DNA, showed that the RAD1/RAD10 complex cuts 5' of the DNA lesion (Bardwell et al., 1994), and the RAD2 protein cuts 3' of the DNA lesion (Harrington and Lieber, 1994).

This scenario is based largely on the known functions of various proteins and on the

dual incision model of nucleotide excision repair in *E. coli*. Currently there is no *in vitro* NER system for yeast. DNA polymerases and DNA ligases involved in repair synthesis in yeast have yet to be identified. The roles of other necessary gene products remain to be studied.

Another important achievement of studies of yeast nucleotide excision repair is a further understanding of transcription-coupled repair. In *E.coli*, the MFD protein is responsible for removing the stalled RNA polymerase (Selby and Sancar, 1993), and the components of NER are not required for viability (Sancar and Sancar, 1988). In yeast, however, many components of NER are critical for viability (Prakash et al., 1993), and this phenomenon has intrigued researchers for a long time. Studies in recent years have shown that DNA helicases critical for NER also participate in other important cellular processes that require unwinding of DNA (Guzder et al., 1993). The two DNA helicases (RAD3 and RAD25) are found to be major components of RNA polymerase II transcription factor b (also named TFIIH), an important factor in the initiation of transcription. Since DNA lesions are removed at a faster rate from the transcribed strand than from the nontranscribed strand, the involvement of these genes in both transcription and DNA repair suggests a key role for these two proteins in preferential repair of the transcribed strand (Guzder et al., 1994a). However, NER is not coupled to transcription in such a simple way as just sharing some components. Studies of various mutants indicated that the repair capacity, helicase activity and transcription ability of RAD3 and RAD25 might be controlled by separate domains. A mutant RAD3 with defective DNA helicase/ATPase activities was shown to be normal in NER (Sung et al., 1988). A mutant

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RAD25 with defective NER ability was fully competent for RNA polymerase II transcription (Park et al., 1992). The exact roles of various genes in both DNA repair and RNA transcription are still not clear.

Recently, Kornberg and coworkers reported evidence showing the existence of a nucleotide excision "repairosome", an interactive complex of all proteins required for NER (Svejstrup et al., 1995). Using antibodies against the core of TFIIH, they isolated a large complex with an apparent mass between 700 KDa and 1000 KDa. This complex contains all the essential proteins known to be required for NER in yeast, such as RAD1/10, RAD 2, RAD4 and RAD14. The investigators went on to suggest that interchange between holo-TFIIH and the repairsome could underlie repair-transcription coupling. However, TFIIH has been shown to be essential only for initiation of RNA transcription by RNA polymerase II, while transcription coupled repair should be coupled to the progression of RNA polymerase. Several models have been suggested to explain the controversy, and the most likely one is that the conformation of a stalled RNA polymerase II at the site of DNA damage resembles that of a transcription initiation complex, and thus recruits the DNA repairosome. More work needs to be done before the mechanisms of transcription coupled repair can be fully understood.

4. Nucleotide excision repair in human cells

Research in understanding NER in human cells was frustrated for a long time because of the lack of an efficient method to isolate the genes involved in NER. A disease with NER deficiency, xeroderma pigmentosum, was first described by Cleaver (1968). Since then, more cases of XP (Kraemer, 1980) and other NER-deficient diseases such as

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Cockayne's Syndrome (CS) (Guzzetta, 1972) have been reported. Complementation experiments using cell lines derived from these patients showed that there are at least 11 complementation groups (Cleaver and Kraemer, 1989; Tanaka et al., 1981), indicating that more than 11 independent genes are involved in the process of NER. However, the early efforts in cloning the genes were fruitless because of the technical difficulty involved in the construction of a complete cDNA or genomic DNA library and then transfecting the library into human cells successfully, since human cells can not maintain the integrity or expression of transfected genes stably.

An alternative approach was used to identify the human genes involved in NER. Beginning in 1982, various Chinese hamster ovary (CHO) cell lines were selected for UV hypersensitivity, and a series of CHO cell lines hypersensitive to UV were obtained (Thompson et al., 1981). Complementation experiments indicated that these mutant cell lines belonged to at least five complementation groups (Busch et al., 1989). Transfection of human genomic DNA into these mutant CHO cell lines, followed by direct selection for UV resistance, identified several human genes that complement these repair deficient phenotypes. These genes were isolated and designated as excision repair cross-complementing (ERCC) genes, including *ERCC1* (Westerveld et al., 1984), *ERCC2* (Weber et al., 1988), *ERCC3* (Weeda et al., 1990a), *ERCC5* (Shiomi et al., 1994) and *ERCC6* (Troelstra et al., 1990).

Since 1989, transfection of these ERCC genes into various XP cell lines showed that *ERCC2, ERCC3, ERCC5* and *ERCC6* genes were equivalent to the *XPD* (Flejter et al., 1992), *XPB* (Weeda et al., 1990b), *XPG* (Shiomi et al., 1994) and *CSB* (CS group B

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complementing) genes (Troelstra et al., 1992) respectively. Within the same time period, two different groups cloned the XPA gene and the XPC complementing genes. The XPA gene was cloned by the complementation of UV sensitivity after large scale transfection of XPA cells with mouse genomic DNA and subsequent isolation of the human counterpart (Tanaka et al., 1989; Tanaka et al., 1990). The XPC gene was cloned by complementation of UV sensitivity after transfecting XPC cells with a normal human cDNA library (Legerski et al., 1992). The repair defect of a subset of XPE cells was found to be complemented by DNA damage binding (DDB) proteins (Keeney et al., 1994). DNA excision repair synthesis in the XPF cell extract was recovered to wild type level by a partially purified protein complex containing ERCC1 (however, ERCC1 alone can not complement the repair defect in XPF cells. (van Vuuren et al., 1993)) (Biggerstaff et al., 1993). Analysis of these various proteins using cell free extracts and other *in vitro* constructed systems led to further understanding of their functions and their corresponding roles in NER. A general picture is depicted, much more complex than that in *E. coli.*

The first step of NER is believed to be the recognition of damaged DNA by the XPA protein (Sugano et al., 1991; Robins et al., 1991; Miura et al., 1991). The defective XPA protein in cell lines derived from XPA patients results in almost no NER activity, indicating that this protein is involved in an early step (Fornace et al., 1976; Tanaka et al., 1977). The XPA gene has been shown to be a human analog of the yeast RAD14 gene (Bankmann et al., 1992), which encodes a protein that binds specifically to UV-damaged DNA. Analysis of the amino acid sequence showed that the XPA protein contains a four cysteine zinc finger motif, suggesting a DNA binding activity (Tanaka et

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al., 1990). It has also been shown that the XPA protein binds preferentially to DNA damaged by UV, cisplatin or OsO_4 (Robins et al., 1991).

As in the yeast RAD14 protein, no other special motif has been found in the XPA protein (Prakash et al., 1993), suggesting the involvement of auxiliary proteins or stimulatory proteins in recognition of DNA damage. Human DNA damage binding (DDB) protein is one such example (Chu and Chang, 1988). DDB is a heterodimer of a 124-kDa polypeptide and a 41-kDa protein (Hwang and Chu, 1993; Keeney et al., 1993), and binds specifically to DNA damaged by UV. Microinjection of DDB into a subset of XPE cells stimulated the repair DNA synthesis to the wild type level (Keeney et al., 1994). It was suggested that DDB functions in an alternative DNA damage recognition pathway, exclusive from the XPA recognition pathway (Chu and Chang, 1988). However, the DNA repair defect in XPE cells is less severe than that in XPA cells (Cleaver and Kraemer, 1989), suggesting that the DDB protein just plays an auxiliary role in DNA damage recognition, whereas the XPA protein serves as the major DNA damage recognition protein (Keeney et al., 1994).

Recently, human replication protein A (RPA) was found to increase the binding of XPA protein about 10 fold to a DNA fragment containing actetylaminofluorene adducts (He et al., 1995), suggesting that RPA may function in conjunction with the XPA protein at the earliest step of DNA damage recognition in excision repair. RPA was first identified as a human single-stranded DNA binding protein (HSSB) (Wold and Kelly, 1988), and later found to be important for DNA replication (Wold and Kelly, 1988; Fairman and Stillman, 1988), DNA recombination (Moore et al., 1991) and DNA excision

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repair (Coverley et al., 1991). Substitution of RPA with a heterologous analog of single-stranded DNA binding protein resulted in repair deficiency (Coverley et al., 1991), suggesting that RPA functions in NER through unique protein-protein contact, not just by the activity of binding to ssDNA. It has been suggested that the local melting of the double helix induced by DNA damage attracts the RPA and then makes the site of the damage a better substrate for the XPA protein (He et al., 1995). RPA is also involved in other steps of NER, which will be discussed later.

The DNA damage recognition is believed to be followed by recruitment of DNA helicases to unwind the double helix. This allows the damaged segment of DNA to be excised, since the XPA protein has no helicase activity (Miura et al., 1991), and only single-stranded DNA endonucleases have been identified as participating in human NER. The XPB (ERCC3) (Weeda et al., 1990b), XPD (ERCC2) (Flejter et al., 1992) and CSB (ERCC6) (Troelstra et al., 1992) proteins have sequence similarities to known DNA helicases and have shown DNA helicase activity *in vitro* (Sung et al., 1993b; Drapkin et al., 1994). The XPB and XPD proteins are found to be components of transcription factor II H (TFIIH) (Schaeffer et al., 1993), part of the basic transcription initiation complex for RNA polymerase II (Buratowski et al., 1989; Zaawel and Reinberg, 1992), suggesting that these proteins possess dual roles, functioning during NER as well as transcription initiation. The XPD protein is the human homolog of the yeast RAD3 protein, which participates in both transcription and DNA repair complexes (Prakash et al., 1993). Using the cDNA of the XPB protein as a probe, Prakash and colleagues isolated a yeast XPB homolog, RAD25 (Park et al., 1992), which also has DNA helicase activity and is

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required for both transcription and NER (Guzder et al., 1991). Both the RAD3 and RAD25 proteins are required for yeast viability, further suggesting the critical role of the XPB and XPD proteins in human cells. Wood and colleagues showed that in an *in vitro* system, purified TFIIH complex is absolutely required for excision repair (Aboussekhra et al., 1995).

The bifunction of XPB and XPD proteins in NER and transcription may offer a simple explanation for transcription-coupled repair in human cells. However, how the cell manages to exchange these shared factors between transcription factors and NER complexes remains to be studied, and defects in either XPB or XPD protein affect the basic DNA repair mechanisms including both the transcription-coupled repair and repair of the genome overall. Furthermore, an extra factor responsible for transcription-coupled repair was identified as ERCC 6, which is defective in CSB patients (Troelstra et al., 1992). Cell lines derived from patients with CSB are proficient in overall genome repair, but lack the preferential repair of actively transcribed genes (Venema et al., 1990b). Transfection of the ERCC6 gene into CSB cells corrected this repair defect of CSB cells. Analysis of the gene sequence suggests that it has a DNA unwinding function (Troelstra et al., 1992). It is thus very likely that the ERCC6 protein serves as a coupling factor, functioning in removing the stalled RNA polymerase at the adducted site and attracting the repair enzymes. However, a recent study by Hanawalt and colleagues suggests that the RNA polymerase may stay in place, while the RNA it is making is partially degraded to allow access for the repair enzymes (Donahue et al., 1994). After the binding of DNA helicases to the DNA damage site, the DNA will be unwound and the exposed single-

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stranded DNA will be clipped out by single-stranded DNA endonucleases: the XPG (ERCC5) protein and the ERCC1/XPF protein complex.

The *ERCC5* gene was first localized and found to be at the same chromosomal location as the cloned *XPG* gene (O'Donovan and Wood, 1993). Furthermore, a purified protein was found to complement the repair defect in cell extracts of both Complementation Group 5 CHO cells and the human XPG cells, indicating that the *ERCC5* gene is the *XPG* gene (O'Donovan and Wood, 1993). This was later confirmed by the cloning of the *XPG* gene (Scherly et al., 1993) and the *ERCC5* gene (Shiomi et al., 1994) by two independent groups and the complementation of the repair defect of XPG cells by transfection of the *ERCC5* gene (Shiomi et al., 1994). The XPG protein is a human homolog of the yeast RAD 2 protein and both were shown to be able to cut single stranded M13 DNA (O'Donovan et al., 1994b). Using a synthetic "spray arm" DNA structure, Wood and colleagues showed that the XPG protein is required for the formation of the 3' incision during NER (O'Donovan et al., 1994a). The absence of evidence for a 5' incision by the XPG cell extract further suggests that either the 3' incision is made before the 5' incision, or that both incisions are made in a coordinated fashion (O'Donovan et al., 1994a).

The other ssDNA endonuclease, ERCC1/XPF protein complex, has not been completely purified and characterized. ERCC1 gene was the first human excision repair gene characterized (van Duin et al., 1986), but no patients have ever been found with a mutation in this gene, suggesting that this gene is very critical for cell viability (van Duin et al., 1989). ERCC1 is a 33-KDa protein but always sediments as a 120-kDa complex in a native glycerol gradient (van Vuuren et al., 1993). This complex is capable of

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complementing activity of CHO complementation groups 1, 4, 11 and XP group F, indicating presence of a preformed complex of ERCC1, ERCC4, ERCC11 and XPF proteins (van Vuuren et al., 1993; Biggerstaff et al., 1993). This complex interacts very tightly because cell extracts of these cell lines can not complement each other (Biggerstaff et al., 1993). ERCC1 has been shown to be a human homolog of yeast RAD1 gene (van Duin et al., 1986). Since the RAD1 protein always exists as a complex with RAD10 protein and this complex makes an incision on the 5' side of the DNA damage (Bardwell et al., 1994), the complex of the ERCC1 protein and the XPF protein and other proteins is very likely to carry out the same function.

Replication protein A may also play a role in this incision step. The locally melted DNA unwound by the DNA helicases can serve as an ideal substrate for RPA binding. The size of the binding site of a molecule of human RPA on ssDNA is ~20-30 nucleotide (Kim et al., 1992; Seroussi et al., 1993), which corresponds well with the distance between the two incisions in UV irradiated DNA (Huang et al., 1992). RPA has also been shown to bind the XPG protein (He et al., 1995). It is possible that RPA might help to target the XPG protein on DNA 5' to the DNA damage.

The XPC protein might also participate in the incision step. Cell lines derived from XPC patients were shown to have an NER system defective in the genome overall subpathway, but normal in the transcription-coupled repair pathway (Kantor et al., 1990; Venema et al., 1990a). The XPC protein was identified as a 125-kDa protein (Legerski and Peterson, 1992). Sequence analysis indicated that it is a human homolog of the yeast RAD4 protein, which is required for NER (Gietz and Prakash, 1988; Legerski and

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Peterson, 1992). However, no obvious DNA binding domains are apparent from the sequence. Hanaoka and colleagues (Matsutani et al., 1994) purified the XPC protein in a complex with two other proteins, HHR 23A and HHR23B, which are human homolog of the yeast RAD23. This protein complex has the activity of binding ssDNA (Matsutani et al., 1994). It may participate in the incision step by binding the unwound DNA or the displaced oligonucleotide. However, it is not clear which of the components is responsible for this property. A ubiquitin-like motif has been identified in the N-terminus of the HHR23B protein, suggesting a role for this N-terminal domain in facilitating the assembly of this protein complex. Recently, Wood and colleagues showed that both the XPC complex and purified TFIIH complex were required to reconstitute the NER reaction in vitro (Aboussekhra et al., 1995). The actual role of the XPC complex remains to be identified.

What happens immediately after the dual incisions is not clear. Whether all these proteins already involved dissociate automatically or are displaced by some other proteins remains to be determined. The human proliferating cellular nuclear antigen (PCNA) is known to play a very important role in the post incision step (Shivji et al., 1992; Toschi et al., 1988). PCNA is an important cofactor for DNA replication, required for locating the 3'-OH of the primer and for the processitivity of DNA polymerases δ and ε (Melendy and Stillman, 1992; Podust et al., 1992). Shivji et al. (1991) showed that PCNA is required for DNA excision repair. Recently, Stillman and colleagues showed that a cyclin -dependent kinase inhibitor, p21, can inhibit PCNA-dependent DNA replication, but not PCNA-dependent excision repair (Li et al., 1994). They suggest that the functions of

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locating a 3'-OH and increasing the processitivity of DNA polymerase are controlled by separate domains. Only the function of locating 3'-OH is required for nucleotide excision repair, since the gap generated by dual incisions of NER is only 29 nucleotide long (Huang et al., 1992) and can be easily filled by DNA polymerases without PCNA.

NER is completed as the final DNA nick is sealed by DNA ligase. It is very likely that the human DNA ligase I is responsible for this step, since cell lines derived from patients with defective DNA ligase I showed hypersensitivity to a wide range of DNA damaging agents, and a retarded rate of joining of Okazaki fragments during DNA replication and strand breaks after exposure to DNA damaging agents (Barnes et al., 1992). However, the participation of the human DNA ligase II and III can not be excluded.

Most of the functions of the proteins described here have only been identified during the past two years. What is clear is that a lot of controversy still exists, such as the actual order of the stepwise binding and releasing of various proteins. Highly specific interactions must exist to use so many different proteins to repair the DNA lesions with such an efficiency and accuracy.

C. Research on nucleotide excision repair at various levels in human cells

Long before the first human repair gene was identified, people had already started to work on various cell lines to understand the characteristics of DNA repair. Over a period of more than 20 years, the research of NER has progressed from the genome overall level to the specific genes to the nucleotide level, leading to more thorough understanding of how various DNA lesions are repaired by NER.

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1. Investigation of DNA repair at the level of the genome overall

DNA repair was first studied on the level of the genome overall using antibodies against specific DNA adducts (Pathak et al., 1986; Wani and D'Ambrosio, 1987) or radioactive carcinogens (Heflich et al., 1980). To use antibodies against a specific type of DNA adducts, cells are exposed to various carcinogens, and at different time points, DNA is isolated and treated with antibodies. The amount of antibodies (usually labeled with radioactive isotopes) bound to DNA is indicative of the amount of the specific DNA adducts formed on DNA, and therefore, the quantitative decrease of the amount of antibodies bound to DNA after repair reflects the extent of repair of the specific adducts. The advantage of this assay is that, at least in theory, it can be used to detect quantitatively the repair of any specific kind of DNA adducts without interference from other DNA adducts, since even if a carcinogen can induce different DNA adducts, monoclonal antibodies can be obtained against a specific kind of them. The disadvantage is that it is hard to generate specific antibodies against DNA adducts. So far only a limited number of antibodies have been successfully used, such as antibodies against UV induced cyclobutane pyrimidine dimers (Roth et al., 1987), UV induced 6-4 pyrimidine pyrimidones (Mitchell et al., 1988; Mori et al., 1988) and BPDE adducts (Nakagawa et al., 1989).

Besides antibodies against DNA adducts, radioactive carcinogens have also been used to detect repair at the level of the genome overall (McCormick and Maher, 1983). After cells are treated with radioactive carcinogens, the genomic DNA is isolated at various time points and the radioactivity remaining on DNA is measured. The decrease of

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radioactivity of the DNA after allowing cell time for repair indicates the removal of DNA adducts by NER. Repair of DNA adducts induced by a certain carcinogen can be measured very accurately in this way.

The studies of DNA repair in the genome overall indicate that different DNA adducts are repaired at different rates in human cells. However, the human genome has long been known to consist of heterogeneous regions (Weintraub and Groudine, 1976). Actively expressed genes are usually free of nucleosomes or contain very open chromatin structures, while other DNA may be packaged into highly condensed chromatin structures, where the accessibility of DNA repair enzymes can be strongly hindered. DNA repair at the genome overall level does not provide any indication of what happens in a specific DNA fragment in the genome, let alone what happens at the nucleotide level.

2. Study of DNA repair at the gene level

A method to detect rates of repair of cyclobutane pyrimidine dimers (CPDs) in specific genes was developed by Bohr et al. (1985). This assay starts with treating cells with UV, isolating the genomic DNA after allowing cells various amounts of time for repair and digesting DNA with restriction enzymes. One half of a restricted DNA sample is treated with T4 endonuclease V (T4EV), which nicks the DNA specifically at the sites of CPDs (Haseltine et al., 1980), while the other half is used as control. Both halves of the sample are then separated in parallel in an alkaline denaturing agarose gel, and transferred to a nylon membrane. After probing the membrane with radioactive probes specific for either strand of a specific gene, one can determine the frequency of cuts by T4EV in the fragment by comparing the amount of the full length intact fragment in the T4EV treated

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sample with that of the control and making use of the Poisson distribution function. Cellular removal of CPDs results in a corresponding lack of nicking by T4EV and an increase in the amount of the full length fragment. The amount of CPDs remaining on this fragment can then be calculated, and the rates of repair of CPDs on a fragment of a specific gene can be determined. Bohr et al. (1985) first discovered that the dihydrofolate reductase (DHFR) gene, an actively expressed gene, was repaired at a faster rate than the genome overall in CHO cells. Later, Mellon et al. (1986) showed that this preferential repair also happened in the *DHFR* gene in human cells and that excision repair was coupled to transcription (Mellon et al., 1987). More studies followed and showed the intragenomic heterogeneity of DNA repair in mammalian cells (Bohr, 1991).

This method not only provided an effective approach to detect DNA repair in any specific gene, but also changed the way to measure the amount of DNA adducts. Instead of directly measuring the amount of DNA adducts, one can incise the damaged DNA at the sites of DNA adducts with a specific enzyme, and quantitate the frequency of cuts as the amount of DNA adducts. The substitution of T4EV with E.coli UvrABC excinuclease greatly expanded the spectrum of this research to virtually all bulky chemical carcinogens (Thomas et al., 1988), since UvrABC excinuclease can excise almost all DNA adducts induced by various bulky chemical carcinogens (Sancar and Sancar, 1988). However, in order for this method to be quantitative, the DNA fragment of interest must be at least 10 Kb long. Thus only an average rate of repair of DNA adducts from such a long fragment can be studied using this method (Bohr, 1991).

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3. Study of DNA repair at the nucleotide level.

The study of excision repair at single nucleotide resolution was first done in *E.coli* by Brash and colleagues (Kunala and Brash, 1992). They treated *E. coli* with UV and digested the genomic DNA with restriction enzymes. An oligomer that had six dTTPs on its 5' end and was designed to be complementary to the 3' end of the desired fragment, was annealed to the DNA and an extension reaction by Taq polymerase enabled the fragment to be extended with [^{32}P]dATP. In this way, only the region of interest was end-labeled. The labeled DNA was cut with T4 endo V and separated on sequencing gels to show the various cuts at specific nucleotide positions. The intensity of each band was indicative of the level of CPDs at that nucleotide position. With this method, they found that the rates of repair varied from site to site. They also found a correspondence between UV mutation hot spots (Schaaper et al., 1987) and the excision repair slow spots, suggesting that site to site variations in the rate of excision can profoundly affect mutation frequency.

