MMR GENES IN THE REPAIR OF DNA DAMAGE INDUCED BY UV AND H2O2

THE ROLE OF MISMATCH REPAIR IN THE REPAIR OF DNA DAMAGE INDUCED BY ULTRAVIOLET RADIATION AND HYDROGEN PEROXIDE

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ABSTRACT

DNA mismatch repair (MMR) recognizes and repairs bases incorrectly incorporated during DNA replication. Germ line mutations in two MMR genes, namely *hMSH2* and *hMLH1*, account for approximately 98% of hereditary non-polyposis colorectal cancers. There is conflicting evidence for the role of *hMLH1* and *hMSH2* in the transcription-coupled repair (TCR) pathway of nucleotide excision repair (NER). Here we have examined the role of these MMR genes in NER using two reporter gene assays. AdHCMVlacZ is a replication-deficient recombinant adenovirus that expresses the β-galactosidase reporter gene under the control of the human cytomegalovirus (HCMV) immediate-early promoter. We have reported a reduced host cell reactivation (HCR) for β-galactosidase expression of UVC-irradiated AdHCMVlacZ in TCR-deficient Cockayne syndrome (CS) fibroblasts compared with normal fibroblasts, indicating that HCR depends, at least in part, on TCR. In addition, we have reported that UVC-enhanced expression of the undamaged reporter gene is induced at lower UVC fluences to cells and at higher levels after low UVC fluences in TCR-deficient compared with normal human fibroblasts, suggesting that persistent damage in active genes triggers increased activity from the HCMV-driven reporter construct. We have examined HCR and UV-enhanced expression of the reporter gene in hMLH1-deficient HCT116 human colon adenocarcinoma cells and HCT116-chr3 cells (the MMR-proficient counterpart of HCT116) as well as hMSH2-deficient LoVo human colon adenocarcinoma cells and their hMSH2-proficient counterpart SW480 cells. We show a greater UV-enhanced expression of the undamaged reporter gene after low UVC exposure in HCT116 compared with

HCT116-chr3 cells and in LoVo compared with SW480 cells. We show also a reduced HCR in HCT116 compared with HCT116-chr3 cells and in LoVo compared with SW480 cells. However, the reduction in HCR was less or absent when cells were pretreated with UVC. These results suggest that detection of an involvement of *hMLH1* and *hMSH2* in TCR is dependent on UVC (254 nm) fluence to cells.

We have also used these two reporter gene assays to examine the role of *hMSH2* and *hMLH1* in the repair of oxidative DNA damage induced by UVA light (335-400 nm) and H_2O_2. UVA and H_2O_2 produce a number of oxidative lesions in DNA (such as 8-hydroxyguanines and thymine glycols) that are repaired by the base excision repair (BER) pathway. We show a reduced HCR for β-galactosidase expression of UVA-treated AdHCMV*lacZ* in *hMSH2*-deficient LoVo human colon adenocarcinoma cells compared to their *hMSH2*-proficient counterpart SW480 cells, but not in *hMLH1*-deficient HCT116 human colon adenocarcinoma cells compared to the *hMLH1*-proficient HCT116-chr3 cells. We also show that pre-treatment of cells with UVA enhances reporter gene expression to higher levels and at lower UVA fluences in LoVo compared to SW480 cells but not in HCT116 compared to HCT116-chr3 cells. These results suggest an involvement of *hMSH2* but not *hMLH1* in the repair of UVA-induced oxidative DNA damage. In contrast, no detectable differences were observed between SW480 and LoVo cells, as well as HCT116-chr3 compared to HCT116 cells, in both of the reporter gene assays that used H_2O_2 as the DNA damaging agent. Based on these results, these findings suggest that neither *hMSH2* nor *hMLH1* play a significant role in the repair of oxidative damage induced by H_2O_2.

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LIST OF ABBREVIATIONS

6-4 PP	pyrimidine (6-4) pyrimidone photoproduct
8-oxoG	8-hydroxyguanine
Ad	adenovirus
AP	apurinic / apyrimidinic site
α-MEM	α-minimal essential medium
β-gal	β-galactosidase
BER	base excision repair
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
CPD	cyclobutane pyrimidine dimer
CPRG	chlorophenol red β-D-galactopyranoside
CS	Cockayne's syndrome
DNA	deoxyribonucleic acid
D_{37}	dose that gives a survival fraction of 0.37 (e^{-1})
FBS	fetal bovine serum
GGR	global genomic repair
GPCR	G-protein coupled receptors
H_2O_2	hydrogen peroxide
HCMV-IE	human cytomegalovirus immediate-early promoter
HCR	host cell reactivation
hHRAD23B	human homologue of the S. cerevisiae protein 23
hMLH1	human mutL homologue 1
hMSH2	human mutS homologue 2
HNPCC	human non-polyposis colorectal cancer
lacZ	gene that codes for the enzyme β-galactosidase
MMR	mismatch repair
MOI	multiplicity of infection
NER	nucleotide excision repair
NO	nitric oxide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
pfu	plaque forming units
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute cell culture media
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulphate
SF	survival fraction
SV40	simian-monkey virus 40
TFIIH	transcription factor-II H
TCR	transcription coupled repair