This method uses the annealing of an oligonucleotide to label the region of interest specifically, which guaranteed the same labeling for all bands on the sequencing gel, so that the intensity of fragments of a certain length is quantitatively indicative of the CPDs' level at a particular site. However, sequencing gels can only separate DNA molecules of up to 600 bp long. *E.coli* has a genome of ~10^6 bp, so it is possible to use this method. A human cell has a genome of 3 x 10^9 bp, so the region of interest which can be analyzed can only be $1/10^7$ of the total DNA, which makes the specific labeling with an oligonucleotide to quantitate the adduct level at each nucleotide position practically

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Ligation mediated polymerase chain reaction (LMPCR) makes it possible to study the DNA repair at the nucleotide level in human cells (Tornaletti and Pfeifer, 1994; Gao et al., 1994; Wei et al., 1995a). LMPCR was first used to detect the level of any cuts with a 5' phosphate in any desired fragment of the genomic DNA (Pfeifer, 1992). DNA is first treated with specific enzymes or reagents to generate such cuts, and a gene specific primer is allowed to anneal at the region of interest and extended with DNA polymerase. The extension terminates at the site of the cut, and a blunt end forms at that site. An asymmetric blunt ended linker with hydroxyl groups at each end is ligated to the blunt end. Use of T4 DNA ligase, which can only ligate a 5' phosphate group with a 3'OH group, allows the linker to be ligated in only one direction. DNA can then be amplified with PCR using the longer oligomer of the linker and a second gene specific primer to allow the amplification of the desired fragments. Separation of the final products allows one to detect the original cuts at particular nucleotide positions and the relative frequencies of these cuts. Pfeifer and colleagues (Tornaletti and Pfeifer, 1994) and Holmquist and colleagues (Gao et al., 1994) used T4 endonuclease V and photolyase to convert CPDs into cuts amplifiable with LMPCR. The relative frequencies of cuts were determined for DNA isolated from cells that had been allowed various time for repair. The decrease in the relative frequency of cuts by T4EV at a particular nucleotide position after DNA repair should reflect the rate of excision repair at that nucleotide position. They showed that the rates of repair of CPDs vary significantly among different sites in human cells.

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In the studies I will describe in this doctoral dissertation, instead of T4EV, I used *E.coli* UvrABC excinuclease, which can excise all DNA adducts induced by bulky carcinogens, to study the repair of BPDE adducts and 1-NOP-induced adducts at the nucleotide level (Wei et al., 1995a; 1995b). I showed that site specific repair also exists for bulky DNA adducts. This use of UvrABC extends the spectrum of this kind of research to essentially all bulky DNA adducts.

LMPCR is an effective tool to study DNA repair at the nucleotide level. It utilizes PCR to amplify the desired fragments to increase the specific signal so that it is possible to detect repair on a 200 bp fragment in a genome of $3x10^9$ bp. However, it has its limitations: for example, it is an indirect method. It actually measures the frequency of cuts by a certain enzyme at various sites. However, the frequency of cuts at a particular site does not necessarily reflect the absolute level of adduct formation at that site. T4EV cuts CPDs and leaves a 5' nucleotide with a base dimer. Photolyase must be used to incise the base dimer so that this 5' end is suitable for ligation. The conversion might not be complete at all sites for both enzymes. UvrABC excises the DNA adducts with variable efficiencies at different nucleotide positions. UvrABC makes incisions at the 4th or 5th phosphodiester bond 3' of DNA adducts (Sancar and Rupp, 1983), which makes it hard to determine the rate of repair at consecutive nucleotide positions. DNA polymerase might drop off before it reaches the cut to generate a blunt end (Garrity and Wold, 1992). The efficiency of the blunt end ligation might be different at various sites. These will eventually result in an altered picture of the original frequencies of cuts at different nucleotide positions.

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Due to these limitations, it is not appropriate to take the frequencies of cuts detected by LMPCR as the quantitative reflection of the distribution of DNA adducts. Nonetheless, it can serve as a quantitative tool to measure the DNA repair at the nucleotide level. T4EV and UvrABC can be expected to cut at a particular site with a constant efficiency. The efficiency of blunt end ligation at a particular site should also be constant. Thus, a decrease in the intensities of the fragment of a certain length in the final LMPCR amplified products should reflect quantitatively the decrease of the adduct level, i.e., the rate of excision repair, at that particular nucleotide position.

D. Effects of protein-DNA interactions on DNA adduct formation and rates of DNA repair

1. Effects of protein-DNA interactions on DNA adduct formation

Ever since researchers started to study the distribution of DNA adducts induced by various carcinogens *in vitro*, they have found that DNA adducts are distributed unevenly and non-randomly (Brash et al., 1987; Yang et al., 1988; Rill and Marsch, 1990; Ponti et al., 1991). On a DNA fragment, there are "hot spots" and "cold spots" for DNA adduct formation, i.e., places where DNA adduct formation is frequent and places where DNA adduct formation is infrequent. Thus it has been suggested that DNA adduct formation is affected by the local structure of DNA, which is determined by the local DNA sequence context. In human cells, most of the DNA either wraps around histone octamers to form nucleosomes or forms tight interactions with other protein factors, such as transcription factors. Protein-DNA interactions limit access of carcinogens, and sometimes change the dynamic structure of the DNA, as revealed by DNA footprinting

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studies. Thus, it is expected that DNA adduct formation in human cells should be affected by various protein-DNA interactions. However, different DNA adducts are induced through different pathways. Various studies have shown that the effects of protein-DNA interactions on the formation of DNA adducts differ, depending on how the adducts are induced, what kinds of protein interactions are involved, and characteristics of the particular carcinogen (e.g., size).

Dimethyl sulfate (DMS) is a small chemical carcinogen, which can directly modify guanine nucleotides by adding an extra methyl group at the N^7 position (Maxam and Gilbert, 1980). It has been shown that binding of transcription factors leads to protection of some guanines from DMS modification (Ephrussi et al., 1985; Kara and Glimcher, 1991), and cleavage of modified nucleotides with piperidine shows a clear footprint where the transcription factor binds, making it an excellent reagent for DNA footprinting. However, due to its small size, the loose interaction between DNA and histone octamers hardly has any effect on its action. DMS has been shown to have the same modification patterns on free DNA, chromatin DNA and chromosomal DNA (Pfeifer et al., 1992; Pfeifer and Riggs, 1991).

Benzo(a)pyrene diol epoxide (BPDE) is the ultimate metabolite of benzo(a)pyrene, an environmental carcinogen. It forms DNA adducts in two steps: it has to intercalate noncovalently to the DNA double helix first and then can form covalent adducts with DNA (MacLeod and Zachary, 1985). Smith and MacLeod (1993) studied BPDE adduct formation on a DNA fragment before and after it wrapped around a reconstituted nucleosome. They found that adduct formation at the core region of the nucleosome was

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inhibited 2.5 fold. This phenomenon was also found in a similar experiment with another carcinogen, aflatoxin-B1 (Moyer et al., 1989). This is expected since these carcinogens are too big to diffuse through the DNA/histone contact and can only access DNA from outside of the nucleosomal structure. However, the relative pattern of distribution of BPDE adducts in DNA that is wrapped around the nucleosome is similar to that on free DNA, suggesting that after intercalation of BPDE, formation of the covalent bound BPDE adduct may be controlled by local DNA structure, unaffected by nucleosomal structures (Smith and MacLeod, 1993).

Another carcinogen, ultraviolet light (UV) is in a fixed direction from a fixed source, differing from chemical carcinogens that diffuse freely in the solution to bind DNA. Thus in a nucleosome structure where DNA is wrapped around histones, nucleotides on the outer side of the DNA double helix should have a shielding effect on the nucleotides in direct contact with the histone octamer. This will result in a highly modulated UV photoproduct (primarily CPDs and (6-4)s) distribution, with an average periodicity of about 10 bases, as shown on purified nucleosome core particles (Brown et al., 1993). What happens in human cells remains to be studied. Pfeifer et al. (1992) determined the distribution of CPDs in the promoter region of the *PGK1* gene at the nucleotide level, and found several CPD hot spots where a transcription factor binds, suggesting that binding of transcription factors changes the dynamic structure of the local DNA, and enhances the UV photoproduct formation.

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2. Effects of protein-DNA interaction on DNA repair

In human cells, DNA repair is carried out by various enzyme complexes. It might be expected that protein interaction with DNA would inhibit DNA repair, since such proteins would inhibit the access of the enzymes to the DNA. This was evidenced in the fact that different regions of the genome have different sensitivity to DNase I (Weintraub and Groudine, 1976). In 1985, Bohr et al. showed that in Chinese hamster ovary cells, an actively transcribed *DHFR* gene was repaired faster than the genome overall, and they defined this phenomenon as preferential repair. Since actively transcribed genes usually have more open chromatin structures, as shown by their sensitivity to DNaseI, it is to be expected that the DNA repair complex can gain easy access to the DNA, and DNA adducts will be repaired faster. In 1986, Mellon et al. showed that in human cells the *DHFR* gene was also repaired faster than the genome overall. In addition, they showed that the preferential repair was largely due to the fast repair of the transcribed strand because of transcription-coupled repair, suggesting that the contribution of the open chromatin structures to preferential repair of active genes is very limited (Mellon et al., 1987; Mellon and Hanawalt, 1989).

However, later studies on DNA repair of specific genes in human cells have shown that preferential repair can not be attributed solely to transcription-coupled repair. Comparison of the rate of DNA repair on the nontranscribed strand of various actively-transcribed genes in human cells and on the nontranscribed regions indicates that DNA adducts on the nontranscribed strand of active genes usually are removed faster than those on the nontranscribed regions, and that different nontranscribed regions are repaired at different

rates. BPDE adducts on the nontranscribed strand of the *HPRT* gene have been shown to be repaired faster than the genome overall (Chen et al., 1992). CPDs on the nontranscribed strand of the *DHFR* gene are also repaired faster than genome overall (Evans et al., 1993). BPDE adducts and CPDs on a nonexpressed loci, 754, are repaired much more slowly than the genome overall (Chen et al., 1992; Evans et al., 1993). DNA adducts on the nontranscribed strand rarely interfere with transcription, and transcription-coupled repair is believed not to be involved in the repair of DNA adducts located on the nontranscribed strand (Bohr, 1991). Thus, these data strongly suggest that chromatin structure plays an important role in determining the rate of repair. A recent study by Bohr and colleagues (Kruk et al., 1995) showed that CPDs in telomeres of human cells are repaired faster than those in the nontranscribed non-coding 754 locus, but more slowly than those in the nontranscribed δ-globin gene, suggesting that different chromatin structures may exist in various nontranscribed regions and affect the accessibility of DNA repair enzymes differently. The effect of protein interactions was also shown at the nucleotide level. Gao et al. (1994) showed that CPDs at the transcription factors' binding sites were removed at a slower rate than the average in the *PGK1* gene promoter region, suggesting that binding of transcription factors inhibits the access of DNA repair machinery and thus slows down DNA repair.

Although DNA/protein interactions may inhibit the access of DNA repair machinery and DNaseI in a similar way, the actions of the two are different. DNaseI directly binds DNA and makes incisions, resulting in an incision pattern with a periodicity of about 10 bp on nucleosomal DNA wrapped around histone octamers (Pfeifer and Riggs, 1991). It

has been shown that rates of excision repair vary significantly at the nucleotide level, however, this variation does not have a periodicity correlated to the nucleosomal structure (Tornaletti and Pfeifer, 1994; Gao et al., 1994). Furthermore, this site-specific repair also occurs on DNA which is nucleosome-free (Wei et al., 1995a; 1995b). This suggests that, consistent with what is known from other studies, DNA repair complexes act in a different and more complex fashion than the direct action of DNaseI.

LIST OF REFERENCES

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Aboussekhra, A., Biggerstaff, M., Shivji, M.K.K., Vilpo, J.., Moncolin, V., Podust, V.N., Protic, M, Hubscher, U., Egly, J.-M.& Wood, R.D. 1995. Mammalian DNA nucleotide excision repair reconstituted with purified protein components. *Cell*, **80**:859-868.

Ames, B.N. 1989. Endogenous DNA damage as related to cancer and aging. *Mutat. Res.*, **214**:41-46.

Au, K.G., Welsh, K.& Modrich, P. 1992. Initiation of methyl-directed mismatch repair. *J. Biol. Chem.*, **267**:12142-12148.

Bailly, V., Sung, P., Prakash, L. & Prakash, S. 1991. DNA RNA helicase activity of RAD3 protein of Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. USA*, **88**:9712-9716.

Bailly, V., Sommers, C.H., Sung, P., Prakash, L. & Prakash, S. 1992. Specific complex formation between proteins encoded by the yeast DNA repair and recombination genes *RAD1* and *RAD10. Proc. Natl. Acad. Sci. USA*, **89**:8273-8277.

Bardwell, A.J., Bardwell, L., Tomkinson, A.E. & Friedberg, E.C. 1994. Specific cleavage of model recombination and repair intermediates by the yeast RAD1-RAD10 DNA endonuclease. *Science*, **265**:2082-2085.

Barnes, D.E., Tomkinson, A.E., Lehmann, A.R., Webster, A.D.B.& Lindahl, T. 1992. Mutations in the DNA ligase I gene of an individual with immunodeficiencies and cellular hypersensitivity to DNA-damaging agents. *Cell*, **69**:495-503.

Biggerstaff, M., Szymkowski, D.E. & Wood, R.D. 1993. Co-correction of the ERCC1, ERCC4 and xeroderma pigmentosum group F DNA repair defects in vitro. *EMBO J.*, **12**:3685-3692.

Bohr, V.A.1991. Gene-specific DNA repair. *Carcinogenesis*, **12**:1983-1992.

Bohr, V.A., Smith, C.A., Okumoto, D.S. & Hanawalt, P.C. 1985. DNA repair in an active gene: removal of pyrimidine dimers from the *DHFR* gene of CHO cells is much more efficient than in the genome overall. *Cell*, **40**:359-369.

Brash, D.E., Seetharam, S., Kraemer, K.H., Seidman, M.M. & Bredberg, A. 1987.

Photoproduct frequency is not the major determinant of UV base substitution hot spots or cold spots in human cells. *Proc. Natl. Acad. Sci. USA*, **84**:82-86.

Bronner, C.E., Morrison, P.T., Warren, G., Smith, L.G., Lescoe, M.K., Kane, M., Earobino, C., Lipford, J., Lindblom, A. Et al. 1994. Mutation in the DNA mismatch repair gene homologue *hMLH1* is associated with hereditary non-polyposis colon cancer. *Nature*, **368**:258-261.

Brown, D.W., Libertini, L.J., Suquet, C., Small, E.W. & Smerdon, M.J. 1993. Unfolding of nucleosome cores dramatically changes the distribution of ultraviolet photoproducts in DNA. *Biochemistry*, **32**:10527-10531.

Buratowski, S., Hahn, S., Guarente, L. & Sharp, P.A. 1989. Five intermediate complexes in transcription initiation by RNA polymerase II. *Cell*, **56**:544-550.

Buratowski, S. 1993. DNA repair and transcription: the helicase connection. *Science*, **260**:37-38.

Busch, D., Greiner, C., Lewis, K., Ford, R., Adair, G. & Thompson, L. 1989. Summary of complementation groups of UV-sensitive CHO cell mutants isolated by large scale screening. *Mutagenesis*, **4**:349-354.

Cairns, J. 1981. The origin of human cancers. *Nature*, **289**:353-357.

Chen, R.-H., Maher, V.M., Brouer, J., van de Putte, P. & McCormick, J.J. 1992. Preferential repair and strand specific repair of benzo(a)pyrene diol epoxide adducts in the *HPRT* gene of diploid human fibroblasts. *Proc. Natl. Acad. Sci. USA*, **89**:5413-5417.

Chu, G. & Chang, E. 1988.Xeroderma pigmentosum group E cells lack a nuclear factor that binds to damaged DNA. *Science*, **242**:564-567.

Cleaver, J.E. 1968. Defective repair replication of DNA in xeroderma pigmentosum. *Nature*, **218**:652-656.

Cleaver, J.E. 1969. Xeroderma pigmentosum: a human disease in which an initial stage of DNA repair is defective. *Proc. Natl. Acad. Sci. USA*, **63**:428-435.

Cleaver, J.E. 1990. Do we know the cause of xeroderma pigmentosum? *Carcinogenesis*, **11**:875-882.

Cleaver, J.E. & Kraemer, K.H. 1989. in *Xeroderma pigmentosum* of *The Metabolic Basic of Inherited Disease*, eds, Soviver, C.R., Beaudet, A.L., Sly, W.S.& Valle, D.(McGraw-Hill, New York) **Vol.II**, 2949-2971.

Coverly, D., Kenny, M.K., Munn, M., Rupp, W.D., Lane, D.P. & Wood, R.D. 1991. Requirement for the replication protein SSB in human DNA excision repair. *Nature*, **349**:538-541.

Cox, B. & Game, J. 1974. Repair systems in Saccharomyces. *Mutat. Res.*, **26**:257-264.

Donahue, B.A., Yin, S., Taylor, J.S., Reines, D.& Hanawalt, P.C. 1994. Transcript cleavage by RNA polymerase II arrested by a cyclobutane pyrimidine dimer in the DNA template. *Proc. Natl. Acad. Sci.USA*, **91**:8502-8506.

Drapkin, R., Reardon, J.T., Ansari, A., Huang, J.-C., Zawel, L., Ahn, K., Sancar, A. & Reinberg, D. 1994. Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II. *Nature*, **368**:769-772.

Ephrussi, A., Church, G.M., Tonegawa, S. & Gilbert, W. 1985. B lineage specific interactions of an immunoglobins enhancer with cellular factors in vivo. *Science*, **227**:134-140.

Evans, M.K., Taffer, B.G., Harris, C.C. & Bohr, V.A. 1993.DNA strand bias in the repair of the p53 gene in normal human and xeroderma pigmentosum group C fibroblasts. *Cancer Res.*, **53**:5377-5381.

Fairman, M.P. & Stillman, B. 1988. Cellular factors required for multiple stages of SV40 DNA replication in vitro. *EMBO J.*, 7:1211-1218.

Feaver, W.J., Svejstrup, J.Q., Bardwell, L., Bardwell, A.J., Buratowski, S., Gulyas, K.D., Donahue, T.F., Friedberg, E.C., Kornberg, R.D. 1993. Dual roles of a multiprotein complex from S. Cerevisiae in transcription and DNA repair. *Cell*, **75**:1379-1387.

Fishel, R., Lescoe, M.C., Rao, M.R., Copeland, N.G., Jenkins, N.A., Garber, J., Kane, M., Kolodner, R. Et el. 1993. The human mutation gene homologue MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell*, **75**:1027-1037.

Flejter, W.L., McDoniel, L.D., Johns, D., Friedberg, E.C.& Schultz, R.A. 1992. Correction of xeroderma pigmentosum complementation group D mutant cell phenotypes by chromosome and gene transfer: Involvement of the human *ERCC2* DNA repair gene. *Proc. Natl. Acad. Sci. USA*, **89**:261-265.

Fornace, A.J., Kohn, K.W.& Kann, H.E. 1976. DNA single strand breaks during repair of UV damage in human fibroblasts and abnormalities of repair in xeroderma pigmentosum. *Proc. Natl. Acad. Sci. USA*, **73**:39-43.

Gao, S., Drouin, R. & Holmquist, G.P. 1994. DNA repair rates mapped along the human *PGK1* gene at nucleotide resolution. *Science*, **263**:1438-1440.

Garrity, P.A. & Wold, B.J. 1992. Effects of different DNA polymerase in ligation-mediated PCR: Enhanced genomic sequencing and in vivo footprinting. *Proc. Natl. Acad. Sci. USA*, **89**:1021-1025.

Gietz, R.D.& Prakash, S. 1988.Cloning and nucleotide sequence analysis of the Saccharomyces cerevisiae *RAD4* gene required for excision repair of UV-damaged DNA. *Gene*, **74**:535-541.

Grilley, M., Welsh, K.M., Su, S.-S.& Modrich, P. 1989. Isolation and characterization of the *Escherichia coli* mutL gene product. *J. Biol. Chem.*, **264**:1000-1004.

Grielley, M., Griggith, J. & Modrich, P. 1993. Bidirectional excision in methyl-directed mismatch repair. *J. Biol. Chem.*, **268**:11830-11837.

Grossman, L. & Thiagalingam. 1993. Nucleotide excision repair: a tracking mechanism in search of damage. *J. Biol. Chem.*, **268**:16871-16874.

Guzder, S.N., Qiu, H., Sommers, C.H., Sung, P., Prakash & Prakash. 1994a. DNA repair gene *RAD3* of *S. Cerevisiae* is essential for transcription by RNA polymerase II. *Nature*, **367**:91-94.

Guzder, S.N., Sung, P., Baily, V., Prakash, L. & Prakash, S. 1994b. RAD25 is a DNA helicase required for DNA repair and RNA polymerase II transcription. *Nature*, **369**:578-581.

Guzzetta, F. 1972. Cockayne-Neil-Dingwall syndrome. In *Handbook of clinical neurology*, eds, Vinken, P.J. & Bruyn, G.W. (North Holland, Amsterdam) **vol 13**, 431-440.

Habraken, Y., Sung, P., Prakash, L. & Prakash, S. 1993. Yeast excision repair gene *RAD2* encodes a single-stranded DNA endonuclease. *Nature*, **366**:365-368.

Hanawalt, P.C. 1994. Transcription-coupled repair and human disease. *Science*, **266**:1957-1958.

Harrington, J.J. & Lieber, M.R.C. 1994. Function domains within FEN-1 and RAD2 define a family of structure-specific endonuclease: Implication for nucleotide excision repair. *EMBO J.*, **8**:1344-1355.

Haseltine, W.A., Gordon, L.K., Lindan, C.P., Grafstrom, R.H., Shaper, L.N. & Grossman, L. 1980. Cleavage of pyrimidine dimers in specific DNA sequences by a pyrimidine dimer DNA glycosylase of *M. Leteus*. *Nature* , **285**:634-641.

He, Z., Henricksen, L.A., Wold, M.S.& Ingles, C.J. 1995. RPA involvement in the damage-recognition and incision steps of nucleotide excision repair. *Nature*, **374**:566-569.

Heflich, R.H., Hazard, R.M., Lommel, L., Scribner, J.D., Maher, V.M.& McCormick, J.J. 1980.A comparison of the DNA binding, cytotoxity and repair synthesis induced in human fibroblasts by reactive derivatives of aromatic amide carcinogens. *Chem. Biol. Interact.*, **29**:43-56.

Higgins, D., Prakash, S., Reynolds, P., Polakowski, R., Weber, S. & Prakash, L. 1983. Isolation and characterization of the *RAD3* gene of *Saccharomyces cerevisiae* and inviability of *RAD3* deletion mutants. *Proc. Natl. Acad. Sci. USA*, **80**:5680-5684.

Hoeijmakers, J.H.J. & Bootsma, D. 1994. Incisions for excisions. *Nature*, **371**:654-655.

Huang, J.-C., Svoboda, D.L., Reardon, J.T. & Sancar, A. 1992. Human nucleotide excision nuclease removes thymidine dimers from DNA by incsing the 22nd phosphodiester bond 5' and the 6th phosphodiester bond 3' to the photodimer. *Proc. Natl. Acad. Sci. USA*, **89**:3664-3668

Hwang, B.J. & Chu, G. 1993.Purification and characterization of a human protein that binds to damaged DNA. *Biochemistry*, **32**:1657-1666.

Husain, J., van Houton, B., Thomas, D., Abdel-Monem, M. & Sancar, A. 1985. Effect of DNA polymerase I and DNA helicase II on the turn over rate of UvrABC excision nuclease. *Proc. Natl. Acad. Sci. USA*, **82**:6774--6778.

Kantor, G.J., Barsalou, L.S. & Hanawalt, P.C. 1990.Selective repair of specific chromatin domains in UV-irradiated cells from xeroderma pigmentosum complementation group C. *Mutat. Res.*, **235**:171-180.

Kara, K.J. & Glimcher, L.H. 1991. In vivo footprinting of MHC class II genes: Bare promoters in the bare lymphocyte syndrome. *Science*, **252**:709-712.

Keeney, S., Chang, G.J. & Linn, S. 1993.Characterization of a human DNA damage protein implicated in xeroderma pigmentosum E. *J. Biol. Chem.*, **268**:21293-21300.

Keeney, S., Exer, A.P.M., Brody, T., Vermeulen, W., Bootsma, D., Hoeijmakers, J.H.J. & Linn, S. 1994. Correction of the DNA repair defect in xeroderma pigmentosum group E by injection of a DNA damage-binding protein. *Proc. Natl. Acad. Sci. USA*, **91**:4053-4056.

Kim, C., Snyder, R.O.& Wold, M.S. 1992. Binding properties of replication protein-A from human and yeast cells. *Mol. Cell. Biol.*, **12**:3050-3059.

Kraemer, K.H. 1980. Xeroderma pigmentosum. In *Clinical Dermatology*, eds, Demis, D.J., Dobson, R.L. and McGuine, J. (Harper and Row, Hagerstown), **4**:1-33.

Kruk, P.A., Rampino, N.J. & Bohr, V.A. 1995. DNA damage and repair in telomeres. Relation to aging. *Proc. Natl. Acad. Sci. USA*, **92**:258-262.

Kunala, S. & Brash, D.E. 1992. Excision repair at individual bases of the *Escherichia coli lacI* gene: relation to mutation hot spots and transcription coupling activity. *Proc. Natl. Acad. Sci. USA*, **89**:11031-11035.

Leach, F.S., Nicolaides, N.C., Paradopoulos, N., Liu, B., Jen, J., Parsons, R., Peitomaki, P., Sistonen, P., Aaltonex, L.A., et al. 1993. Mutations of a *mutS* homolog in hereditary nonpolyposis colorectal cancer. *Cell*, **75**:1215-1223.

Lehmann, A.R., Arlett, C.F., Broughton, B.C., Harcourt, S.A., Steingrims-dottir, H., Stefanini, M., Taylor, A.M.R., Natarajan, A.T., Green, S., King, M.D., MacKie, R.M., Stephenson, J.B.P. & Tohure, J.L. 1988. Trichothiodystrophy, a human DNA repair disorder with heterogeneity in the cellular response to ultraviolet light. *Cancer Res.*, **48**:6090-6096.

Legerski, R.& Peterson, C. 1992. Expression cloning of a human DNA repair gene involved in xeroderma pigmentosum group C. *Nature*, **359**:70-73.

Li, R., Waga, S., Hannon, G.J., Beach, D. & Stillman, B. 1994. Differential effects by the *p21* CDK inhibitor on PCNA CDK inhibitor on PCNA-dependent DNA replication and repair. *Nature*, **371**:534-537.

Lin, J.-J. & Sancar, A. 1992. (A)BC excinuclease: the *E.coli* nucleotide excision repair enzyme. *Mol. Microbiol.*, **6**:2219-2224.

Lindahl, T., Sedgwick, B., Sekiguchi, M. & Nakabeppu, Y. 1988. Regulation and expression of the adaptive response to alkylating agents. *Annu. Rev. Biochem.*, **57**:133-157.

Lohman, T.M. 1992. *Escherichia coli* DNA helicases: mechanisms of DNA unwinding. *Mol. Microbiol.*, **6**:5-18.

MacInnes, M.A.& Mudgett, J.S. 1990.Cloning of the functional human excision repair gene *ERCC-5*:Potential gene regulatory features conserved with other human repair genes. *Prog. Clin. Biol. Res.*, **340**:265-274.

MacLeod, M.C. & Zachary, K. 1985.Catalysis of carcinogen-detoxification by DNA: Comparison of enantiomeric diol epoxides. *Chem. Biol. Interact.*, **54**:45-55.

Markowitz, S., Wang, J., Mijeoff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R.S., Zborowska, E., Kinzler, K.W., Volgestein, B., Brattain, M. & Wilson, J.K.V. 1995. Inactivation of the Type II TGF-β receptor in colon cancer cells with microsatellite

instability. *Science*, **268**:1336-1338.

Matsutani, C., Sugasawa, K., Yanagisawa, J., Sonoyma, T., Ui, M., van der Spek, D.J., Bootsma, D., Hoeijmakers, J.H.J. & Hanaoka, F. 1994. Purification and cloning of a nucleotide excision repair complex involving the xeroderma pigmentosum group C protein and a human homologue of yeast *RAD23*. *EMBO J.*, **13**:1831-1843.

Maxam, A.M.& Gilbert, W. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.*, **65**:499-560

Mazur, S.J. & Grossman, L. 1991.Dimerization of *Escherichia coli* UvrA and its binding to undamaged and ultraviolet damaged DNA. *Biochemistry*, **30**:4432-4443.

McCormick, J.J. & Maher, V.M. 1983. Role of DNA lesions and DNA repair in mutagenesis and transformation of human cells, in *Human carcinogensis* (Academic Press, Inc.), chapter 16, 401-429.

Meehan, T. & Straub, K. 1979.Double-stranded DNA selectively binds benzo(a)pyrene diol epoxide. *Nature*, **277**:410-412.

Melandy, T. & Stillman, B. 1992. In *Nucleic Acids and Molecular Biology*, eds, Eckstein, F. & Lilly, D.M.J. (Spinger, Berlin and Heidelberg) **vol.6**, 129-158.

Mellon, I. & Hanawalt, P.C. 1989. Induction of the *Escherichia coli* lactase operon selectively increases repair of its transcribed DNA strand. *Nature*, **342**:95-98.

Mellon, I., Bohr, V.A., Smith, C.A.& Hanawalt, P.C. 1986. Preferential DNA repair of an active gene in human cells. *Proc. Natl. Acad. Sci. USA*, **83**:8878-8882.

Mellon, I., Spivak, G.& Hanawalt, P.C. 1987. Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian *DHFR* gene. *Cell*, **51**:241-249.

Meselson, M. 1988. in *Recombination of the genetic material*, ed., K. B. Low (Academic Press, San Diego), pp. 91-113.

Mitchell, D.L., Humphrey, R.M., Adair, G.M., Thompson, L.H. & Clarkson, J.M. 1988. Repair of (6-4) photoproducts correlates with split-dose recovery in UV-irradiated normal and hypersensitive rodent cells. *Mutat. Res.*, **193**:53-63.

Miura, N., Miyamoto, I., Asuhina, H., Satokata, I., Tanaka, K. & Okada, Y. 1991. Identification and characterization of XPAC protein: the gene product of the human *XPAC* (xeroderma pigmentosum group A complementing) gene. *J. Biol. Chem.*, **266**:19786-19789.

Modrich, P. 1994. Mismatch repair, genetic stability, and cancer. *Science*, **266**:1959-1960.

Moore, S.P., Erdile, L., Kelley, T. & Fishel, R. 1991. The human homologous pairing protein HPP-1 is specifically stimulated by the cognate single-stranded binding protein hRP-A. *Proc. Natl. Acad. Sci. USA*, **88**:9067-9071.

Mori, T., Matsunaga, T., Hirose, T.& Nikaido, O. 1988. Establishment of a monoclonal antibody recognizing ultraviolet light induced (6-4) photoproducts. *Mutat. Res.*, **194**:263-270.

Moyer, R., Marien, K., van Holde, X. & Bailey, G. 1989. Site specific aflatoxin B1 adduction of sequence positional nucleosome core particles. *J. Biol. Chem.*, **264**:12226-12231.

Naegeli, H., Bardwell, L. & Friedberg, E.C. 1992. The DNA helicase and adenosine triphosphatase activities of yeast RAD3 protein are inhibited by DNA damage. A potential mechanism for damage-specific recognition. *J. Biol. Chem.*, **267**:392-398.

Naegeli, H., Bardwell, L. & Friedberg, E.C. 1993. Inhibition of RAD3 DNA helicase activity by DNA adducts and abasic sites: Implication for the role of a DNA helicase in damage-specific incision of DNA. *Biochemistry*, **32**:613-621.

Nakagawa, M., Nakatsuru, Y. & Ishikawa, T. 1989. Immunohistochemical detection of carcinogen-DNA adducts and DNA repair in mouse skin. *J. Invest. Dermatol.*, **92**:2755-2795

Nakai, S. & Matsumoto, S. 1967. Two types of radiation-sensitive mutants in yeast. *Mutat. Res.*, **4**:129-136.

Naumovski, L.& Friedberg, E.C. 1994. A DNA repair gene required for the incision of damaged DNA is essential for viability in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. USA* , 80:4818-4821.

Nicolaides, N.C., Papadopoulas, N., Liu, B., Wei, X.F., Carter, K.C., Ruber, S.M., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D.& Fraser, C.M. 1994. Mutations of two *PMS* homologous in heretidary nonpolyposis colon cancer. *Nature*, **371**:75-79.

O'Donovan, A.,& Wood, R.D. 1993. Identical defects in DNA repair in xeroderma pigmentosum group G and rodent ERCC group 5. *Nature*, **363**:185-188.

O'Donovan, A., Davies, A.A., Moggs, J.G., West, S.C.& Wood, R.D. 1994a. XPG endonuclease makes the 3' incision in human DNA nucleotide excision repair. *Nature*, **371**:432-435.

O'Donovan, A., Scherly, D., Clarkson, S.G. & Wood, R.D. 1994b. Isolation of active recombinant XPG protein, a human DNA repair endonuclease. *J. Biol. Chem.*, **269**:15965-15968.

Orren, D.K. & Sancar, A. 1989. The (A)BC excinuclease of *Escherichia coli* has only the UvrB and UvrC subunits in the incision complex. *Proc. Natl. Acad. Sci. USA*, **86**:5237-5241.

Orren, D.K., Selby, C.P., Hearst, J.E. & Sancar, A. 1992. Post-incision steps of nucleotide excision repair in *Escherichia coli. J. Biol. Chem.*, **267**:780-788.

Papadopoulos, N., Nicolaides, N.C., Wei, Y.F., Ruben, S.M., Carter, K.C., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., Adams, M.D., et al. 1994. Mutation of a mutL homologue in hereditary colon cancer. *Science*, **263**:1625-1628.

Park, E., Guzder, S.N., Koxen, M.H., Jaspers-Dekker, I. & Weeda, G. 1992. *RAD25 (SSL2)*, the yeast homolog of the human xeroderma pigmentosum group B DNA repair gene, is essential for viability. *Proc. Natl. Acad. Sci. USA*, **89**:11416-11420.

Parsons, R., Li, G.M., Longley, M.J., Fang, W.H., Papadopoulos, N., Jen, J., de la Chapelle, A., Kinzler, K.W., Vogelstein, B. & Podrich, P. 1993. Hypermutability and mismatch repair defiency in RER$^+$ tumor cells. *Cell*, **75**:1227-1235.

Pathak, M.A., Zarebska, Z., Mihm, M.C. Jr, Jarzabek, C.M., Chorzelski, T.& Jablonska, S. 1986. Detection of DNA psoralen photoadducts in mammalian skin. *J. Invest. Dermatol.*, **86**:308-315

Pfeifer, G.P.1992. Analysis of chromatin structure by ligation mediated PCR. *PCR method and appl.*, **2**:107-111.

Pfeifer, G.P. & Riggs, A.D. 1991. Chromatin differences between active and inactive X chromosome revealed by genomic footprinting of permeabilized cells using DNaseI and ligation-mediated PCR. *Genes & Dev.*, **5**:1102-1113.

Pfeifer, G.P., Drouin, R., Riggs, A.D.& Holmquist, G.P. 1992. Binding of transcription factors creats hot spots for UV photoproducts in vivo. *Mol. Cell. Biol.*, **12**:1798-1804.

Podust, V.N., Georgaki, A., Strack, B. & Hubscher, U. 1992.Calf thymus RF-C as an essential component for DNA polymerase delta and epsilon holoenzymes function. *Nucleic Acids Res.*, **20**:4159-4165.

Ponti, M., Forrow, S.M., Souhani, H.L., D'Incalci, M. & Hartley, J.A. 1991. Measurement of the sequence specificity of covalent DNA modification by antineoplastic agents using Taq DNA polymerase. *Nucleic Acids Res.*, **19**:2929-2934.

Prakash, S., Sung, P. & Prakash, L. 1993. DNA repair genes and proteins of *Saccharomyces cerevisiae*. *Annu. Rev. Genet.*, **27**:53-70.

Reynolds, R.J. & Friedberg, E.C. 1981. Molecular mechanisms of pyrimidine dimer excision in *Saccharomyces cerevisiae*: incision of ultraviolet-irradiated deoxyribonucleic acid in vivo. *J. Bacteriol.*, **146**:692-704.

Rill, R.L.& Marsh, G.A. 1990. Sequence preference of covalent DNA binding by anti(+)- and anti(-)-benzo(a)pyrene diol epoxide. *Biochemistry*, **29**:6050-6058.

Robins, P., Johes, C.J., Biggerstaff, M., Lindahl, T. & Wood, R.D. 1989. Complementation of DNA repair in xeroderma pigmentosum group A cell extracts by a protein with affinity for damaged DNA. *EMBO J.*, **10**:3913-3921.

Roth, M., Multer, H.& Boyle, J.M. 1987. Immunochemical determination of an initial step in thymine dimer excision repair in xeroderma pigmentosum variant fibroblasts and biopsy material from the normal population and patients with basal cell carcinoma and melanoma. *Carcinogenesis*, **8**:1301-1307.

Sancar, A. & Rupp, W.D. 1983.A novel repair enzyme: UVRABC excision nuclease of *Escherichia coli* cuts a DNA strand on both sides of the damaged region. *Cell*, **33**:249-260.

Sancar, A. & Sancar, G.R. 1988. DNA repair enzymes. *Annu. Rev. Biochem.*, **57**:29-67.

Sancar, A. & Tang, M.-S. 1993. Nucleotide excision repair. *Photochem. Photobio.*, **57**:905-921.

Sancar, G.B. 1990. DNA photolyases:physical properties, action mechanisms, and roles in dark repair. *Mutat. Res.*, **236**:147-160.

Schaaper, R.M., Dunn, R.L. & Glickman, B.W. 1987.Mechanisms of ultraviolet-induced mutation. Mutation spectra in the *Escherichia coli* lacI gene for a wild type and an excision-repair-deficient strain. *J. Mol. Biol.*, **198**:187-202.

Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeulen, W. Hoeijmakers, J.H.J., Chambon, P. & Egly, J.M. 1993. DNA repair helicase: a component of BTF2(TFIIH) basic transcription factor. *Science*, **260**:58-63.

Scherly, D., Nouspikel, T., Corlet, J., Ucla, C., Bairoch, A.& Clarkson, S.G. 1993. Complementation of the DNA repair defect in xeroderma pigmentosum group G cells by a human cDNA related to yeast *RAD2*. *Nature*, **363**:182-185.

Seeberg, E. & Steinum, A. 1982. Purification and properties of the UvrA protein from *E.*

coli. Proc. Natl. Acad. Sci. USA, **79**:988-992.

Selby, C.P. & Sancar, A. 1990. Transcription preferentially inhibits nucleotide excise repair of the template strand in vitro. *J. Biol. Chem.*, **265**:21330-21336.

Selby, C.P. & Sancar, A. 1991. Gene and strand-specific repair in vitro: partial purification of a transcription-repair coupling factor. *Proc. Natl. Acad. Sci. USA*, **88**:8232-8236.

Selby, C.P. & Sancar, A. 1993. Molecular mechanism of transcription-repair coupling. *Science*, **260**:53-58.

Seroussi, E. & Lavi, S. 1993. Replication protein A is the major single-stranded-DNA binding protein detected in mammalian cell extracts by gel retardation assays and UV cross-linking of long and short single-stranded DNA molecules. *J. Biol. Chem.*, **268**:7147-7154.

Shiomi, T., Harada, Y., Saito, T., Shiomi, N., Okuno, Y.& Yamaizumi, M. 1994. An *ERCC5* gene with homology to yeast *RAD2* is involved in group G xeroderma pigmentosum. *Mutat. Res.*, **314**:167-175.

Shivji, M.K.K., Kenney, M.K. & Wood, R.D. 1992. Proliferating cell nuclear antigen is required for DNA excision repair. *Cell*, **69**:367-374.

Smith, B.L. & MacLeod, M.C. 1993. Covalent binding of the carcinogen benzo(a)pyrene diol epoxide to Xenopus laevis 5S DNA reconstituted into nucleosomes. *J. Biol. Chem.*, **268**:20620-20629.

Strauss, B., Rabkin, S., Sagher, D. & Moore, P. 1982. The role of DNA polymerase in base substitution mutagenesis on noninstructional templates. *Biochimie*, **64**:829-838.

Sugano, T., Uchida, T. & Yamazumi, M. 1991. Identification of a specific protein defective in group A xeroderma pigmentosum cells. *J. Biochem.* (Tokyo), **110**:667-674

Sung, P., Prakash, L., Matson, S.W.& Prakash, S. 1987. RAD3 protein of *Saccharomyces cerevisiae* is a DNA helicase. *Proc. Natl. Acad. Sci. USA*, **84**:8951-8955.

Sung, P., Higgins, D., Prakash, L. & Prakash, S. 1988. Mutation of lysine-48 to arginine in the yeast RAD3 protein abolishes its ATPase and DNA helicase activities but not the ability to bind ATP. *EMBO J.*, 7:3263-3269.

Sung, P., Reynolds, P., Prakash, L. & Prakash, S. 1993a.Purification and characterization of the Saccharomyces cerevisiae RAD1/RAD10 endonuclease. *J. Biol. Chem.*, **266**:26391-26399.

Sung, P., Bailly, V., Weber, C., Thompson, L.H., Prakash, L. & Prakash, S. 1993b.Human xeroderma pigmentosum group D gene encodes a DNA helicase. *Nature*, **365**:852-855.

Svejstrup, J.Q., Wang, Z., Feaver, W.J., Wu, X., Bushnell, D.A., Donahue, T.F., Friedberg, E.C. & Korberg, R.D. 1995. Different forms of TFIIH for transcription and DNA repair: Holo-TFIIH and a nucleotide excision repairsome. *Cell*, **82**:21-28.

Tanaka, K., Hayakawa, H., Sekiguchi, M. & Okada, Y. 1977. Specification of T4 endonuclease V on damaged DNA in xeroderma pigmentosum cells in vivo. *Proc. Natl. Acad. Sci. USA*, **74**:2958-2962.

Tanaka, K., Kawai, K., Kumahara, Y., Ikenega, M. & Okada, Y. 1981. Genetic complementation groups in Cockayne syndrome. *Somatic Cell Genet.*, **7**:445-462.

Tanaka, K., Satokata, I., Ogita, Z., Uchido, T. & Okada, Y. 1989. Molecular cloning of a mouse DNA repair gene that complements the defect of group A xeroderma pigmentosum. *Proc. Natl. Acad. Sci. USA*, **86**:5512-5516.

Tanaka, K., Miura, N., Satokata, I, Miyamoto, I., Yoshida, M., Satoh, Y., Kondo, S., Yasui, A., Okayama, H. & Okada, Y. 1990. Analysis of a human DNA excision repair gene involved in group A xeroderma pigmentosum and containing a zinc finger domain. *Nature*, **348**:73-76.

Thomas, D.C., Morton, A.G., Bohr, V.A. & Sancar, A. 1988. General method for quantitating base adducts in specific mammalian genes. *Proc. Natl. Acad. Sci. USA,* **85**:3723-3727.

Thompson, L.H., Busch, D.B., Brookman, K., Mooney, C.L. & Glaser, D.A. 1981. Genetic diversity of UV-sensitive DNA repair mutants of Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA*, **78**:3734-3737.

Todo, T., Takemori, H., Ryo, H., Ihara, M., Matsunaga, T., Nikaido, O., Sato, K.& Nomura, T. 1993. A new photoreactivating enzyme that specifically repairs ultraviolet light-induced (6-4) photoproducts. *Nature*, **361**:371-374.

Tornaletti, S. & Pfeifer, G.P. 1994. Slow repair of pyrimidine dimers at *p53* mutation hot spots in skin cancer. *Science*, **263**:1436-1438.

Toschi, C. & Bravo, R. 1988. Changes in cyclin/proliferating cell nuclear antigen distribution during DNA repair synthesis. *J. Cell Biol.*, **107**:1623-1628.

Troelstra, C., Odijk, H., de Wit, J., Westerveld, A., Thompson, L.H., Bootsma, D. & Hoeijmakers, J.H.J. 1990. Molecular cloning of the human excision repair gene *ERCC-6*. *Mol. Cell. Biol.*, **10**:5806-5813.

Troelstra, C., van Cool, A., de Wit, J., vermeuten, W., Bootsma, D. & Hoeijmakers, J.H.J. 1992. ERCC6, a member of a subfamily of putative helicase, is involved in Cockayne's syndrome and preferential repair of active genes. *Cell*, **71**:939-953.

van Duin, M., de Wit, J., Odijk, H., Westerveld, A., Yasui, A., Koken, M.H.M., Hoeijmakers, J.H.J. & Bootsma, D. 1986. Molecular cloning and characterization of the human excision repair gene *ERCC-1*: cDNA cloning and amino acid homology with the yeast DNA repair gene *RAD10*. *Cell*, **44**:913-923.

van Duin, M., Vredeveldt, G., mayne, L.V., Vermeulen, W., Klein, B., Weeda, G., Hoeijmakers, J.H.J. & Westveld, A. 1989. The cloned human DNA excision repair gene *ERCC-1* fails to correct xeroderma pigmentosum complementation groups A though I. *Mutat. Res.*, **217**:83-92.

van Houten, B. 1990. Nucleotide excision repair in *Escherichia coli*. *Microbiol. Rev.*, **54**:18-51.

van Vuuren, A.J., Appeldoorn, E., Odijk, H., Yasui, A., Jaspers, N.G.J., Bootsma, D. & Hoeijmakers, J.H.J. 1993. Evidence for a repair enzyme complex involving ERCC1 and complementing activities of ERCC4, ERCC11 and xeroderma pigmentosum group F. *EMBO J.*, **12**:3693-3701.

Venema, J., van Hoffen, A., Natarajon, A.T., van Zeeland, A.A.& Mullanders, L.H.F. 1990a. The residual repair capacity of xeroderma pigmentosum complementation group C fibroblasts is highly specific for transcriptionally active gene. *Nucleic Acids Res.*, **18**:443-448.

Venema, J., Mullenders, L.H., Natarajan, A.F., van Zeeland, A.A. & Mayne, L.V. 1990b. The genetic defect in Cockayne's Syndrome is associated with a defect in repair of UV-induced DNA damage in transcriptionally active DNA. *Proc. Natl. Acad. Sci. USA*, **87**:4707-4711.

Visse, R., de Ruijter, M., Moolenaar, G.F. & van de Putte, P. 1992. Analysis of UvrABC endonuclease reaction intermediates on cisplatin-damaged DNA using mobility shift gel electrophoresis. *J. Biol. Chem.*, **267**:6736-6742.

Volgestein, B. & Kinzler, K.W. 1992. p53 function and dysfunction. *Cell*, **70**:523-526.

Wani, A.A. & D'Ambrosio, S.M. 1987. Immunological quantitation of O^4-ethylthymidine in alkylated DNA: repair of minor miscoding base in human cells. *Carcinogenesis*, **8**:1137-1144.

Weber, C.A., Salazar, E.P., Stewart, S.A. & Thompson, L.H. 1988. Molecular cloning and biological characterization of a human gene, *ERCC-2*, which corrects the nucleotide

excision repair defection in CHO UV5 cells. *Mol. Cell. Biol.*, **8**:1137-1146.

Weeda, G., van Ham, R.C.A., Masurel, R., Westerveld, A., Odijk, H., de Wit, J., Bootsma, D., van der Eb, A.J. & Hoeijmakers, J.H.J. 1990a. Molecular cloning and biological characterization of the human excision repair gene *ERCC-3*. *Mol. Cell. Biol.*, **10**:2570-2581.

Weeda, G., van Ham, R.C.A., Vermulen, W., Bootsma, D., van der Eb, A.J.& Hoeijmakers, J.H.J. 1990b. A presumed DNA helicase encoded by *ERCC-3* is involved in the human repair disorders xeroderma pigmentosum and Cockayne's Syndrome. *Cell*, **62**:777-791.

Wei, D., Maher, V.M. & McCormick, J.J. 1995(a). Site-specific rates of excision repair of benzo(a)pyrene diol epoxide adducts in the hypoxanthine phosphoribosyltranscferase gene of human fibroblasts: Correlation with mutation spectra. *Proc. Natl. Acad. Sci. USA*, **92**:2204-2208.

Wei, D., Maher, V.M. & McCormick, J.J. 1995(b) Effect of adduct conformation and local DNA secondary structure on rates of repair of polycyclic adducts in human cells. Manuscript submitted.

Weintraub, H. & Groudine, M. 1976.Chromosomal subunits in active genes have an altered conformation. *Science*, **193**:848-856.

Westerveld, A., Hoeijmakers, J.H.J., van Duin, M., de Wit, J., Odijk, H., Pastink, A., Wood, R.D. & Bootsma, D. 1984. Molecular cloning of a human DNA repair gene. *Nature*, **310**:425-428.

Wold, M.S. & Kelly, T.J. 1988. Purification and charicterization of replication protein A, a cellular protein required for in vitro replication of Simian virus 40 DNA. *Proc. Natl. Acad. Sci. USA*, **85**:2523-2327.

Yang, L.L., Maher, V.M.& McCormick, J.J. 1980. Error free excision of the cytotoxic, mutagenic N^2-deoxyguanosine DNA adducts formed in human fibroblasts by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene. *Proc. Natl. Acad. Sci. USA*, **77**:5933-5937.

Yang, J.-L., Maher, V.M. & McCormick, J.J. 1988. Kinds and spectrum of mutations induced by 1-nitrosopyrene adducts during plasmid replication in human cells. *Mol. Cell. Biol.*, **8**:3364-3372.

Zawel, L. & Reinberg, D. 1992. Advances in RNA polymerase II transcription. *Curr. Biol.*, **4**:488-452.

CHAPTER II

Site specific rates of excision repair of benzo(a)pyrene diol epoxide adducts in the hypoxanthine phosphoribosyltransferase gene of human fibroblasts--Correlation with mutation spectra

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ABSTRACT

When synchronized populations of repair-proficient diploid human fibroblasts were treated with (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE) in early S phase, just as the hypoxanthine phosphoribosyltransferase (*HPRT*) gene was being replicated, 5% of the induced base substitutions were found at nucleotide 212 and 5% at 229 in exon 3. However, when the population was treated in early G_1 phase to allow 12 or more hr for repair prior to the onset of S, 21% of the substitutions were found at nucleotide 212 and 10% at 229. No such cell cycle-dependent difference in distribution of base substitutions occurred in excision repair deficient cells. To test whether the increase in the relative frequency of mutations resulted from inefficient repair at these sites, we adapted ligation mediated PCR to measure the rates of removal of BPDE adducts from individual sites in exon 3 of the *HPRT* gene. Cells were treated with 0.5 μM BPDE in early G_1 phase and harvested immediately or after being allowed 10, 20 and 30 hr for repair. DNA was analyzed for the original distribution of BPDE adducts in the region of interest (the nontranscribed strand of exon 3) and those remaining after repair. The sites of adduct formation were determined using *Escherichia coli* UvrABC excinuclease to excise the adducts, and annealing a 5' biotinylated gene specific primer to the DNA and extending it with Sequenase 2.0 to generate a blunt end at the site of each cut. A linker was ligated to the blunt end, and the desired fragments were isolated from the rest of the genomic DNA by use of magnetic beads and amplified by PCR and analyzed on a sequencing gel. The distribution of fragments of particular lengths

indicated the relative number of BPDE adducts initially formed or remaining at specific sites in the gene. The rates of repair at individual sites varied widely along exon 3 of the *HPRT* gene and were very slow at nucleotides 212 and 229, strongly supporting the hypothesis that inefficient DNA repair plays an important role in the formation of mutation hot spots.

INTRODUCTION

Previous studies in this laboratory on the relationship between DNA repair and mutations induced by carcinogens in synchronized populations of diploid human fibroblasts show that the frequency of 6-thioguanine (6-TG) resistant mutants induced by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE) in nucleotide excision repair proficient cells is highest in cells treated in early S phase just as the *HPRT* gene is being replicated, and much lower in cells treated in early G_1 phase so that the cells will have 12 or more hr for repair before they begin DNA replication (Yang et al., 1982; Watanabe et al., 1985; Chen et al., 1990). This is not true for cells from a xeroderma pigmentosum (XP) complementation group A patient, which are virtually incapable of nucleotide excision repair; the frequency of mutants induced in XP cells treated in G_1 phase is the same as in cells treated in S phase (Yang et al., 1982; Chen et al., 1991). Analysis of the kinds and locations of mutations induced by BPDE in the *HPRT* gene of repair proficient cells treated in early S phase and G_1 phase (Chen et al., 1990) showed that the kinds of mutations did not differ significantly, but there was a significant difference in the frequency of the mutations at specific sites (see Figure 1). Of 20 base substitutions derived from cells treated in early S phase, just as the *HPRT* gene was being replicated, only one was located at nucleotide 212 and one at 229 within exon 3 of the *HPRT* gene. However, of 19 base substitutions from cells treated in G_1 phase and allowed at least 12 hours for excision repair before DNA replication, four (21%) were at nucleotide 212 and two (10%) were at 229. If repair occurs with the same

Fig.1: Number of mutants with a base substitution in the region of interest, i.e., the 184 nucleotide of the nontranscribed strand of exon 3 of the *HPRT* gene, observed in 19 mutants analyzed from cells treated at early G1 phase (filled bars), and 20 mutants analyzed from cells treated at early S phase(blank bars).

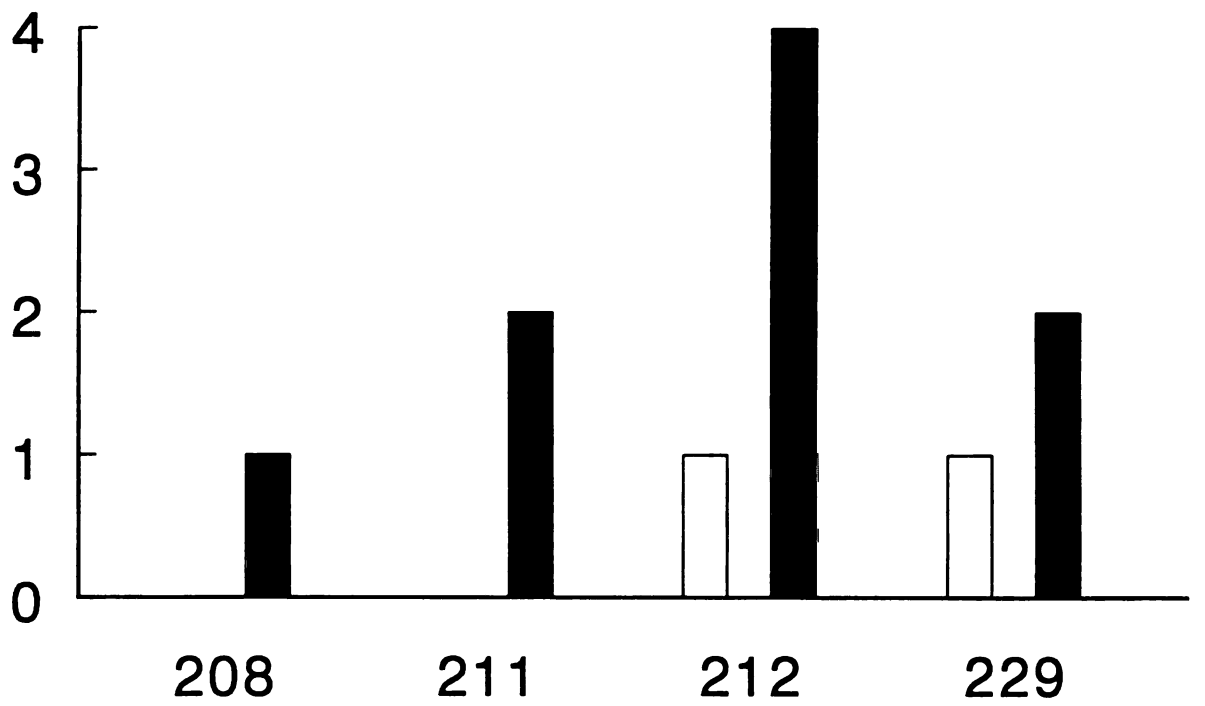

Fig. 1

efficiency at each site, the relative frequency of mutations at each site should not change. We hypothesize that the hot spots seen in the G_1 cells resulted from inefficient repair of BPDE adducts in those sites so that after excision repair has occurred for a certain period of time, the adducts remaining in those sites represents a higher fraction than originally present. The fact that no such increase in relative frequency of mutations at particular sites was observed in a similar study using XP cells from complementation group A supported this hypothesis (Chen et al., 1991).

Recently, Tornaletti and Pfeifer (1994) and Holmquist and colleagues (Gao et al., 1994) showed that the rates of excision repair of cyclobutane pyrimidine dimer, induced by UV_{254} irradiation, vary at the nucleotide level along the human *p53* and *PGK1* gene, respectively. Using ligation mediated PCR (LMPCR), Tornaletti and Pfeifer (1994) were able to detect the relative change in the frequency of UV photoproducts at individual sites and show that seven out of the eight mutation hot spots found by Brash and his colleagues (Brash et al., 1991; Ziegler et al, 1993) in the *p53* gene of basal cell and squamous cell carcinomas, are sites where repair is slow. These investigators suggest that this slow rate of repair plays an important role in the formation of the mutation hot spots found in the tumors. Underlying this conclusion is the assumption that the rate of repair in the epithelial cells in skin is similar to that seen in confluent fibroblasts in culture, and that such cells have adequate time for repair after irradiation before the particular mutations occur.

The present study was designed to determine whether inefficient repair at particular sites in the *HPRT* gene could explain the relative increase in frequency of mutations

observed in diploid human fibroblasts in culture treated with BPDE in early G_1 phase compared to the same cells treated just as the gene is being repaired. To do so, we synchronized repair proficient fibroblasts, and treated them with BPDE in early G_1 phase to allow adequate time for repair, harvested them immediately or after various times, extracted the DNA and assayed it for the relative frequency of adducts in the region of interest (the nontranscribed strand of exon 3 of the *HPRT* gene). *Escherichia coli* UvrABC excinuclease was used to excise the BPDE adducts generating breaks in DNA that leave terminal 5'-phosphate groups (Seeberg et al, 1983; van Houten et al, 1986; Tand et al, 1992; Sancar and Rupp, 1983). The relative number of cuts at particular sites was determined using LMPCR. The decrease of the number of cuts occurring in DNA at each specific site after BPDE- treated cells were allowed time for repair indicated the rates of excision repair at that site during the period of repair.

Using this method, we were able to quantitate the rate of repair of BPDE adducts in exon 3 of the *HPRT* gene at the nucleotide level. Our data indicated that the rate of removal of adducts from various sites differ significantly, and as predicted by the mutation spectra data of Chen et al. (1990), the rates of repair at the two sites that showed a relative increase in mutation frequency (cell cycle dependent hot spots) were among the slowest.

MATERIALS AND METHODS

Cell Culture and Synchronization. Diploid human male fibroblasts from neonatal foreskin (McCormick and Maher, 1981), designated SL68, were cultured in Eagle's medium containing 10% supplemented bovine calf serum (Hyclone, Logan, UT). Cells were driven into the G_0 state by density inhibition and mitogen deprivation (Chen et al, 1990). To stimulate the cells to reenter the cell cycle, they were trypsinized and plated at 1.7 x 10^4 cells/cm^2 in fresh medium.

BPDE Treatment and Post-Treatment Incubation. For the DNA repair study, the cells in early G_1, 5 hr after release from G_0, were rinsed twice with phosphate-buffered saline and treated with 0.5 μM BPDE for 1 hr as described (Chen et al., 1990). Cells were lysed immediately or incubated in fresh medium for the indicated time and then lysed. To study the initial distribution of BPDE adducts formed in cells treated in early S and in early G1 phase, two synchronized populations were treated simultaneously: one 17 hr after releasing from G_0 and the other 5 hr after releasing from G_0. The two populations were rinsed twice with phosphate-buffered saline, treated with 13 μM BPDE for 1 hr as described (Chen et al., 1990) and lysed immediately.

Isolation and Purification of DNA. Cells were washed with Tris-buffered saline and lysed in buffer containing 10 mM Tris.HCl (pH 8.0), 0.5% SDS and 60 μg/ml RNase A as described (Sambrook et al., 1989). After 1 hr incubation, proteinase K was added to a final concentration of 150 μg/ml, and the sample was incubated at 50°C for 3 hr before being extracted with phenol/chloroform (1:1, vol/vol) using Phase Lock Gel tubes (5

Prime-3 prime, Inc., West Chester, PA). The DNA was precipitated with ethanol, redissolved in 10 mM Tris (pH 8.0), 1 mM EDTA (TE 8.0), and was incubated overnight with restriction enzymes *BglI* and *SpeI* (Boeringer Mannheim Biochemicals, Indianapolis, IN) to decrease viscosity and with RNase A (10 μg/ml) to eliminate any trace amount of contaminating RNA. The DNA was once again purified and the concentration was measured using a spectrophotometer.

UvrABC Excision Reaction. The UvrA, UvrB and UvrC protein subunits were kindly provided by Dr. Pieter van de Putte of Leiden University, Leiden, the Netherlands. DNA (12 μg from cells treated with 0.5 μM BPDE and 6 μg from cells treated with 13 μM BPDE) from cells that had been allowed various lengths of time to repair BPDE adducts was used for analysis. UvrABC digestion was performed as described (Chen et al., 1992) except that the buffer contains 100 mM NaCl, 1 mM ATP and no BSA. The reaction was stopped as described (Chen et al., 1992) and the samples were extracted with phenol/chloroform and purified with Centricon-30 microconcentrators (Amicon Inc., Beverly, MA). The purified DNA was precipitated with ethanol and redissolved in 11 μl H_2O.

Generating a Sequence Ladder. A genomic DNA fragment containing exon 3 of the *HPRT* gene was amplified using a primer from intron 2 (CCTTATGAAACATGAGCGCAAAGG) and a primer from intron 3 (CTCACTGTAACCAAGTGAAATGAAA). The latter was designated gene specific primer 2. The amplified fragment was purified with Centricon-30 tube, subjected to the Maxam-Gilbert sequencing method (Maxam and Gilbert, 1980), and amplified by

LMPCR as described below.

Extension of Primer and Linker Ligation. Extension and ligation were done essentially as described (Mueller and Wold, 1989), except gene specific primer 2, used as the first primer, was biotinylated at the 5' end. The annealing temperature was adjusted to 52°C. The linker consisted of a longer oligomer (GTCTTATGTATGTATCTCGAATGCT) and a shorter oligomer (AGCATTCGAGAT).

Isolation of the Desired Fragments with Magnetic Beads. DNA was mixed with 75 µg Dynabeads M280-streptavidin (Dynal A/S, Oslo, Norway) and the desired fragments were isolated using a magnet following the manufacturer's instructions.

Polymerase Chain Reaction. The purified DNA fragments were amplified in a mixture containing 35 mM Tris.HCl (pH 8.55), 2.75 mM $MgCl_2$, 60 mM KCl, 16 mM NaCl, 0.2 mM dNTPs, 15 pmol each of gene specific primer 3 (GCAAGTATGGTTTGCAGAGATTC), which is 3' to primer 2, and the longer oligomer of the linker and 2.5 units of Taq DNA polymerase (Perkin Elmer Corp., Norwalk, CT) in a total volume of 50 µl. The amplification was performed by 22 cycles of denaturation (94°C, 1 min), annealing (48°C, 1 min) and extension (72°C, 2 min). After the final cycle, one more unit of Taq polymerase was added and the sample was incubated for another 7 min at 72°C. Amplified DNA samples were purified by Centricon-30 tubes and the final volume of the parallel samples was adjusted to be equal.

Annealing and Extending a ^{32}P-labeled Primer. A gene specific sequencing primer (ACCTAGTGTTGCCACTA), 3' to primer 3, was labeled with γ-^{32}P-ATP (New England Nuclear, Boston, MA) by T4 polynucleotide kinase (United States Biochemicals Corp.

(USB), Cleveland, OH) following the manufacturer's instructions. Labeled primer (0.5 pmol) was mixed with 6 µl purified LMPCR product and 1µl 0.5% NP-40 and 2 µl Sequenase Reaction Buffer (USB) and incubated at 95°C for 5 min. The samples were chilled on ice for 5 min and 5 µl reaction mixture (20 mM DTT, 1 mM dNTPs, 2 units of Sequenase 2.0) was added. The samples were incubated at 37°C for 10 min and the reaction was stopped by adding 5 µl Stop Solution (USB). After that, the samples were heated at 95°C for 3 min and loaded into a prewarmed 6% sequencing gel. After electrophoresis, the gel was dried and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Determining the Initial Distribution of BPDE Adducts in Cells Treated at Early G1 Phase or S Phase. One possible explanation for the difference Chen et al. (3) observed in the spectrum from cells treated at early G1 phase and that from cells treated in early S phase is that the initial distribution of BPDE adducts in the two populations differs significantly because of differences in chromatin structure. To determine if this were the case, we prepared two populations of synchronized cells, one in early S and the other in early G1 phase and treated them at the same time with the same sample of BPDE to avoid any variations in the concentration of BPDE. This protocol involved releasing cells from G_0 at two different times prior to the simultaneous exposure to BPDE. The control population was only exposed to DMSO. Immediately after treatment, the cells were lysed, and the DNA was extracted and digested with *Spe*I and *Bgl*I to generate a 2kb fragment containing the region of interest.

The DNA was then treated with UvrABC excinuclease to introduce an incision at 8th nucleotide 5' to the site of a BPDE adduct and a incision at 4th or 5th nucleotide 3' to the adduct, with the majority at 4th nucleotide(Tang et al., 1992; Sancar and Rupp, 1983). The samples were denatured and 5' biotinylated gene specific primer 2 was annealed and extended to the site of cut with Sequenase 2.0. After ligation of the asymmetric blunt-ended linker, the DNA was mixed with Dynabeads. A magnet was used to attract the Dynabeads carrying the desired fragments of DNA, and the rest of the DNA was washed away. The fragments of interest were then released from the strand attached to the

Fig.2. Autoradiogram showing the initial distribution of BPDE adducts in synchronized cell populations treated in early G1 or S phase, as analyzed using UvrABC and LMPCR. Cells were treated with 13 μM BPDE, a dose yielding an adduct level of approximately 0.1 in the 184 nucleotides of the nontranscribed strand of exon 3 of the *HPRT* gene. The labeled products were amplified from 6 μg genomic DNA from cells treated in S (lane S) or G1 phase (lane G1) or treated with DMSO only (lane U). The nucleotide positions of the region of interest are shown. Lane seq-G contains the products of the G reaction of the Maxam-Gilbert sequencing method performed on a DNA fragment containing exon 3 followed by LMPCR, as described in the text. The bands in Lane Seq-G are 4 nucleotides longer than the corresponding UvrABC generated products.

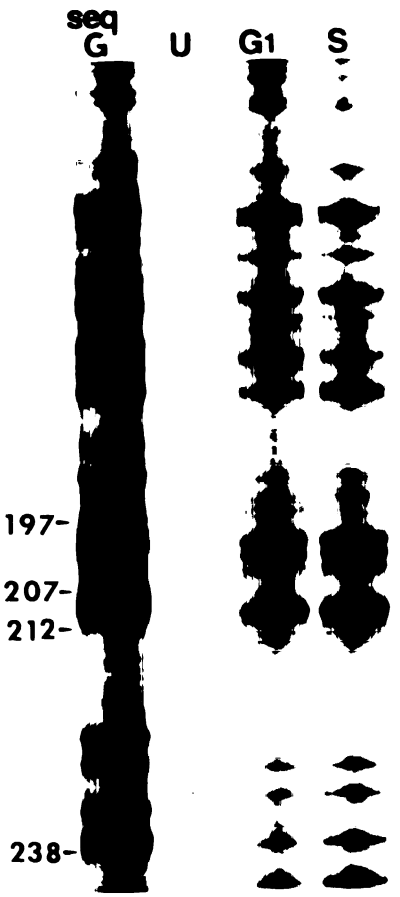

Fig.2

Dynabeads by alkali denaturation and amplified by 22 cycles of PCR as described in the Methods section.

The result is shown in Figure 2. The DNA from cells treated at early G1 phase (5 hr after release from G_0) or S phase (17 hr after release) showed identical patterns, indicating the difference observed in mutation spectra was not due to difference in BPDE binding patterns. The bands correspond to those of the G lane of the sequencing ladder, but the lengths of the fragments are four or five nucleotides shorter, with the vast majority being four nucleotides shorter. The band patterns in Figure 2 indicate that the majority, if not all, of the BPDE adducts involve guanine, in agreement with previous reports (Conney, 1982). In experiments in which a DNA fragment containing exon 3 was treated in vitro with BPDE, digested with UvrABC, and similarly analyzed, we also found that the vast majority of the cuts generated 3' of the BPDE adducts in the region of interest were at the fourth nucleotide (data not shown). Lane U of Figure 2 shows that there was no nonspecific UvrABC excision of untreated DNA, indicating that UvrABC specifically recognizes BPDE adducts.

Determining the Rate of Excision Repair of BPDE Adducts Using LMPCR. To determine if the difference in mutation spectra between cells treated in early S or G1 phase results from inefficient repair of BPDE adducts at those particular sites, we treated populations of human cells in early G_1 phase with 0.5 μM BPDE and used LMPCR to analyze the initial distribution of BPDE adducts in exon 3 of the *HPRT* gene and the fraction remaining at each site after time for repair. Chen et al. (1992) showed that the same cells treated with 1.2 μM BPDE were fully capable of nucleotide excision repair.

If DNA synthesis were to occur during the time allowed for repair, this would give an apparent decrease in frequency of adducts. Extensive studies in this laboratory show that under the conditions used, untreated cells enter S-phase approximately 15 hr after release from G_0 (Watanabe et al., 1985; Konze-Thomas et al, 1982). However, BPDE causes a dose-dependent delay in DNA synthesis. Before beginning our repair study, we compared cells treated with 0.5 μM BPDE and control cells exposed to DMSO for the amount of incorporation of tritiated thymidine into DNA during 30 hr. This value was less than 0.5% of the incorporation in the control (data not shown), indicating that during the period of interest, the extent of replication would be too small to affect measurements of loss of BPDE adducts.

To quantify adduct levels using LMPCR, amplification must be in the exponential range (Kalinowski et al., 1992). To determine if this were the case under our conditions, we mixed 6 μg of DNA isolated from cells immediately after 0.5 μM BPDE treatment with 6 μg of control DNA and analyzed it with UvrABC and LMPCR using our standard conditions. Similar analysis was carried out using 12 μg of DNA from the treated cells. The intensities of the various bands with the former were approximately half that of the corresponding bands in the latter (data not shown), indicating that after 22 cycles of PCR, the amplification was still in the exponential range.

We used LMPCR to analyze the rate of repair in cells treated with 0.5 μM BPDE in early G1 and allowed 0, 10, 20 and 30 hr for repair. The results are shown in Figure 3. The DNA from these populations showed patterns similar to those observed in the samples shown in Figure 2, i.e., the lengths of the fragments are 4 nucleotides shorter

Fig.3: Autoradiogram illustrating extent of repair in the nontranscribed strand of exon 3 of the HPRT gene of cells treated with 0.5 μM BPDE, a dose giving an adducts level of 0.005 per exon 3 strand.. Lane G and CT contain the products of the G and CT reactions of the Maxam-Gilbert sequencing method as described in text; Lane U shows analysis of the DNA from cells treated with DMSO only; Lanes 0, 10, 20 and 30 show analysis of BPDE adducts in DNA isolated from cells treated in early G1 phase and harvested immediately or after incubation for the indicated number of hours. The nucleotide positions of cuts generated by UvrABC are indicated by numbers; The corresponding bands in the G sequencing ladder are indicated by arrow heads.

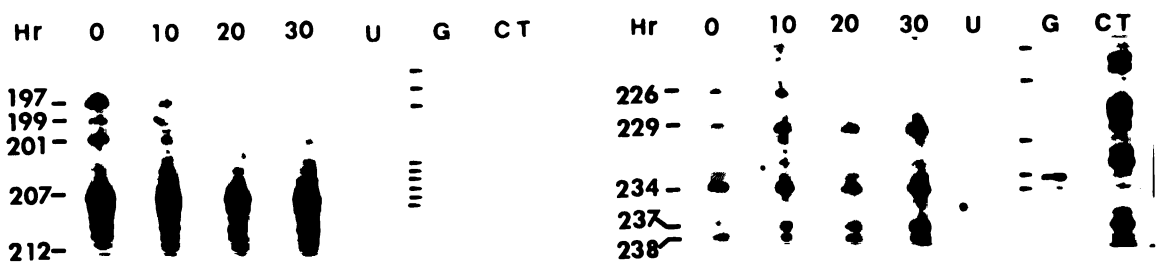
Hr
0
10
20
30
U
G
C T
197
199
201
207
212
Hr
0
10
20
30
U
G
C T
226
229
234
237
238

Fig. 3

than the corresponding fragments in the G lane of the sequencing control. The intensity of the bands shows that binding at certain sites, e.g., 207, was more frequent than at other sites, e.g., 212. Repair of adducts at specific sites was shown by a progressive decrease in the intensities of the corresponding gel bands.

Evidence of Site-specific Repair of BPDE Adducts in Human Cells. The rates of excision repair at individual nucleotides were determined by measuring the intensities of the bands in Figure 3 with the PhosphorImager and comparing the relative changes for each of the corresponding bands. The results are shown in Figure 4. The rate of repair varied along the strand and the differences were reproducible in three independent experiments. The average rate of repair of adducts from the nontranscribed strand of exon 3 was about 52% removed after 30 hr. However, at some sites a significant fraction of the adducts had been removed after only 10 hr, e.g., 85% removed at position 199, while at other sites such as 212 and 229, only 25% and 19% of the original adducts had been removed even after 30 hr. At these positions, i.e., 212 and 229, the rates of repair were among the slowest. These are the two sites where the relative mutation frequency increased in cells having time for repair compared to cells having little or no time for repair. These data strongly support the hypothesis that failure to remove adducts from specific sites before DNA replication occurs plays an important role in the formation of the mutation hot spots.

Fig.4: Rates of repair of BPDE adducts in the region of interest. The intensities of the bands in Figure 3 were quantified using a PhosphorImager. The intensities at time 0, representing the initial level of adducts, were taken as 100% (First bar).The relative percentage of BPDE adducts remaining at each site after 10 hr (Second bar), 20 hr (Third bar) and 30 hr (Fourth bar) are shown. The standard deviations were calculated from three independent experiments. The standard deviations for site 237 have been omitted because in one experiment out of three, i.e., the one shown in Figure 3, the intensity of the bands increased with time for repair.

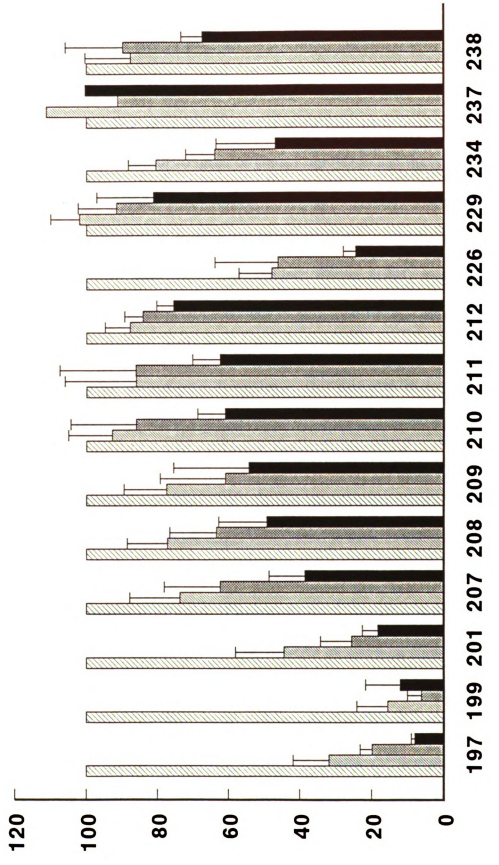
120
100
80
60
40
20
0
197 199 201 207 208 209 210 211 212 226 229 234 237 238

Fig. 4

DISCUSSION

We determined the initial distribution of BPDE adducts at the nucleotide level in the nontranscribed strand of exon 3 of the *HPRT* gene of human fibroblasts and the rate at which they are repaired at each site and found that, although the initial distribution of adducts in cells treated at G1 phase or S phase is essentially the same, some sites are repaired significantly slower than other sites. Tornaletti and Pfeifer (1994) recently showed that the rate of repair of cyclobutane pyrimidine dimers from the *p53* gene of human fibroblasts also differed from one site to another and that those which were repaired slowly did not share a common primary sequence. Our data also show this. Holmquist and coworkers (Gao et al., 1994) reported that some of the sites in the *PGK1* gene that are repaired much more slowly than other sites are located within transcription factor binding sites in the promoter. It is highly unlikely that transcription factors bind to exon 3 of the *HPRT* gene, but nucleosomes might be expected to form in that region and chromatin folding may also cause steric hindrance to the action of the repair enzymes and account for some of the slower repair rates. Changes in DNA secondary structure induced by photoproducts or bulky adducts are considered to be an initiating signal for nucleotide excision repair (Sancar and Sancar, 1988; van Houten, 1990). One possibility to account for the very rapid rates we observed at certain sites, and the one that we favor, is that the degree of distortion caused by a BPDE adduct at certain sites is greater than at others, and that this contributes significantly to the differences we observed.

Our data strongly support the hypothesis that the cell-cycle dependent mutation hot

spots induced by BPDE, i.e, at sites 212 and 229, are the result of inefficient repair, since the same initial distribution of BPDE adducts in cells at G1 phase or S phase indicates that the formation of those hot spots cannot be attributed to different BPDE binding. After 30 hr of time for repair, 75% of the adducts at position 212 and 81% of those at 229 were still present. These percentages are much higher than the average, i.e, about 48% left on, and are significantly higher than the 8% and 12% left on at positions 197 and 199. Nucleotide 211 may also represent a site where there was a relative increase in the frequency of mutations induced by BPDE in cells with time for repair; of 20 base substitutions from cells treated in S phase, none were located there, compared to 2 out of 19 from cells treated in G_1 phase (Fig. 1). This increase may reflect inefficient repair, since 63% of the adducts remained at that site after 30 hr (Fig. 4).

Not all the sites in exon 3 where the initial adduct level was high or where repair was slow show up as mutation hot spots. A major reason is that the selection system used to detect mutants, i.e., resistance to 6-TG, affects the chance of finding a particular mutation. Mutations at certain sites, e.g., 210, 234 and 237, will not change the amino acids sequence and thus cannot be detected, even though the initial adduct levels at these sites were significant and the rates of repair at those sites were average or slower than average. A base substitution at site 238, a site which showed a significant amount of adduct formation (Fig. 3) and very slow repair (Fig. 4), would change the amino acid sequence, but a search of the human *HPRT* mutation database of Dr. Neal Cariello at the University of North Carolina shows that no base substitution at this site have been reported. The same argument holds true for site 207. The initial adduct level was very high at this site

and the rate of repair is close to average, but in the database only one base substitution has ever been reported in this site and this mutation was a tandem mutation (207-208 GG>TT). Since a G>T at 208 is selectable, it is very likely that the mutation reported at 207 was silent. This strongly suggests that substitutions at these two sites do not significantly affect the function of the protein and therefore cannot be detected by the 6-TG resistance assay.

Even for mutations that do affect the function of the HPRT protein, it is likely that different mutations at various sites affect the protein function to a different extent and can be recovered with different efficiencies. Most of the base substitutions induced by BPDE are G>T mutations. It is possible that a G>T mutation at some sites damages the function of HPRT protein less than a G>A or G>C mutation. If so, the mutation frequency at these sites will be low because the majority of the mutations at these sites are hard to detect using 6-TG assay. All of these selection biases can affect the final mutation spectrum obtained. That explains why in cells treated at S phase, with little or no time for repair before replication of *HPRT*, no mutations were found at certain sites where initial adduct levels were high and yet, 1 mutation each out of 20 mutations was found in site 212 and 229, where BPDE adduct level is relatively low. Thus, selection can make it difficult to correlate DNA damage spectra with mutation spectra. This may especially be the case when the end point is a tumor, because tumors arise as the result of multiple mutations and multiple steps of selection.

In addition to the strong effect that selection can have, many other factors play a role in converting DNA damage into a spectrum of mutations. Rate of repair at individual sites

in the gene is only one of these. The original distribution of damage is also critical. What is more, when DNA polymerases encounter a damage, e.g., a BPDE adduct, local sequence context, the conformation of the adduct and the specific DNA polymerase itself will determine the possibility that the polymerase can bypass the lesion, and whether or not the result is a mutation. For example, a context in which a BPDE adduct has a high chance of being bypassed in an error-free manner or a high chance of failing to be bypassed at all will give a low mutation frequency even if the level of adducts at that site is high. If the polymerase fails to bypass, this incompletely replicated DNA may eventually be repaired by other error prone or error free mechanisms.

The advantage of our system is that it allows one to compare the difference between the mutation spectrum of cells mutagenized when the majority are replicating or about to replicate the target gene and that of the same cells exposed many hours prior to that time with the rates of repair at individual nucleotides in cells exposed under the same conditions. Excision repair deficient XP cells do not show a cell cycle-dependent change in mutation hot spots (Chen et al, 1991). Therefore, the major molecular basis for the changes in mutation spectra observed in repair proficient cells is very likely the differences in rate of repair at individual nucleotides. The chance of a cell with a certain mutation at a particular site in the HPRT gene surviving 6-TG selection should be constant. Therefore, if one observes an increase in relative frequency of mutations at a particular site in one population of mutagenized repair-proficient cells compared to another and there is no significant difference in the initial distribution of DNA damage in the two populations, the observed difference in mutation spectra should reflect adducts

remaining at particular sites due to inefficient repair at those positions.

In summary, our data show that the initial distribution of BPDE adducts in the nontranscribed strand of the exon 3 of the *HPRT* gene in diploid human fibroblasts was non-random, and the rate of repair of these adducts at individual sites varied significantly. The rates of repair at the cell-cycle dependent mutation hot spots in that region, i.e, at positions 211, 212 and 229, were among the slowest, indicating that inefficient repair plays an important role in the formation of such mutation hot spots. The results of our study also indicate that UvrABC excinuclease can be used in LMPCR assays to quantify rates of excision repair. This should greatly expand the range of investigations of repair that can be carried out.

ACKNOWLEDGEMENT

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REFERENCES

Brash, D.E., Rudolph, J.A., Simon, J.A., Lin, A., McKenna, G.J., Baden, H.P., Halperin, **A.J. &** Ponten, J. 1991. A role for sunlight in skin cancer: UV-induced *p53* mutations in **squam**ous cell carcinoma. *Proc. Natl. Acad. Sci. USA*, **88**:10124-10128.

Chen, R.-H., Maher, V.M. & McCormick, J.J. 1990. Effect of excision repair by diploid **human** fibroblasts on the kinds and locations of mutations induced by (±)-7β,8α-**dihyd**roxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene in the coding region of the *HPRT* gene. *Proc. Natl. Acad. Sci. USA*, **87**: 8680-8684.

Chen, R.-H., Maher, V.M. & McCormick, J.J. 1991. Lack of a cell cycle-dependent strand **bias** for mutations induced in the *HPRT* gene by (±)-7β,8α-dihydroxy-9α,10α-epoxy-**7,8,9,**10-tetrahydrobenzo(a)pyrene in excision repair-deficient human cells. *Cancer Res.*, **51:25**87-2592.

Chen, R.-H., Maher, V.M., Brouwer, J., van de Putte, P. & McCormick, J.J. 1992. **Prefe**rential repair and strand specific repaair of benz(a)pyrene diol epoxide adducts in the *HPRT* gene of diploid human fibroblasts. *Proc.Natl. Acad. Sci. USA*, **89**:5413-5417.

Conney, A.H. 1982. Induction of microsomal enzymes by foreign chemicals and **carcin**ogenesis by polycyclic aromaticc hydrocarbons. *Cancer Res.*, **42**:4875-4917.

Gao, S., Drouin, R. & Holmquist, G.P. 1994. DNA repair rates mapped along the human *PGK1* gene at nucleotide resolution. *Science,* **263**:1438-1440.

Kalinowski, D.P., Illenye, S. & Van Houten, B. 1992. Analysis of DNA damage and **repair** in murine leukemia L1210 cells using a quantitative polymerase chain reaction. *Nucleic Acids Res.*, **20**:3485-3494.

Konze-Thomas, B., Hazard, R.M., Maher, V.M. & McCormick, J.J. 1982. Extent of **excision** repair before DNA synthesis determines the mutagenic but not the lethal effect **of UV** radiation. *Mutat. Res.*, **94**:421-434.

Maxam, A.M. & Gilbert, W. 1980. Sequencing end-labeled DNA with base-specific **chemical** cleavage. *Methods Enzymol.*, **65**:499-560.

McCormick, J.J. & Maher, V.M. 1981. in *DNA Repair: A Laboratory Manual of Research Procedures, eds.* Friedberg, E.C. & Hanawalt, P.C. (Dekker, New York), **Vol.1B**, 501-**521.**

Mueller, P.R. & Wold, B. 1989. In vivo footprinting of a muscle specific enhancer by

ligation mediated PCR. *Science*, **246**:780-786.

Sambrook, J., Fritsch, E.F. & Maniatis, T. 1989. in *Molecular Cloning: A Laboratory Manual.* (Cold Spring Harbor Lab., Cold Spring Harbor, NY) 9.16-9.18.

Sancar, A. & Rupp, W.D. 1983. A noval repair enzyme: UVRABC excision nuclease of *Escherichia coli* cuts a DNA strand on both sides of the damaged region. *Cell*, **33**:249-260.

Sancar, A.& Sancar, G.B. 1988. DNA repair enzymes. *Ann. Rev. Biochem.*, **57**: 29-67.

Seeberg, E., Steinium, A.L., Nordenskjold, N., Soderhaus, S. & Jerstrom, B. 1983. Strand-break formation in DNA modified by benzo(a)pyrene diol epoxide. Quantitative cleavage by *Escherichia coli* UvrABC endonuclease. *Mutat.Res.*, **112**:139-145.

Tang, M., Pierce, J.R., Doisy, R.P., Nazimiec, M.E. & MacLeod, M.C. 1992. Difference and similarities in the repair of two benzo(a)pyrene diol epoxide isomers induced DNA adducts by uvrA, uvrB, aand uvrC gene products. *Biochemistry*, **31**:8429-8436.

Tornaletti, S. & Pfeifer, G.P. 1994. Slow repair of pyrimidine dimers at *p53* mutatio hot spots in skin cancer. *Science*, **263**:1436-1438.

Van Houten, B., Masker, W.E., Carrier, W.L. & Regan, J.D. 1986. Quantitation of carcinogen-induced DNA damage and repair in human cells with the UVRABC excision nuclease from *Escherichia coli. Carcinogenesis*, **7**:83-87.

Van Houten, B. 1990. Nucleotide excision repair in *Escherichia coli. Microbiological Reviews*, **54**:18-51.

Watanabe, M., Maher, V.M. & McCormick, J.J. 1985. Excision repair of UV-or benzo(a)pyrene diol epoxide-induced lesions in xeroderma pigmentosum variant cells is "error free". *Mutat. Res.*, **146**: 285-294.

Yang, L.L., Maher, V.M. & McCormick, J.J. 1982. Relationship between excision repair and the cytotoxic and mutagenic effect of the "anti"7,8-diol-9,10-epoxide of benzo(a)pyrene in human cells. *Mutat. Res.*, **94**: 435-447.

Ziegler, A., Leffell, D.J., Kunala, S., Sharma, H.W., Gailani, M., Simon, J.A., Halperin, A.J., Baden, H.P., Shapiro, P.E., Bale, A.E. & Brash, D.E. 1993. Mutation hot spots due to sun light in the *p53* gene of nonmelanoma skin cancer. *Proc. Natl. Acad. Sci. USA*, **90**:4216-4220.

Chapter III

Effect of the adduct conformation and the local conformation of DNA on rates of repair of polycyclic adducts in human cells

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SUMMARY

To assess the effect of the adduct conformation and the local conformation of the DNA where the adduct has formed on the rate of nucleotide excision repair in human cells, we determined the rate of repair of 1-nitrosopyrene (1-NOP)-induced adducts in exon 3 of the hypoxanthine phosphoribosyltransferase (*HPRT*) gene at the nucleotide level using ligation mediated polymerase chain reaction, and compared the results with those we obtained previously using benzo(a)pyrene diol epoxide (BPDE). The principal DNA adduct formed by either agent involves guanine. Although the average rate of excision repair of 1-NOP adducts in exon 3 was 2~3 times faster than that of BPDE adducts, at a particular nucleotide, the rate of repair of 1-NOP adducts was slower or faster than that of BPDE adducts or, in some cases, equal to that of BPDE adducts. These results strongly suggest that the rate of repair at each position depends on both the specific adduct conformation and the local DNA conformation. The results provide experimental support for the recent hypothesis by Sancar and colleagues [*Proc. Natl. Acad. Sci. USA* **91**, 12213 (1994)] that the efficiency of excision repair depends on the ability of the adducted position to support a higher affinity interaction between the recognition subunits and the DNA.

INTRODUCTION

It is well known that procaryotic and eucaryotic cells rely on nucleotide excision repair (NER) to remove a wide range of DNA damage caused by various agents, including UV irradiation and such polycyclic carcinogens as benzo(a)pyrene diol epoxide (BPDE) and N-acetoxy-2-acetylaminofluorene (N-AcO-AAF) (Sancar, 1994; Lin and Sancar, 1992; Sancar and Tang, 1993). Studies using loss of *E. coli* UvrABC excinuclease sensitive sites (van Houten et al., 1986) or loss of radiolabeled polycyclic DNA adducts (Heflich et al., 1980; Yang et al., 1980) show that various types of DNA damage are removed from the overall genome of human cells at very different rates. To account for such differences in rate, it has generally been hypothesized that the structure of the photoproduct or adduct, i.e., the degree of distortion it causes in the DNA helix, is the major determining factor.

More recently it was found that actively-transcribed genes are repaired faster than non-transcribed genes (preferential repair) (Chen et al., 1992), and what is more, nucleotide excision repair of damage in the transcribed strand of such genes is faster than in the nontranscribed strand (strand specific or transcription-coupled repair) (Hanawalt, 1994). These observations focused attention on the contribution of the chromatin structure in determining the rate of NER and the role of the transcription process itself. Nevertheless, comparative data on the rate of repair of various kinds of damage in the individual strands of the HPRT gene of human fibroblasts in culture show that the rate of excision repair of UV induced photoproducts (cyclobutane pyrimidine dimers (CPD) and pyrimidine-6-4-

pyrimidones (6-4s)) (9) or adducts formed by 1-nitrosopyrene (1-NOP) (McGregor et al., 1995) is much faster than excision of BPDE adducts (Chen et al., 1992). Therefore, the conformation of the damage itself appears to play a dominant role in determining the rate of repair.

However, very recent studies in human fibroblasts of the rates of excision repair of UV-induced CPD (Tornaletti and Pfeifer, 1994; Gao et al., 1994) or BPDE adducts (Wei et al., 1995) at the nucleotide level (site-specific repair) in particular genes indicate that the conformation of the photoproduct or adduct cannot be the sole determinant. This is because the rate of excision of a specific type of CPD, e.g., a cytosine-cytosine dimer, or of the guanine-BPDE adduct varied significantly from one position in the gene of interest to another position in that gene, sometimes by more than 10-fold. No consensus primary DNA sequence could be found to account for this difference. Holmquist and his colleagues (Gao et al., 1994) proposed that the presence of transcription factors at specific sites interfered with excision repair because they found out that the region in the *PGK1* gene where the repair of CPD is the slowest is also the region where transcription factors bind. Since most of the human DNA is considered to be wrapped around histone octamers to form nucleosomes, Tornaletti and Pfeifer (1994) hypothesized that the nucleosomal structure causes steric hindrance to the repair enzymes, and makes rates of repair vary at different sites.

In the present study, we examined the question of the relative contribution of the adduct conformation and that of the local conformation of the DNA, including nucleosome pattern effects and possible transcription factors etc., by determining the rate

of excision of 1-NOP adducts at the nucleotide level in exon 3 of the HPRT gene and comparing the results with those of BPDE adducts we recently obtained in the same region under the same conditions (Wei et al., 1995). The principal adducts of both polycyclic compounds involve guanine, with BPDE binding primarily at the N^2 position and 1-NOP at the C8 position of guanine. If steric hindrance to the action of the excision repair enzyme complex at specific positions in the region of interest results from various forms of protein-DNA interaction, it might be expected that the contribution of these factors to the rate of repair at a specific position in the gene will be constant. Then, substituting one structurally related adduct for another at that specific position ought not to affect the pattern of site-specific repair.

To carry out such a study, we synchronized normal human fibroblasts, treated them with 1-NOP in early G1 phase, determined the rates of repair at the nucleotide level using ligation mediated PCR and *E. coli* UvrABC excinuclease and compared the results with those we obtained previously with BPDE (Wei et al., 1995). The results showed that in the region of interest, 1-NOP adducts were repaired at least twice as fast as the BPDE adducts. However, repair of 1-NOP adducts at some sites was almost as slow as that of BPDE adducts or even slower, indicating that neither the adduct conformation nor the local DNA conformation alone is the sole determinant of the rate of repair at a particular site. These data strongly support the hypothesis that the efficiency of NER depends not only on the effect of the adduct conformation itself, but also on the local DNA conformation.

MATERIALS AND METHODS

Cell culture and synchronization. Diploid human male fibroblasts from neonatal foreskin, designated SL68 (McCormick and Maher, 1981), were cultured in Eagle's minimal essential medium containing 10% supplemented bovine calf serum. Cells were driven into the G0 state by density inhibition and mitogen deprivation (Watanabe et al., 1985). To stimulate the cells to reenter the cell cycle, they were trypsinized and plated at 1.7 X 10^4 cells/cm^2 in fresh medium containing serum.

Carcinogen treatment and post treatment incubation. 1-nitrosopyrene, kindly provided in solid form by Dr. Frederick Beland at National Center for Toxicology Research (Jefferson, AK), was dissolved in anhydrous DMSO, stored in -80°C freezer under nitrogen gas and thawed only prior to use. Five hr after the cells were released from G0, the medium was removed, the cells were rinsed twice with phosphate-buffered saline, and serum-free medium was added. The cells were treated with 0.8 μM 1-NOP by adding appropriate volume of 1-nitrosopyrene stock solution and incubating for 1 hr at 37°C. The cells were rinsed and lysed immediately or incubated in fresh medium containing serum for the time before being lysed.

Determination of extent indicated of DNA synthesized. Cells were incubated in fresh medium containing serum and ^{3}H-Tdr (6.7 Ci/mmol. DuPont) for 10 hr and then lysed and DNA were extracted and purified as described (Wei et al., 1995). The specific activity of DNA (cpm/μg of DNA) was determined and the amount of the newly synthesized DNA was calculated.

Determination of the rates of excision repair of 1-NOP adducts at the nucleotide level. DNA was isolated from the cell lysates and purified with phenol/chloroform as described (Wei et al., 1995). Unless otherwise specified, 12 μg of purified DNA was cut with SpeI and BglI and then digested with UvrABC excinuclease, kindly provided by Dr. Pieter van de Putte at Leiden Univerisity (Leiden, the Netherlands), to excise the 1-NOP adducts as described (Chen et al., 1992). The frequency of UvrABC cuts at each site was then measured using ligation mediated PCR as described (Wei et al., 1995). Briefly, the UvrABC digested DNA was purified and redissolved in 11 μl of H_2O. Then DNA was denatured and a gene specific primer (primer 2) containing a biotin at 5' end was allowed to anneal and extended by Sequenase 2.0. A specially designed linker was ligated and the desired fragments were isolated with 75 μg of Dynabeads M-280 Streptavidin following the manufacturer's instructions. The purified fragments were then amplified with 22 cycles of PCR, using the longer oligomer of the linker and a second gene specific primer (primer 3), located 3' of primer 2. Amplified DNA was purified with phenol/chloroform and precipitated in ethanol along with 10 μg yeast tRNA, and redissolved in 15 μl solution containing 10 mM Tris·HCl (pH 7.5) and 1 mM EDTA. Six microliter of the final PCR product was labeled using a ^{32}P-labeled gene specific sequencing primer and separated on a sequencing gel. The intensity of the bands was analyzed using a PhosphorImager (Molecular Dynamics).

RESULTS

Determination of DNA replication during the time allowed for repair. Any semiconservative DNA synthesis occurring within the period allowed for repair would give an apparent decrease in the frequency of adducts. Extensive studies in this laboratory show that under the conditions used, cells synchronized by release from density inhibition and serum deprivation begin DNA synthesis after ~15 hr (Watanabe et al., 1985). However, treating cells with carcinogens in early G1 phase causes a dose-dependent delay in the onset of DNA synthesis (Yang et al., 1982). Since we treated cells 5 hr after release from G0, DNA replication during the next 10 hr should be negligible. To determine the extent of daughter strand DNA synthesized during 10-20 hr after treatment, we treated cells in early G1 with 0.8 μM 1-NOP or with the solvent DMSO alone (control) and measured the amount of incorporation of ^{3}H-Tdr into DNA during the period. This value for the 1-NOP treated cells was ~2% of the incorporation in the control cells (data not shown). Therefore we used 12.2 μg DNA from this sample to ensure the same amount of parental DNA to start with.

Rates of repair of 1-NOP adducts in exon 3 of the *HPRT* gene. To determine the rates of repair of 1-NOP adducts at the nucleotide level on exon 3 of the *HPRT* gene, we treated synchronized populations of excision repair-proficient diploid human fibroblasts in early G1 phase for 1 hr with 0.8 μM 1-NOP and lysed the cells immediately or after allowing 10 hr or 20 hr for repair. DNA was extracted and excision by E. coli UvrABC excinuclease was used to determine the fraction of adducts remaining in each position in

Fig.1: Autoradiogram illustrating extent of repair in the nontranscribed strand of exon 3 of the *HPRT* gene of cells treated with 0.8 μM 1-NOP (A) or 0.5 μM BPDE (B). Lane G contain the products of the G reaction of the Maxam-Gilbert sequencing method as described (13); Lane U in (B) shows analysis of the DNA from cells treated with DMSO only; Lanes 0, 10, 20 and 30 show analysis of 1-NOP adducts (A) or BPDE adducts (B) in DNA isolated from cells treated in early G1 phase and harvested immediately or after incubation for the indicated number of hours. The nucleotide positions of cuts generated by UvrABC are indicated by numbers. The corresponding bands in the G sequencing ladder are indicated by arrow heads. The obvious band located above position 207 in (A) is indicative of a 1-NOP adenine adduct formed at site 205, a very minor adduct for 1-NOP.

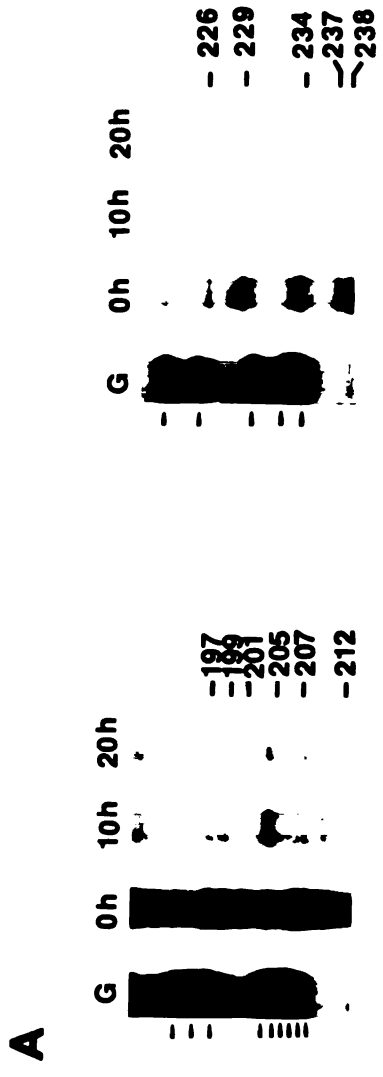
A
G
0h
10h
20h
197
199
201
205
207
212
G
0h
10h
20h
226
229
234
237
238

Fig. 1

B

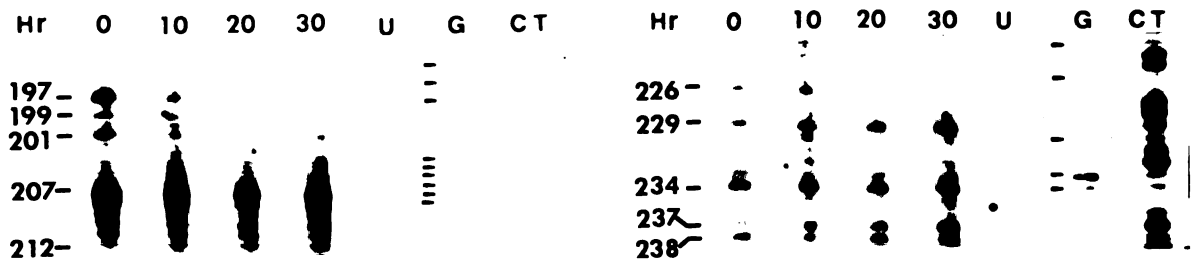
Hr 0 10 20 30 U G C T
197
199
201
207
212
Hr 0 10 20 30 U G C T
226
229
234
237
238

Fig. 1

the DNA region of interest. The cuts generated by UvrABC were mapped with ligation mediated PCR as described (Wei et al., 1995). The results are shown in Figure 1A. The results we obtained previously at the same region of the *HPRT* gene with BPDE adducts are shown in Fig. 1B for purpose of comparison. UvrABC excinuclease can be expected to excise a specific adduct at a specific site with constant efficiency. Therefore, the decrease in frequency of cuts at a particular site should accurately reflect the decrease of the adduct level at that site, i.e., the rate of repair. The average rate of repair of 1-NOP adducts in the region of interest, i.e., positions 197-238 in exon 3 of the *HPRT* gene, was approximately 67% excised in 10 hr and 78% excised in 20 hr. The corresponding average rate for BPDE adducts is 22% in 10 hr and 34% in 20 hr (Wei et al., 1995).

Comparison of site-specific repair of 1-NOP adducts and BPDE adducts in the region of interest. The site specific rate of repair of 1-NOP adducts differed from that of BPDE adducts (Fig. 2). At some positions in exon 3, the 1-NOP adducts were repaired faster than BPDE adducts. Even at positions at which BPDE adducts were repaired the most quickly, i.e., 197, 201 and 226 (20%, 26%, 46% remaining after 20 hr, respectively), the repair of 1-NOP adducts was significantly faster than that of BPDE adducts (only 7%, 8%, 6% left on after 20 hr, respectively). At positions where BPDE adducts are excised much less quickly, e.g., positions 207 and 237-238 (63% and 92% remaining after 20 hr, respectively), 1-NOP adducts were repaired significantly faster than BPDE adducts (only 11% and 29% left on after 20 hr, respectively). These data strongly suggest that the conformation of the adduct plays a major role in determining the rate of excision repair.

However, 1-NOP adducts were not repaired faster than BPDE adducts at every position.

Fig.2: Relative percentage of 1-NOP-induced adducts (filled bars) and BPDE adducts (blank bars) remaining in the region of interest after 20 hr of repair. The intensities of the bands in Figure 1 were quantified using a PhosphorImager, and the relative decrease in intensity of each of the corresponding bands was calculated. Two independent experiments were performed for 1-NOP and three were performed for BPDE. The rates of repair reported represent the mean. The intensities at time 0, representing the initial level of adducts, were taken as 100%.

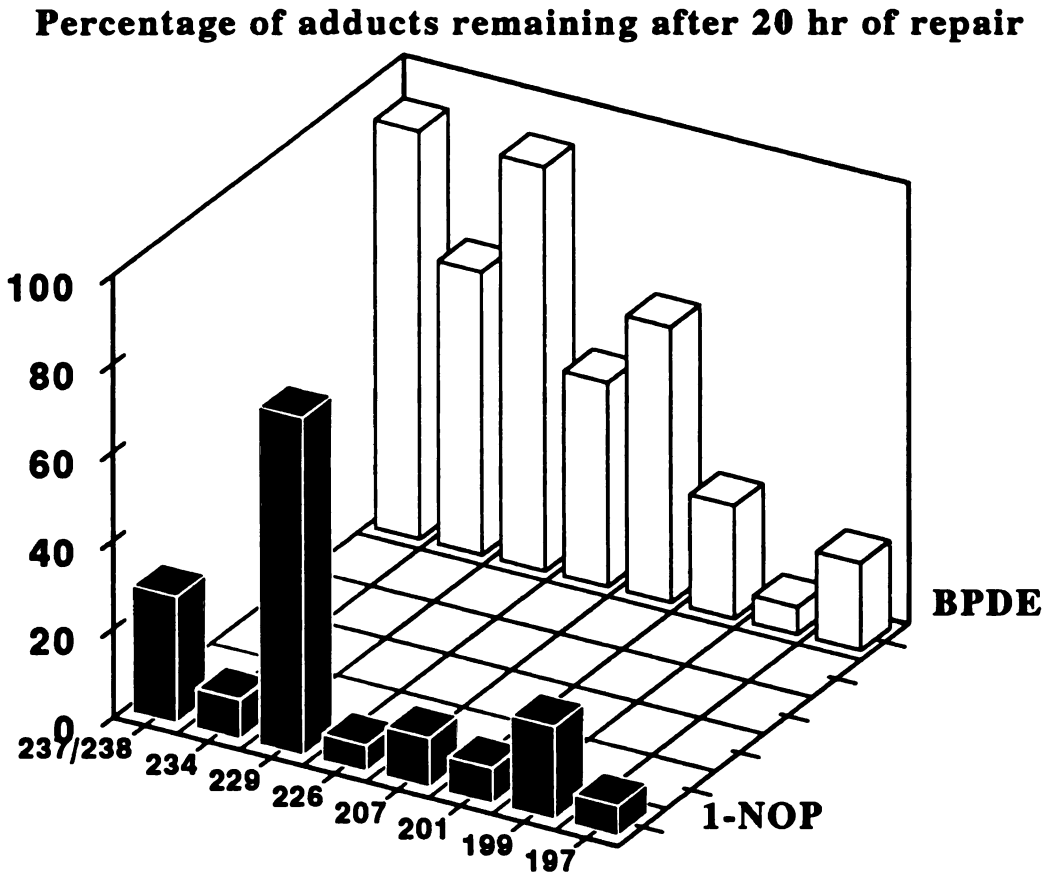
Percentage of adducts remaining after 20 hr of repair
100
80
60
40
20
0
237/238
234
229
226
207
201
199
197
BPDE
1-NOP

Fig. 2

At position 229, where BPDE adducts were repaired very slowly (91% remaining after 20 hr), the repair of 1-NOP adducts was comparably slow (76% left on after 20 hr), and at position 199, 1-NOP adducts were repaired even more slowly than BPDE adducts, i.e., 21% of 1-NOP adducts remained after 20 hr, compared to only ~6% of BPDE adducts. These data indicate that although on an overall level, 1-NOP adducts are repaired faster than BPDE adducts, when one examines individual nucleotide positions in the gene, the rate of repair of 1-NOP adducts can be slower or faster than that of BPDE adducts at that same site, or equal to the rate of repair of BPDE adducts. Therefore, even though the conformation of the adduct plays a major role, other factors having to do with the local conformation of the DNA are critical for determining the rate of NER.

Not only did the rate of repair of the two kinds of adducts at a specific position, e.g., 207, differ significantly (Fig. 1), but their relative patterns of site-specific repair (Fig. 2) also differed. In the pattern for BPDE adducts, the repair rate at position 199 is very rapid compared to the overall average, which was 34% removed in 20 hr; at positions 237-238, it is much slower than average; and at positions 207 and 234, it is equal to average. In contrast, in the pattern for 1-NOP adducts, the rate at position 199 was equal to the average, which was ~78% removed in 20 hr; at positions 237-238, it was also average; but at position 207 and position 234 it was much more rapid than the average. Therefore, we conclude that the contribution of the local conformation of DNA to the rates of excision repair at specific sites varies among different DNA adducts.

DISCUSSION

We have investigated the rates of excision repair of 1-NOP adducts at the nucleotide level on the nontranscribed strand of exon 3 of the *HPRT* gene in normal human fibroblasts. We found that the majority of the adducts involved guanine, and that the rates of repair at different sites varied significantly. By comparing the site specific rate of repair of 1-NOP adducts to that of BPDE adducts in the same region, we found out that although on average the 1-NOP adducts were repaired at least two times faster than BPDE adducts, at some sites, 1-NOP adducts were repaired as slowly as or even slower than BPDE adducts, indicating that the rates of repair depend on the adduct conformation as well as the local conformation of the DNA where the adduct has formed.

A comparative study in a human embryonic cell line showed that the tritiated 1-NOP adducts were removed 2-3 times faster that tritiated BPDE adducts from the genomic DNA (Yang et al., 1988). A similar result was found when McGregor et al (1995) measured the rate of repair of 1-NOP adducts in a 20 kb fragment of the *HPRT* gene in normal human fibroblasts and compared it to the repair of BPDE (Chen et al., 1992). But the reasons underlying this difference are not clear. BPDE, a reactive intermediate of benzo(a)pyrene, principally binds to the N^2 position of guanine. The adduct is pentacyclic and lies in the minor groove of the DNA molecule with the pyrenyl ring oriented toward the 5' end of the modified strand, where it causes little helical distortion (Cosman et al., 1992). 1-NOP, a partially reduced metabolite of 1-nitropyrene, undergoes a further intracellular reduction step in huamn fibroblasts before it forms an unstable reactive

intermediate that binds principally to the C8 position of guanine to form the stable tetracyclic adduct N-(deoxyguanosine-8-yl)-1-aminopyrene. This adduct lies in the major groove of the DNA and causes little helical distortion (Nolan et al., 1994). It is possible that an adduct located in the major groove is more accessible to the NER machinery than an adduct located in the minor groove, and thus is repaired faster. If this is the case, it could explain why the repair of 1-NOP adducts was much faster than that of BPDE adducts at nucleotide positions such as 207 and 226, but it cannot explain why 1-NOP adducts were repaired as slowly as BPDE adducts at position 229 and even more slowly than BPDE adducts at position 199.

We also showed that the contribution of the local conformation of DNA to rates of excision repair at specific sites varies among different types of DNA adducts. Thus, it is unlikely that the site specific rate of repair is a mere reflection of the local DNA conformation or protein interaction. What matters is the final local conformation adopted by the DNA after formation of the adduct. Therefore, we conclude that site specific rates of repair reflect the combined effect of the adduct conformation and the specific local conformation of the DNA where the adduct has formed.

The model recently proposed by Sancar and colleagues for the human excinuclease system (Huang et al., 1994) hypothesizes that excision repair in human cells starts with nonspecific binding of the recognition subunits to DNA, and this transient, nonspecific binding results in changes in the local DNA conformation, such as DNA kinking and unwinding. It proposes that if the presence of an adduct facilitates this conformational change, a higher affinity interaction between the recognition subunits and DNA occurs,

and this long-lived complex allows binding of the nuclease subunits that induce the double incision. This model implies that the final conformation of the adducted site determines the efficiency of nucleotide excision repair. Our data in Figures 1 and 2, showing that the rate of excision repair is determined by the adduct conformation and the specific local conformation of DNA where the adduct is located, provide experimental support for this model and also explain why no consensus primary DNA sequence was found for either rapidly or slowly repaired sites in DNA (Tornaletti and Pfeifer, 1994; Gao et al., 1994; Wei et al., 1995).

An unanswered question has been how nucleotide excision repair can recognize and repair such a wide range of DNA damage. Our results, indicating that the signal for nucleotide excision repair is the special DNA conformation adopted at a specific site as a result of adduct formation, may shed light on that question. The nucleotide excision repair system may detect and repair not just a specific kind of DNA damage, but rather any section of DNA that has adopted a dynamic conformation that differs from that of the standard conformation of DNA.

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REFERENCES

Chen, R.-H., Maher, V.M., Brouwer, J., van de Putte, P. & McCormick, J.J. 1992. Preferential repair and strand specific repaair of benz(a)pyrene diol epoxide adducts in the *HPRT* gene of diploid human fibroblasts. *Proc.Natl. Acad. Sci. USA*, **89**:5413-5417.

Cosman, M., Santos, C.D.L., Fiala, R., Hingerty, B.E., Singh, S.B., Ibanez, V., Margulis, L.A., Live, D., Geacintov, N.E., Broyde, S., & Patel, D.J. 1992. Solution cconformation of the major adduct between the carcinogen (+)-anti-benzo(a)pyrene diol epoxide and DNA. *Proc. Natl. Acad. Sci. USA*, **89**:1914-1918.

Gao, S., Drouin, R., Chen R. & Holmquist, G.P. 1994. DNA repair rates mapped along the human *PGK1* gene at nucleotide resolution. *Science,* **263**:1438-1440.

Hanawalt, P.C. 1994. Transcription-coupled repair and human disease. *Science*, **266**:1957-1958.

Huang, J.-C., Hsu, D.S., Kazantsev, A. & Sancar, A. 1994. Substrate spectrum of human excinucleases: Repair of abasicc sites, methylated bases, mismatches and bulky adducts. *Proc. Natl. Acad. Sci. USA,* **91**:12213-12217.

Heflich, R.H., Hazard, R.M., Lommel, L., Scribner, J.D., Maher, V.M. & McCormick, J.J. *1980.* A comparison off the DNA binding, cytotoxity and repair synthesis induced in human fibroblasts by reactive derivative of aromatic amid carcinogens. *Chem. Biol. Interactions*, **29**:43-47.

Lin, J.-J. & Sancar, A. 1992. (ABC) excinuclease: the Escherichia coli nucleotide excision repair enzyme. *Mol. Microbiol.*, **6**:2219-2224.

McCormick, J.J. & Maher, V.M. 1981. in *DNA Repair: A Laboratory Manual of Researcch Procedures,* eds. Friedberg, E.C. & Hanawalt, P.C. (Dekker, New York), **Vol. 1B**, 501-521.

McGregor, W.G., Mah, C.-M., Chen, R.-H., Maher. V.M. & McCormick, J.J. 1995. Rapid, strand specific repair of 1-nitrosopyrene adducts but not those of N-acetoxy-2-acetylaminofluorene in the hypoxanthine phosphoribosyltransferase (HPRT) gene of diploid human fibroblasts. *Proc. Amer. Assoc. Cancer Res.*, **36**:142.

Nolan, J.J., Vyas, R.R., Hingerty, B.E., Broyde, S. & Basu, A.K. 1994. *Proc. Amer. Assoc. Cancer Res.*, **35**: 650 .

Sancar, A. 1994. Nucleotide excision repair. *Science*, **266**:1954-1956.

Sancar, A. & Tang, M.S. *1993*. Nucleotide excision repair. *Photochem. Photobiol.*, **51**:905-921.

Tornaletti, S. & Pfeifer, G.P. 1994. Slow repair of pyrimidine dimers at *p53* mutatio hot spots in skin cancer. *Science*, **263**:1436-1438.

Tung, B., McGregor, W.G., Wang, Y.-C., Maher, V.M.& McCormick, J.J. 1995 Comparison of rates of excision of major UV photoproducts in the hypoxanthine phosphoribosyltranscferase (HPRT) gene of normal and xeroderma pigmentosum variant (XPV) cells. *Mutat. Res.*, in press.

Van Houten, B., Masker, W.E., Carrier, W.L. & Regan, J.D. 1986. Quantitation of carcinogen-induced DNA damage and repair in human cells with the UVRABC excision nuclease from *Escherichia coli. Carcinogenesis*, **7**:83-87.

Watanabe, M., Maher, V.M. & McCormick, J.J. 1985. Excision repair of UV-or benzo(a)pyrene diol epoxide-induced lesions in xeroderma pigmentosum variant cells is "error free". *Mutat. Res.*, **146**: 285-294.

Wei, D., Maher, V.M. & McCormick, J.J. 1995. Site specific rates of excision repair of benz(a)pyrene diol epoxide adducts in the hypoxanthine phosphoribosyltransferase gene of human fibroblasts: Correlation with mutation spectra. *Proc. Natl. Acad. Sci. USA*, **92**:2204-2208 .

Yang, L.L., Maher, V.M. & McCormick, J.J. 1980. Error free excision of the cytotoxic, mutagenic N^2-deoxyguanosine DNA adducts formed in human fibroblasts by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro-benzo(a)pyrene. *Proc. Natl. Acad. Sci.* USA, **77**:5933-5937.

Yang, L.L., Maher, V.M. & McCormick, J.J. 1982. Relationship between excision repair and the cytotoxic and mutagenic effect of the "anti"7,8-diol-9,10-epoxide of benzo(a)pyrene in human cells. *Mutat. Res.*, **94**: 435-447.

Yang, J.-L., Maher, V.M. & McCormick, J.J. 1988. Kinds aand spectrum of mutations induced by 1-nitrosopyrene adducts during plasmid replication in human cells. *Mol. Cell. Biol.*, **8**:3364-3372.

CHAPTER IV

Comparison of the distribution of benzo(a)pyrene diol epoxide adducts at the nucleotide level in DNA of human cells at various stages of the cell cycle and in DNA treated in vitro

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SUMMARY

(±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE) is the principal reactive metabolite of the carcinogenic environmental pollutant benzo(a)pyrene. To determine the effect of the cell cycle on the distribution of BPDE adducts in normal human fibroblasts, we synchronized the cells by release from density inhibition (G0), and treated them with BPDE in early G1 phase, at the onset of S phase, in late S phase or at G2/M border. Cell synchronized at G1/S border with aphidicolin or at metaphase with colcemid were also treated with BPDE. Analysis of the distribution of BPDE adducts in the nontranscribed strand of exon 3 of the hypoxanthine phosphoribosyltransferase gene, using *E. coli* UvrABC excinuclease and ligation mediated polymerase chain reaction, showed that the relative distribution in the cellular DNA was essentially the same across the cell cycle. Furthermore, for almost all nucleotide positions, the distribution of BPDE adducts was similar to that found when purified DNA was treated with BPDE in vitro. The exception was that adduct formation in vivo at a region of six consecutive guanines, i.e., nucleotide 207-212, was strongly enhanced compared to that seen with DNA treated in vitro. No evidence of nucleosomal structures or other protein-DNA interactions could be found within the region of interest. Therefore, we conclude that the enhancement of BPDE adduct formation at nucleotide 207-212 in vivo reflects the physiological environment in the cell nucleus and that this effect is unchanged throughout the cell cycle.

INTRODUCTION

The ultimate reactive metabolite of benzo(a)pyrene is the (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE) (Marquardt et al., 1976), and the (+)-enantiomer is the form that has been shown in mouse skin carcinogenesis studies to lead to malignant transformation (Slaga et al., 1979). The major DNA adduct formed by BPDE in vivo and in vitro is the addition of BPDE residue to the N^2 position of guanine (Weinstein et al., 1976). In vitro studies on the mechanism of carcinogenic interaction with DNA suggest that BPDE first intercalates noncovalently into the base pairs of DNA, and then the intercalated BPDE either is hydrolyzed to nonreactive products (tetrols) or covalently binds to DNA (MacLeod and Zachary, 1985; MacLeod, 1990). In fact, only a small fraction of intercalated BPDE actually becomes covalently bound.

The distribution of BPDE adducts at the nucleotide level in DNA treated in vitro has been studied with DNA polymerases (Thrall et al., 1992), *E.coli* UvrABC excinuclease (Tang et al., 1992) or laser irradiation (Smith and MacLeod, 1993). These studies show that the BPDE adducts are distributed in a nonrandom and consistent pattern. However, the distribution of BPDE adducts in DNA at the nucleotide level in mammalian cells had not previously been studied. We (Wei et al., 1995) adapted ligation mediated PCR (LMPCR) (Mueller and Wold, 1989; Pfeifer et al., 1989) in combination with the use of UvrABC excinuclease to carry out a study with DNA from diploid human fibroblasts treated with BPDE. That study also showed that BPDE adducts are distributed in a non-random, consistent manner.

Indirect evidence predicts that the complicated environment in intact cells will result in patterns of DNA adduct formation by bulky carcinogens that differ from the pattern seen with DNA in vitro. Examples include the finding that a positional isomer of BPDE, 9r,10t-dihydroxy-7c,8c-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene could not form stable adducts in vivo, but formed adducts with purified DNA almost as efficiently as BPDE (Baird and Diamond, 1977), and that the bulky carcinogen cisplatin forms adducts at an extra site in the DNA of intact cells, a site where no obvious adducts are formed in vitro (Grimaldi et al., 1994). The finding by MacLeod and Selkirk (1982) that Mg^{2+} inhibits the rate of the hydrolysis of BPDE suggests that other molecules in the nucleus may also affect BPDE adduct formation. To see if this were the case, we used LMPCR to investigate the pattern of DNA adduct formation at the nucleotide level in vivo in cells exposed to BPDE at various times during the cell cycle, and compared the results with those we obtained when we treated purified DNA with BPDE in vitro.

We found that the relative patterns of BPDE adduct formation in the region of interest were essentially the same across the cell cycle, and at most sites were very similar to that pattern observed in vitro. However, in vivo there was a much stronger binding of BPDE in a region of six consecutive guanines. DNA footprinting experiments showed that this difference was not the result of nucleosomal structures or other protein-DNA interactions within the region of interest. We conclude that under the physiological conditions in the nucleus of human cells, BPDE binding can be strongly enhanced in certain regions, such as in a run of guanines, and that this effect remains constant throughout the cell cycle.

EXPERIMENTAL PROCEDURES

Cell culture and synchronization. Diploid human male fibroblasts derived from a neonatal foreskin, designated SL68 (McCormick and Maher, 1981), were cultured in Eagle's minimal essential medium containing 10% supplemented bovine calf serum. Cells were synchronized into G0 by density inhibition and mitogen deprivation (Konze-Thomas et al., 1982) and released by being trypsinized and plated at 1.7 X 10^4 cells/cm^2 in fresh medium containing serum. A second cell population similarly released from G0 was incubated in fresh medium containing 0.2 μg/ml aphidicolin (Sigma) for at least 24 hr to block the cells at the onset of S phase, and the medium was replaced with fresh complete medium, lacking aphidicolin, half an hour before the treatment. A third population released from G0 was cultured in fresh medium with serum for about 25 hr, and the medium was then replaced by medium containing 0.4 μg colcemid (Gibco/BRL) for another 20 hr to block the cells at metaphase.

Treatment of cells with BPDE. BPDE, obtained as a dry powder from Chemsyn Science Laboratory, was dissolved in anhydrous DMSO immediately before use. Before treatment, the medium on the cells was removed, the cells were rinsed twice with phosphate-buffered saline (PBS), and serum-free medium was added. An appropriate volume of the BPDE stock solution was added by pipetteman. The cells were incubated at 37°C for 1 hr, and the cells were then rinsed and lysed immediately.

Determination of the distribution of BPDE adducts in vivo at the nucleotide level. DNA was isolated from the cell lysates and purified as described (Wei et al., 1995), and

6 μg of DNA per sample was cut with SpeI and BglI (Boehringer Mannheim). UvrABC excinuclease, kindly provided by Dr. Pieter van de Putte at Leiden University (Leiden, the Netherlands), was used to excise the BPDE adducts as described (Chen et al., 1992). UvrABC cuts in the region of interest were then mapped using ligation mediated PCR as described (Wei et al., 1995). Briefly, the UvrABC-digested DNA was purified, redissolved in H_2O, and denatured. A gene specific primer (primer 2) containing a biotin at the 5' end was allowed to anneal and was extended to the site of cut by Sequenase 2.0 (United States Biochemicals). A linker was ligated, and the desired fragments were isolated using Dynabeads M-280 Streptavidin (Dynal). Unless otherwise noted, the purified fragments were amplified by 22 cycles of PCR, using the longer oligomer of the linker and a second gene specific primer (primer 3), located 3' of primer 2, and were purified with a Centricon-30 microconcentrater (Amicon). Six microliters of the final PCR product was labeled using a ^{32}P-labeled gene specific sequencing primer and separated on a sequencing gel. The intensity of the bands was analyzed using a PhosphorImager (Molecular Dynamics).

Determining the amount of ^{3}H-BPDE adduct formation on a DNA fragment containing exon 3. A genomic DNA fragment containing the region of interest was amplified with an intron primer and gene specific primer 2 as described (Wei et al., 1995). The DNA was purified, digested with DdeI to generate a fragment of 354 bp, and was treated with ^{3}H-BPDE (1744.2 mCi/mmol, Chemsyn Science Laboratory) at a concentration of 13.7 μM. The DNA was then purified as described (Yang et al., 1987), and the concentration was measured on a spectrophotometer. An appropriate amount of DNA was counted on

a scintillation counter to determine the radioactivity remaining bound to the DNA. The frequency of BPDE adducts formed on each DNA fragment was calculated as described (Yang et al., 1987). The frequency on either strand of the DNA fragment was estimated to be 50%.

Analyzing the distribution of BPDE adducts on this DNA fragment with Sequenase 2.0. Gene specific primer 3 was labeled with γ-^{32}P-ATP (6000Ci/mmol, New England Nuclear) and T4 polynucleotide kinase (United States Biochemicals) following the manufacturer's suggestions. The DNA fragment with a known amount of BPDE adducts was denatured and annealed with the labeled primer and extended with Sequenase as described (Wei et al., 1995). The sample was then run on a sequencing gel and analyzed with a PhosphorImager.

Analyzing the efficiency of UvrABC excision on this DNA fragment. The DNA fragment with ^{3}H-BPDE adducts was digested with UvrABC and purified using the conditions described by Wei et al (1995). The DNA was separated on a sequencing gel and electroblotted onto a nylon membrane with a homemade electroblotter following the instructions of Pfeifer and Riggs (1993). After completion of transfer, the DNA was crosslinked to the membrane with a UV crosslinker (Stratagene). Gene specific primer 3 labeled as described above was then used to probe the membrane, using the same conditions described by Pfeifer and Riggs (1993), except that the hybridization temperature was decreased to 40°C. After overnight hybridization, the membrane was washed at room temperature as described (Pfeifer and Riggs, 1993), exposed to a PhosphorImager screen, and analyzed.

In vivo DNA footprinting. For dimethyl sulfate (DMS) footprinting, the cells were rinsed twice with PBS, and the serum free medium containing 0.1% DMS (Sigma) was added, and the cells were incubated for 10 min at room temperature. Cells were washed again and lysed immediately. DNA was extracted and purified as described (Wei et al., 1995). The DNA sample was treated with 1M piperidine (Sigma) at 90°C for 30 min and purified and analyzed using LMPCR as described (Wei et al., 1995). As a control, purified untreated genomic DNA from SL68 cells was subjected to the guanine specific cleavage reactions in the standard Maxam-Gilbert protocol and analyzed using LMPCR as described (Wei et al., 1995). For the purpose of comparison, all samples were separated on a sequencing gel in parallel .

For micrococcal nuclease (MNase) footprinting, we adapted the protocol of Pfeifer and Riggs (1991). Briefly, cells were trypsinized, washed with PBS, and resuspended in solution I (150 mM sucrose, 80 mM KCl, 35 mM Hepes at pH 7.4, 5 mM K_2HPO_4, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$) with 0.05% lysolecithin (Sigma) for 1 min at 37°C. Cells were washed once with solution I lacking lysolecithin, and treated for 5 min with 20 units/ml of MNase (Promega) in 150 mM sucrose, 50 mM Tris·HCl (pH 7.5), 50 mM NaCl, and 2 mM $CaCl_2$. The treatment solution containing MNase was removed, the cells were lysed, and DNA was extracted and purified as described (Wei et al., 1995). As a control, purified untreated genomic DNA from the same cell line was similarly treated with micrococcal nuclease and purified. Both the control and the sample DNA were digested with BglI and SpeI and purified. Since DNA fragments generated by MNase contain 5'-OH group, which prevents ligation of the linker, the DNA was phosphorylated

with T4 polynucleotide kinase in the presence of 0.2 mM ATP according to the manufacturer's instructions. DNA was purified again and analyzed with LMPCR as described (Wei et al., 1995).

RESULTS

The distribution of BPDE adducts in exon 3 of the HPRT gene across the cell cycle. As part of a study on the effect of DNA repair on the spectrum of mutations induced by BPDE, we showed that the initial distribution of BPDE adducts on the nontranscribed strand of exon 3 of the *HPRT* gene in normal human fibroblasts was essentially the same for cells treated in early G1 phase or in early S phase (Wei et al., 1995). In the present study, using a series of methods to achieve cell synchrony, we have investigated the distribution of BPDE adducts in the same region of DNA in such cells treated at various phases of the cell cycle. In the first method, cells were released from G0 by being plated at a lower density in fresh complete medium containing serum, and treated with BPDE 5, 17, 25, 30 hr after release. Under the culture conditions used, at these time points, the majority of the cells were in early G1 phase, early S phase, late S phase, and at the G2/M border, respectively (data not shown). In the second method, cells released from G0 were further synchronized at the G1/S border (i.e., at the very beginning of S phase) with aphidicolin, and treated with BPDE 30 min after release from this block. Aphidicolin stops cells at the beginning of S phase by inhibiting DNA polymerases (Pegrali-Noy et al., 1980), and studies by Grossmann et al. (1985) in this laboratory indicate that replication of the *HPRT* gene occurs during the first 1 hr or 1.5 hr after release from aphidicolin. In the third method, cells were synchronized with colcemid, which stops cells at metaphase by disrupting the microtubules and thus inhibiting the formation of the spindle (Sele et al., 1977). Staining the cells with Hoechst reagents showed that under

the conditions we used, about 70% of the cells were at the metaphase at the time they were treated with BPDE. (data not shown.)

A BPDE dose of 13 μM was chosen because at this dose, no DNA replication or DNA repair occurs (data not shown). If either such process were to occur during the 1 hr BPDE treatment, it would interfere with the analysis. A 13 μM dose yields approximately 1 adduct per 2000 nucleotides (Chen et al., 1992), and in our analysis we examine the BPDE adduct formation at the nucleotide level within a 200 bp fragment. Therefore, the average frequency is 0.1 per fragment.

After BPDE treatment, DNA was extracted and digested with *E.coli* UvrABC excinuclease to excise the BPDE adducts, and a gene specific primer was annealed and extended with Sequenase 2.0 to generate a blunt end at the site of each cut. A linker was ligated to the blunt end, and the desired fragments were amplified by PCR and labeled with a third primer. The labeled product was separated on a sequencing gel. The distribution of fragments of particular lengths indicated the relative number of cuts generated at these sites by UvrABC and, therefore, the relative level of BPDE adducts formation at these sites.

The results are shown in Figure 1, along with the sequencing ladders. Virtually every BPDE adduct involved guanine. The most striking observation in Figure 1 is that the intensities of the corresponding bands of all six samples are similar, indicating that the relative distribution of BPDE adducts for cells at various stages of the cell cycle was essentially the same. This strongly suggests that the permeability of BPDE was the same for cells at the various stages of the cell cycle, that BPDE was able to diffuse freely in

Fig. 1. Autoradiogram showing the distribution of BPDE adducts in exon 3 of the *HPRT* gene when cells were treated with BPDE at various stages of the cell cycle or when purified DNA was treated in vitro. Cells were treated for 1 hr with 13 μM BPDE, a dose yielding an adduct level of approximately 0.1 in the 184 nucleotides of the nontranscribed strand of exon 3 of the *HPRT* gene. Treatment was at 5 hr (lane 5), 17 hr (lane 17), 25 hr (lane 25) or 30 hr (lane 30) after release from G0; or 30 min after release from aphidicolin synchronization; or after colcemid treatment. DNA was isolated and subject to UvrABC digestion and LMPCR to determine the distribution of BPDE adducts. A purified DNA fragment containing exon 3 (lane Fra) or purified genomic DNA (lane Gen) treated with BPDE in vitro was also analyzed with UvrABC and LMPCR to determine the distribution of BPDE adducts. The final LMPCR products were labeled and separated on a sequencing gel. The region of six consecutive guanines, i.e., nucleotide 207-212, is shown. Lane seq-G contains the products of the G reaction of the Maxam-Gilbert sequencing method performed on a DNA fragment containing exon 3 followed by LMPCR as described (Wei et al., 1995). The bands in Lane Seq-G are 4 nucleotides longer than the corresponding UvrABC generated products (Wei et al., 1995).

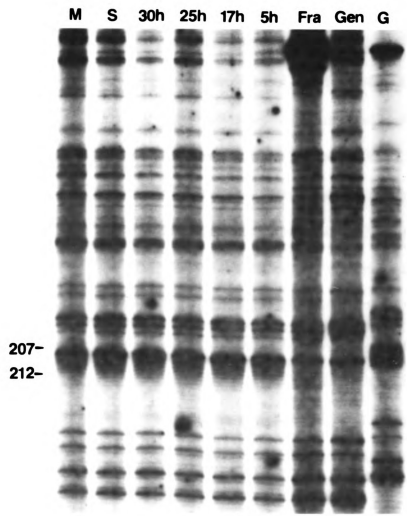

Fig. 1

cells at every stage of the cell cycle, and that even though the nuclear location of the *HPRT* gene can be expected to change from G1 phase to metaphase, BPDE had the same binding specificity to a particular guanine nucleotide.

In the samples from cells synchronized with aphidicolin or colcemid, the intensities of all the bands were slightly higher than those in the other lanes. It is possible that cells treated with these chemicals allowed BPDE to diffuse more easily, resulting in a slightly higher local concentration of BPDE in the nucleus and therefore a higher frequency of BPDE adduct formation, or that these chemicals increased the chance of BPDE intercalation or conversion of intercalated BPDE to the covalently bound form.

Comparison of the BPDE adduct formation in vivo with that in vitro in the region of interest. The fact that the pattern of BPDE adduct formation in cells was essentially the same across the cell cycle suggested that the binding pattern was determined mainly by local DNA sequence. If so, the pattern of BPDE adduct formation in DNA treated in vivo should be the same as that seen when the same DNA is purified and treated with BPDE in vitro. To test this, we determined the pattern of BPDE adduct formation in vitro on a purified DNA fragment containing exon 3 of the *HPRT* gene. A genomic DNA fragment was amplified with PCR as described in Method and Materials and digested with restriction enzyme DdeI to generate a fragment of 354 bp. The DNA fragment was purified and treated with BPDE and analyzed using UvrABC and LMPCR. As shown in the lane of Figure 1 labeled Fra, the relative adduct formation at almost every site was similar to what had been found when intact cells were treated with BPDE. However, the relative level of BPDE adduct formation in the run of six guanines, nucleotide 207-212,

was significantly lower than seen in DNA from cells, indicating that the potential for BPDE covalent binding at this region was enhanced in vivo.

To test the possibility that this lower binding in the region 207-212 was an artifact caused by using the amplified DNA fragment, we performed the same analysis on genomic DNA treated with BPDE in vitro. Genomic DNA was purified and digested with restriction enzymes SpeI and BglI to decrease the viscosity. The DNA was then treated with 13 μM BPDE and analyzed using LMPCR. The result, shown in the lane of Figure 1 labeled Gen, indicated that the distribution of BPDE adducts in the purified genomic DNA treated in vitro was the same as that found in the PCR-amplified DNA fragment. A similar experiment was also done with purified, non-restricted, intact genomic DNA (Mw>100 kDa), and the same result was obtained (data not shown). These results indicate that the enhancement of the relative level of BPDE adducts at nucleotides 207-212 observed when cellular DNA was treated in vivo did not reflect a difference in the length of DNA treated, but rather reflected the difference in conditions in which the DNA was treated: i.e., the fact that the DNA was in a special microenvironment of the nucleus in vivo, rather than dissolved in TE buffer in vitro.

Determining whether nucleosomes are present in the region of interest. One possible explanation for BPDE adduct formation at nucleotide 207-212 being enhanced in vivo is that the region of six guanines is located at the terminal of a nucleosome formed at exon 3 of the *HPRT* gene. Smith and MacLeod (1993), using a reconstructed nucleosome, showed that covalent binding of BPDE at the terminal 20-30 bp of nucleosomal DNA is enhanced 2.5 fold relative to the central portion. If the region of six guanines in exon3

of the *HPRT* gene were located at the terminal of a nucleosome, BPDE adduct formation there might well be enhanced relative to that at the rest of exon 3. To test this, we carried out in vivo DNA footprinting to determine if there were any nucleosomal structure or other protein-DNA interactions in the region of interest.

Dimethyl sulfate (DMS) is the reagent most commonly used for in vivo footprinting, and its small size allows it to diffuse easily through cell membranes to react with DNA in the nucleus, so no manipulation of the cells is required prior to treatment. Therefore, we carried out in vivo footprinting by treating normal fibroblasts in G1 phase with DMS, extracting the DNA, cutting it with piperidine as described, and analyzing the products using LMPCR. The results are shown in Fig. 2a. Comparison of the amplified pattern with that of the highly purified genomic DNA shows that they are essentially the same, indicating that there was no obvious nucleosomal structures or other protein-DNA interaction at the region of interest. We found a similar pattern in cells treated with DMS at metaphase (Fig 2a), which is consistent with other studies showing that DMS is small enough to diffuse freely through the condensed chromosome structure (Pfeifer and Riggs, 1991).

DMS is only effective in detecting protein-DNA contacts in DNA sequences containing Gs in the major groove of the DNA double-helix, and Pfeifer et al. (1992) showed that use of this chemical to detect nucleosomal structure is not optimal, probably because it can penetrate protein-DNA complexes. To confirm the DMS footprinting data, we also used micrococcal nuclease to detect the possible presence of nucleosomes in the region of interest.

Fig. 2. Autoradiograms showing the in vivo DNA footprinting with dimethyl sulfate (A) or micrococcal nuclease (B). In A, purified genomic DNA treated with dimethyl sulfate (DMS) in vitro (lane Con) or DNA from cells treated with DMS at G1 phase (lane G1) or metaphase (lane M) was analyzed with piperidine and LMPCR. In B, purified genomic DNA digested with micrococcal nuclease (Con) or DNA from cells permeabilized with lysolecithin and treated with micrococcal nuclease at G1 phase (lane G1) was phosphorylated with T4 polynucleotide kinase and analyzed with LMPCR as described in Materials and Methods, except that DNA was amplified by 27 cycles of PCR instead of 22 cycles. Lane G shows the G sequence ladder as in Lane Seq-G in Fig. 1. The region of six consecutive guanines, i.e., nucleotide 207-212, is shown. The patterns of the in vivo DNA footprints are similar to those obtained with purified genomic DNA. Although some extra modifications or incisions were seen in the DNA footprinting patterns when cells were treated compared to those found when purified genomic DNA was treated, they do not represent a pattern typical for a nucleosome. Rather, they probably result from differences in the environment of the DNA in cells and in TE buffer.

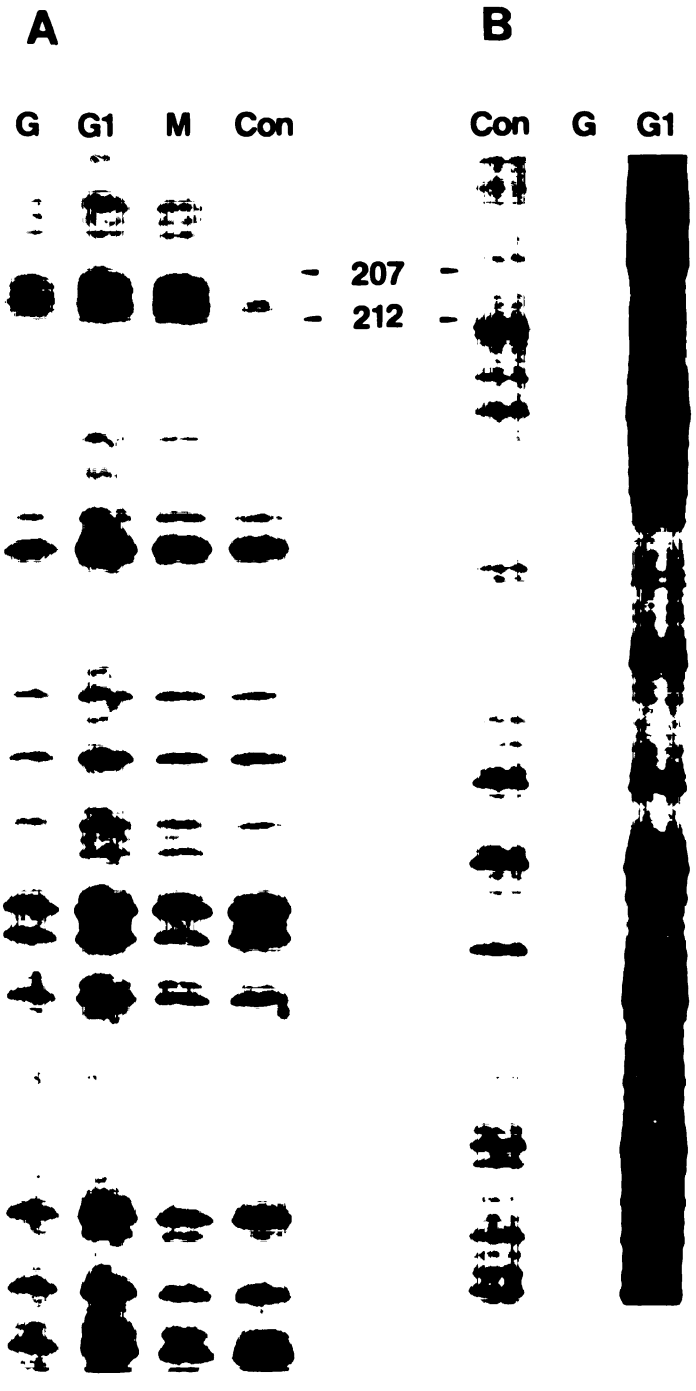

Fig.2

Micrococcal nuclease is widely used to study the nucleosomal structure because of its preference for incision of nucleosome-free DNA (Zhang et al., 1983). This protein cannot readily penetrate cell membranes to incise DNA in the nucleus. Pfeifer and Riggs (1991) showed that better in vivo footprinting can be achieved using cells permeabilized by lysolecithin than using isolated nuclei. Therefore, we first permeabilized populations of normal fibroblasts, synchronized in G1 phase, using lysolecithin and treated them with micrococcal nuclease. The results, shown in Figure 2b, indicated that the incision pattern of micrococcal nuclease on genomic DNA in vivo was very similar to that on highly purified genomic DNA in vitro. Since micrococcal nuclease is expected to incise preferentially at the linker region in the presence of a nucleosome and generate an incision pattern different from that on pure genomic DNA, we conclude that there is no nucleosome formation at exon 3 of the *HPRT* gene. We also obtained similar results in DNA footprinting using DNase I and hydroxyl free radicals (data not shown). The lack of a nucleosome structures or other protein-DNA interaction in the region of interest suggests that the strong enhancement of BPDE adduct formation at nucleotide 207-212 in vivo compared to that in vitro is caused by the special physiological environment that exists in the cell nucleus, and is not present in the TE buffer pH 8.0. The data further indicate that this effect is very consistent throughout the cell cycle.

Evidence of a special secondary DNA structure. Several studies on the distribution of BPDE adducts in purified DNA treated in vitro using interference with polymerization to detect the presence of adducts (Dittrich and Krugh, 1991; Thrall et al., 1992) or using photodestruction by laser radiation (Boles and Holgon, 1986) have shown that long runs

of guanine are hot spots for BPDE adduct formation. This suggests that such runs form a special localized DNA secondary structure that is especially favorable to BPDE binding. As shown in Figure 1, our results using LMPCR to detect the distribution of BPDE adducts in the run of six guanine in exon 3 of the *HPRT* gene of purified DNA treated in vitro do not show such preferential binding.

To further investigate the distribution of BPDE adducts in this region, we made use of two other approaches. A fragment of DNA containing exon 3 was amplified and cut with DdeI to generate a fragment of 354 bp, so that BPDE adduct formation at every nucleotide position could be analyzed later on a sequencing gel. The purified DNA fragment was then treated with generally tritiated BPDE. After extensive phenol/chloroform extraction and purification, the DNA concentration and the radioactivity remaining on the DNA was measured. Calculation indicated that the level of binding was approximately 0.56 BPDE adducts per DNA fragment (data not shown). Since the two strands of this DNA fragment contain approximately equal numbers of guanines, we estimated the level of binding to be 0.28 BPDE adducts per strand of the fragment.

To test whether primer extension by Sequenase can be used to quantitatively determine BPDE adduct formation at the nucleotide level in exon 3, as was shown for a poly(G) region by Thrall et al. (1992), we denatured the DNA fragment containing this known amount of ^{3}H-BPDE adduct. A ^{32}P labeled gene-specific primer was annealed and extended with Sequenase 2.0. The product was separated on a sequencing gel, in parallel with a sequencing control. The results (Figure 3a) showed that Sequenase 2.0 stopped one

nucleotide prior to each guanine exclusively. Quantitation of the gel using a PhosphorImager indicated that the frequency of full length DNA fragments generated by Sequenase from the BPDE treated DNA was approximately 74%, while with the untreated DNA, essentially all the ^{32}P-labeled fragments were of full length (data not shown). Thus, our data indicate that Sequenase was stopped quantitatively by BPDE adducts. It is obvious from Fig. 3a that when termination of Sequenase extension was used as the measure of BPDE adduct levels, the region of six consecutive guanines (nucleotide 207-212) was a hot spot for BPDE modification, and furthermore, there was selective modification of guanines at the 5' end of this polyd(G) region, which is consistent with the previous finding of Thrall et al. (1992).

UvrABC has been shown to excise BPDE adducts with close to 100% efficiency (Tang et al., 1992; van Houten et al., 1986; Seeberg et al., 1983), but the efficiency of UvrABC excision has also been shown to vary according to the local DNA secondary structure (Seeberg and Fuchs, 1990). If this region of six consecutive guanines adopts a special secondary structure, such a secondary structure might have an impact on the efficiency of UvrABC excision. To test this, we used UvrABC to digest another aliquot of the same DNA fragment treated with tritiated BPDE that was used in the Sequenase extension study, and we separated the products on a sequencing gel. The DNA was then electroblotted onto a nylon membrane and probed with a ^{32}P-labeled oligomer complementary to the 3' end of the desired strand, so that the efficiency of UvrABC incision on the 3' side of the BPDE adducts on this strand could be determined. The result is shown in Figure 3b. Image analysis showed that the frequency of full length

fragments was approximately 75%. When the artifactual decrease of the efficiency of UvrABC cutting at either end of the DNA fragment is taken into consideration, these results indicate that UvrABC excised the BPDE adducts with close to 100% efficiency, consistent with previous observations. As shown in Fig. 3b, more frequent cuts are observed in the region of six Gs than at many other sites. However, the level of BPDE adduct formation in that region as detected by UvrABC cutting was only about 50-60% of the level detected using Sequenase (data not shown).

To confirm this observation, the ^{32}P-labeled primer 3 was annealed to this same DNA fragment treated with BPDE that had or had not been digested by UvrABC, and extended with Sequenase. In the DNA that had not digested by UvrABC, Sequenase stopped one nucleotide before each guanine. At virtually every guanine site in exon 3 in the DNA that was digested with UvrABC, extension by Sequenase was terminated four nucleotides 3' of the DNA adduct, as a result of UvrABC excision (data not shown). However, at nucleotide position 207-212, only approximately half of the termination shifted. This further confirmed that UvrABC excision was impeded by the local secondary structure in this region. The fact that this incomplete excision by UvrABC was not observed in any other guanine position in exon 3 (data not shown), supports our conclusion that a special secondary structure is adopted by the region of six consecutive guanines.

Fig. 3. Autoradiograms showing the distribution of BPDE adducts in a DNA fragment treated with ^{3}H-BPDE determined using Sequenase 2.0 (A) or UvrABC (B). A DNA fragment containing exon 3 of the *HPRT* gene was treated with ^{3}H-BPDE. One aliquot of this DNA was annealed with a ^{32}P-labeled gene-specific primer 3 and extended with Sequenase 2.0 (lane BP). Two aliquots of this DNA were digested with UvrABC, separated on a sequencing gel, electroblotted onto a nylon membrane and hybridized with the ^{32}P-labeled primer 3 (lane R1 and lane R2). Lanes C, G, A contain the products of C, G, A sequencing reactions when the gene-specific primer 3 was used to sequence the DNA fragment according to the standard Sanger method. Therefore, lane C indicated the position of every guanine in the complementary strand (nontranscribed strand).

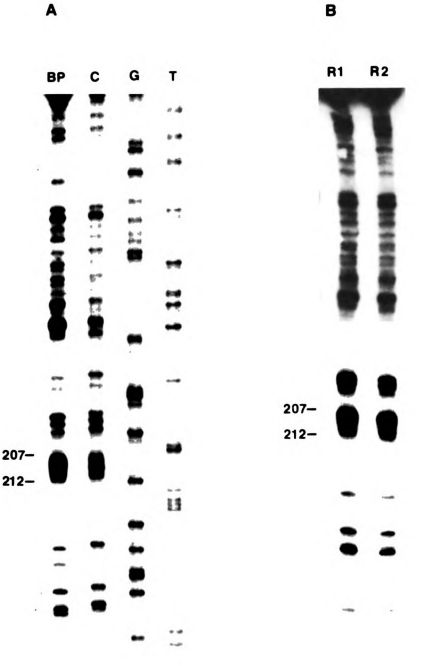

Fig. 3

DISCUSSION

Our present investigation of the distribution of BPDE adducts on the nontranscribed strand of exon 3 of the *HPRT* gene in normal human fibroblasts at the level of single nucleotide resolution showed that the distribution pattern was essentially unchanged across the cell cycle. This suggests that the action of BPDE (intercalation into DNA, conversion to the covalent bound form) is unaffected by the changing cytoskeletal structure and physiological conditions throughout the cell cycle, and that BPDE molecules are small enough to diffuse freely through the highly condensed chromosomal structure, as is the case for dimethyl sulfate. The relative distribution of BPDE adducts in vivo at most sites in the region of interest in the *HPRT* gene from cells was very similar to that found at those sites for purified DNA treated in vitro, except the region of six consecutive guanines. This latter region, i.e., nucleotide 207-212, was a hot spot for BPDE adduct formation for DNA in vitro, but an even "hotter" spot for BPDE adduct formation in vivo. Because no nucleosomal structures or other protein-DNA interactions were found at exon 3 of the *HPRT* gene, we conclude that this difference in binding was due to the special physiological conditions in the cell nucleus.

Cellular DNA is believed to exist in the form of nucleosomes. DNA is wrapped around core histone octamers to form nucleosomes, and then coiled into a chromatin fiber with a width of 30 nm. The existence of a histone octamer at a particular locus would inhibit the action of the DNA endonucleases (Pfeifer and Riggs, 1991, 1993; Smith and MacLeod, 1993), and cause a periodic cutting pattern by hydroxyl free radical (Powers

and Bina, 1991). However, such a pattern was not detected in exon 3 of the *HPRT* gene in our study, strongly suggesting that no nucleosome structure is present in this region. Our data do not exclude the possibility that the nucleosome structure is not single-phased (Zhang et al., 1983; Zhang and Horz, 1984), and therefore no clear nucleosome structure can be detected. But multiphased nucleosomes cannot account for the observed difference in the pattern of BPDE adduct formation at the region of six guanines in vivo compared to that seen in vitro, since this region should not always be located at the terminal end of a nucleosome.

Our finding of a similar pattern of binding among cells at metaphase and the other phases of the cell cycle indicates that the highly folded chromosomal structure has little effect on the accessibility of the genomic DNA to BPDE. Pfeifer and Riggs have shown that the chromosomal structure has no effect on the modification of DNA by DMS (1991), suggesting that the free space between the condensed chromosome structure is big enough to allow small molecules such as DMS to diffuse without difficulty. The molecular weight of BPDE is 301 Da, only 2.4 times that of DMS. Therefore, it is not surprising that BPDE is also small enough to diffuse freely through the chromosomal coils.

We showed that the DNA was selectively adducted by BPDE, i.e., that adducts formed at some sites significantly more frequently than at other sites, and that the pattern of relative distribution of BPDE adducts was very similar for DNA exposed to BPDE in vivo or in vitro. This finding strongly suggests that the potential to form covalent BPDE adducts is, for the most part, determined by the local DNA sequence and remains the

same under different physiological conditions. No predictable pattern of modification could be found at either single guanine sites or guanine dinucleotide sites. However, we did find that the region of six consecutive guanines was a hot spot for BPDE adduct formation with preference for binding to guanines at the 5' end of the run. This result is consistent with what has been observed by other groups (Thrall et al., 1992; Boles and Hogan, 1986; Dittrich and Krugh, 1991), suggesting that a long run of Gs adopts a special conformation favoring the BPDE adduct formation in this specific manner. It has been shown that poly d(G)·poly d(C) DNA can adopt a special DNA conformation similar to that of the conventional A-DNA (Arnott and Selsing, 1974; Wang et al., 1982). The present study showed that, except at the region of six consecutive guanines, UvrABC can excise BPDE adducts in exon 3 of the HPRT gene with close to 100% efficiency. This further supports our hypothesis that the DNA conformation at this region is unique.

A particularly intriguing result is our finding that the potential for BPDE adduct formation at this "hot spot" was strongly enhanced in vivo compared to that in vitro, and that this enhancement was constant across the cell cycle. No nucleosome structures or other protein-DNA interactions were found to account for the observed enhancement. Since we did a direct comparison between cellular DNA treated in vivo, which exists in a physiological microenvironment of the nucleus, and purified genomic DNA treated in vitro, which was dissolved in TE buffer (pH8.0), we attribute the enhancement observed in vivo to the special conditions in the nucleus.

A variety of ions, organic molecules and macromolecules exist in the nucleus, making it a totally different environment than that provided by TE buffer. The presence of Mg^{2+}

and Ca^{2+} has been shown to cause a low-temperature structural transition in the loop of a DNA analog of the yeast tRNA anticodon (Guenther et al., 1992) and to make DNA with a poly d(G)· poly d(C) tract adopt a non-B DNA structure (Kohwi, 1989). Na^{+} and K^{+} can induce structural transitions in oligonucleotides containing four repeats of a telomeric d(G) rich sequence to a folded conformation with G-G base pairs (Raghurama and Cech, 1990). Therefore, these factors might cause conformational transitions in DNA located in the nucleus of the cell. Although our data indicate that this nuclear environment had no effect on the relative distribution of BPDE adducts at the majority sites in the nontranscribed strand of exon 3 of the human *HPRT* gene, it might be expected to affect the noncovalent intercalation and subsequent covalent binding of BPDE at certain sites, such as a region of poly d(G)s. It is very likely that the same factors that account for the hot spot in vitro are strongly enhanced in vivo and responsible for the even more pronounced BPDE adduct formation at this site in vivo. We suggest that the local DNA conformation changes to favor more BPDE intercalation and more BPDE covalent binding at this site. Evidence supporting this hypothesis is the fact that the selective modification by BPDE of guanines at the 5' end of this polyd(G) region in vitro, i.e., BPDE adduct level being highest at nucleotide 207 (5' end) and gradually decreasing to the lowest at nucleotide 212 (3' end), was observed to occur in almost the same manner in vivo. The consistent binding pattern in this region across the cell cycle indicates that this contributing effect remained constant throughout every phase.

Our LMPCR data compare the relative distribution of BPDE adducts in vivo and in vitro, but cannot be used to compare the absolute frequency of BPDE adducts formed in

DNA. Therefore it is always possible that the observed enhancement at the region of six guanines in vivo reflects a general inhibition of BPDE adduct formation at the other guanines of exon 3 in vivo. However, this is unlikely since no nucleosomal structures or other protein-DNA interactions were found to produce such inhibition. It is also highly unlikely that a particular factor can inhibit BPDE adduct formation at virtually every site of the genomic DNA while still keeping the relative distribution pattern untouched. Therefore, we favor the hypothesis that the region of six consecutive guanines is particularly favorable to adduct formation by BPDE.

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REFERENCES

Arnott, S. & Selsing, E. 1974. The structure of polydeoxyguanylic acid with polydeoxycytidylic acid. *J. Mol. Biol.* **88**, 551-552.

Baird, W.M.& Diamond, L. 1977. The nature of benzo(a)pyrene-DNA adducts formed in hamster embryo cells depends on the length of time of exposure to benzo(a)pyrene. *Biochem. Biophys. Res. Commun.* **77**, 162-167.

Boles, T.C.& Hogan, M.E. 1986. High-resolution mapping of carcinogen binding sites on DNA. *Biochemistry* **25**, 3039-3043.

Chen, R.-H., Maher, V.M.& McCormick, J.J. 1990. Effect of excision repair by diploid human fibroblasts on the kinds and locations of mutations induced by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene in the coding region of the HPRT gene. *Proc. Natl. Acad. Sci. USA* **87**, 8680-8684.

Chen, R.-H., Maher, V.M.& McCormick, J.J. 1991. Lack of a cell cycle-dependent strand bias for mutations induced in the *HPRT* gene by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene in excision repair deficient human cells. *Cancer Res.* **51**, 2587-2592.

Chen, R.-H., Maher, V.M.& McCormick, J.J. 1992. Preferential repair andd strand specific repair of benzo(a)pyrene diol epoxide adducts in the *HPRT* gene of diploid human fibroblasts. *Proc. Natl. Acad. Sci. USA* **89**, 5413-5417.

Dittrich, K.A.& Krugh, T.R. 1991. Mapping of (±)-anti-benzo(a)pyrene diol epoxide adducts to human-*Ha-rasI* protooncogene. *Chem. Res. Toxic.* **4**, 227-231.

Grimaldi, K.A., McAdam, S.R.& Hartley, J.A. 1994. DNA damage by anti-cancer agents resolved at the nucleotide level of a single copy gene: evidence for novel binding site for cisplatin in cells. *Nucleic Acids Res.* **22**, 2311-2317.

Grosovsky, A.J.& Little, J.B. 1983. Mutagenesis and lethality following S-phase irradiation of xeroderma pigmentosum and normal human diploid fibroblasts with ultraviolet light. *Carcinogenesis* **4**, 1389-1393.

Grossmann, A., Maher, V.M.& McCormick, J.J. 1985. The frequency of mutants in human fibroblasts UV-irradiated at various times during S-phase suggests that genes for thioguanine- and diphtheria toxin- resistance are repaired early. *Mutat. Res.* **152**, 67-76.

Guenther, R.H., Hardin, C.C., Sierzputowska-Gracz, H., Dao, V. & Agris, P.F. 1992. A magnesium-induced conformational transition in the loop of a DNA analog of the yeast tRNA (Phe) antocodon is dependent on RNA-like modifications of the bases of the stem.

Biochemistry **31**, 11004-11011.

Kohwi, Y. 1989. Non-B DNA structure: preferential target for the chemical carcinogen glycidaldehyde. *Carcinogenesis* **10**, 2035-2042.

Konze-Thomas, B., Hazard, R.M., Maher, V.M. & McCormick, J.J. 1982. Extent of excision repair before DNA synthesis determines the mutagenic but not the lethal effect of UV-irradiation. *Mutat. Res.* **94**, 421-434.

Lee, M.S.& Garrand, W.T. 1991. Transcription-induced nucleosome "splitting": an underlying structure for DNaseI sensitive chromatin. *Embo J.***10**, 607-615.

MacLeod, M.C. 1990. The importance of intercalation in the covalent binding of benzo(a)pyrene diol epoxide to DNA. *J. Theor. Biol.* **142**, 113-122.

MacLeod, M.C. & Selkirk, J.K. 1982. Physical interactions of isomeric benzo(a)pyrene diol-epoxides with DNA. *Carcinogenesis* **3**, 287-292.

MacLeod, M.C.& Zachary, Z. 1985. Catalysis of carcinogen-detoxification by DNA: Comparison of enantiomeric diol epoxide. *Chem.-Biol. Interact.* **54**, 45-55.

Marquardt, H., Grover, P.L. & Sims, P. 1976. In vitro malignant transformation of mouse fibroblasts by non-K-region dihydrodiols derived from 7-methylbenz(a)anthracene, 7,12-dimethylbenz(a)anthracene, and benzo(a)pyrene. *Cancer Res.* **36**, 2059-2064.

McCormick, J.J. & Maher, V.M. 1981. in *DNA repair: A Laboratory Manual of Research Procedures.* eds. Friedberg, E.C.& Hanawalt, P.C. (Dekker, New York), **Vol.1B**, 501-521.

Mueller, P. & Wold, B. 1989. In vivo footprinting of a mouse specific enhancer by ligation mediated PCR. *Science* **246**, 780-786.

Pedrali-Noy, G., Spadari, S., Faures, A., Miller, A.O.A, Kruppa, J.& Koch, G. 1980. Synchronization of Hela cell cultures by inhibition of DNA polymerase alpha with aphidocolin. *Nucleic Acids Res.* **8**, 377-387.

Pfeifer, G.P.& Riggs, A.D. 1991. Chromatin differences between active and inactive X chromosome revealed by genomic footprinting of permeabilizzed cells using DNaseI and ligation mediated PCR. *Genes & Dev.* **5**, 1102-1113.

Pfeifer, G.P.& Riggs, A.D. 1993. in *Methods in Molecular Biology,* **vol.15***: PCR protocols: Current Methods and Applications*, 153-168.

Pfeifer, G.P., Steigerwald, S.D., Mueller, P.L., Wold, B. & Riggs, A. 1989. Genomic

sequencing and methylation analysis by ligation mediated PCR. *Science* **246**, 810-813.

Pfeifer, G.P., Drouin, R., Riggs, A.D.& Holmquist, G.P. 1992. Binding of transcription factors creats hot spots for UV photoproducts in vivo. *Mol. Cell. Biol.* **12**, 1798-1804.

Powers, J.H.& Bina, M. 1991. In vitro assembly of a positioned nucleosome near the hypersensitive region in simian virus 40 chromatin. *J. Mol. Biol.* **221**, 795-803.

Raghuraman, N.K. & Cech, T.R. 1990. Effect of monovalent cation-induced telomeric DNA structure on the binding of oxytrichia telomeric protein. *Nucleic Acids Res.* **18**, 4543-4552.

Seeberg, E., Steinium, A.L., Nordenskjoid, N., Soderhaus, S.& Jerstrom, B. 1983. Strand-break formation in DNA modified by benzo(a)pyrene diol epoxide. Quantitative cleavage by Escherichia coli UvrABC endonuclease. *Mutat. Res.* **112**, 139-145.

Sele, B., Jalbert, P., van Cutsem, B., Lucas, M., Mouriquard, C.& Bouchez, R. 1977. Distribution of human chromosomes on the metaphase plate using banding techniques. *Hum. Genet.* **39**, 39-61.

Slaga, T.J., Brachen, W.J., Gleason, G., Levin, W., Yagi, H., Jerina, D.M.& Conney, A.H. 1979. Marked differences in the skin tumor-initiating activities of the optical enantiomers of the diastereomeric benzo(a)pyrene-7,8-diol-9,10-epoxides. *Cancer Res.* **39**, 67-71.

Smith, B.L.& MacLeod, M.C. 1993. Covalent binding of the carcinogen benzo(a)pyrene diol epoxide to *Xenopus laevis* 5S DNA reconstituted into nucleosomes. *J. Biol. Chem.* **268**, 20620-20629.

Tang, M., Pierce, J.R., Doisy, R.P., Nazimiec, M.E.& MacLeod, M.C. 1992. Difference and similarities in the repair of two benzo(a)pyrene diol epoxide isomers induced DNA adducts by uvrA, uvrB and uvrC gene products. *Biochemistry* **31**, 8429-8436.

Thrall, B.D., Mann, D.B., Smerdon, M.J.& Springer, D.L. 1992. DNA polymerase, RNA polymerase and exonuclease activation on a DNA sequence modified by benzo(a)pyrene diol epoxide. *Carcinogenesis* **13**, 1529-1534.

van Houten, B., Masker, W.E., Carrier, W.L.& Regan, J.D. 1986. Quantitation of carcinogen-induced DNA damage and repair in human cells with the UVRABC excision nucclease from *Escherichia coli*. *Carcinogenesis* **7**, 83-87.

Wang, A.H., Fujii, S., van Boom, J.H. & Rich, A. 1982. Molecular structure of the octamer d(GGCCGGCC): modified A-DNA. *Proc. Natl. Acad. Sci. USA* **79**, 3968-3972.

Watanabe, M., Maher, V.M.& McCormick, J.J. 1985. Excision repair of UV- or

benzo(a)pyrene diol epoxide- induced lesions in xeroderma pigmentosum variant cells is "error-free". *Mutat. Res.* **146**, 285-294.

Wei, D., Maher, V.M. & McCormick, J.J. 1995. Site-specific rates of excision repair of benzo(a)pyrene diol epoxide adducts in the hypoxanthine phosphoribosyltransferase gene of human fibroblasts: Correlation with mutation spectra. *Proc. Natl. Acad. Sci. USA* **92**, 2204-2208.

Weinstein, I.B., Jefferey, A.M., Jennette, K.W., Blobstein, S.H., Harvey, R.G., Harris, C., Autrup, H., Kasai, H. & Nakanishi, K. 1976. Benzo(a)pyrene diol epoxides as intermediates in nucleic acid binding in vitro and in vivo. *Science* **193**, 592-595.

Yang, J.-L., Maher, V.M. & McCormick, J.J. 1987. Kinds of mutations formed when a shuttle vector containing adducts of (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene replicates in human cells. *Proc. Natl. Acad. Sci. USA* **84**, 3787-3791.

Zhang, X.Y. & Horz, W. 1984. Nucleosomes are positioned on mouse satellite DNA in multiple highly specific frames that are correlated with a diverged subrepeat of nine base-pairs. *J. Mol. Biol.* **176**, 1105-129.

Zhang, X.Y., Fittler, F.& Horz, W. 1983. Eight different highly specific nucleosome phases on alpha-satellite DNA in the African green monkey. *Nucleic Acids Res.* **11**, 4287-4306.