Tg	thymine glycol
TNFR	tumour necrosis factor receptor
TTD	trichothiodystrophy
UV	ultraviolet radiation
XP	xeroderma pigmentosum

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Chapter 1:

Literature Review

(1) Ultraviolet Radiation

The spectrum of solar UV radiation and its subsequent biological effects is a relatively complex one, which has led to the division of UV into the following three categories based on their wavelengths: UVC (200-280 nm), UVB (280-320 nm), and UVA (320-400 nm). Since UVC is filtered out by the ozone layer in the stratosphere, little if any reaches the earth's surface (Robert *et al*, 1996) and thus is generally regarded not to have any significance on the environment or on human health. Thus UVA and UVB make up the rest of the ultraviolet portion of solar radiation, with UVA being around 20 fold more intense than UVB in sunlight (Agar *et al*, 2004).

1.1 Effects on Human Skin

Chronic exposure to ultraviolet radiation (UV) from the sun has been unambiguously linked to various visible skin lesions and other effects such as tanning, sunburning, photoaging, and the development of various cancers. Indeed with the relaxed attitude towards sun-protection and tanning that is still prevalent in our society (in spite of numerous reports which advise otherwise), melanomas constitute a common form of cancer. An estimated 4200 new cases of melanoma will develop in Canada in 2004 (Canadian Cancer Society, 2004), many of these which are likely preventable through limiting sun exposure. It has been widely believed that the UVB portion of sunlight is primarily responsible for eliciting many of these effects on the skin, since based on incident energy, UVA induces much less direct energy to DNA than UVB and therefore has been considered less carcinogenic (Agar *et al*, 2004). However, several

lines of evidence have suggested UVA as a significant source of concern in public health, considering the widespread use of UVB-blocking sunscreens and tanning beds that utilize high-intensity UVA sources (Douki *et al*, 2003). UVA has been shown to cause adverse skin effects usually associated with UVB exposure, such as skin erythema, photoaging (Wamer and Wei, 1997), and melanoma (Setlow *et al*, 1993). Additionally, it was recently reported that mutations in the basal cell layer in human squamous tumours are predominantly induced by UVA rather than UVB, presumably due to greater attenuation of incident UVB radiation by the upper layers of the human epidermis (Agar *et al*, 2004).

1.2 Effects on Cells and DNA; Photosensitization

Much of the adverse skin effects caused by exposure to ultraviolet radiation, especially with respect to the progression of skin cancer, can be attributed to the cytotoxic and mutagenic effects of UV-induced DNA damage. Even though UVC radiation itself presents little biological relevance to humans, it has been widely used as an experimental tool to efficiently induce the formation of bulky lesions that distort the DNA double helix, such as cyclopyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4 PP). These lesions, which are primarily removed by the nucleotide excision repair (NER) pathway (Friedberg, 2001), have been implicated in cell death, mutations, and carcinogenesis (Kurosaki *et al*, 2003). Many UVC light sources emit at a maximum wavelength of 254 nm, which is close to the maximum absorbance wavelength of the DNA bases. Therefore the induction of these bulky lesions is mainly due to the direct absorption of UVC photons by the DNA (Ravanat *et al*, 2001).

UVB, which represents the most energetic portion of sunlight that reaches the earth, also causes the formation of CPDs and 6-4 PPs through the direct excitation of DNA bases, although not as efficiently as UVC (Douki *et al*, 1999). In contrast, UVA produces formations of these particular bulky lesions at a much lower rate upon exposure, since incident UVA radiation is only weakly absorbed by DNA. Rather, UVA has been more characterized as a major cause of DNA oxidative damage caused by the absorption of various cellular chromophores such as riboflavin, porphyrin, nicotinamide adenine dinucleotide phosphate (NADPH) (Robert *et al*, 1996), and 3-hydroxypyridines (Wondrak *et al*, 2004). These chromophores act as photosensitizers in initiating type I or type II photo-oxidation reactions (see Fig. 1; reviewed in Foote, 1991; Ravanat *et al*, 2001). Type I is characterized by one-electron oxidation or hydrogen abstraction processes, which upon reaction with molecular oxygen (O_2) produce various reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-) and the hydroxyl radical ($OH^\bullet$). The $OH^\bullet$ radical is extremely reactive towards biological molecules and thus generates a multitude of modifications in DNA such as base damage, sugar damage, and DNA-protein crosslinks (Jaruga and Dizdaroglu, 1996). Type II involves the production of singlet oxygen ($^1O_2$), which in turn has been found to only react with guanine residues, leading to the production of various guanine oxidation products, most notably 8-hydroxyguanine (8-oxoG). This exclusive reaction between $^1O_2$ and guanine is due to guanine exhibiting the lowest ionization potential of all DNA bases (Douki *et al*, 2003). Indeed the formation of 8-oxoG has been routinely used as a marker for oxidative DNA damage from exogenous sources such as UVA.

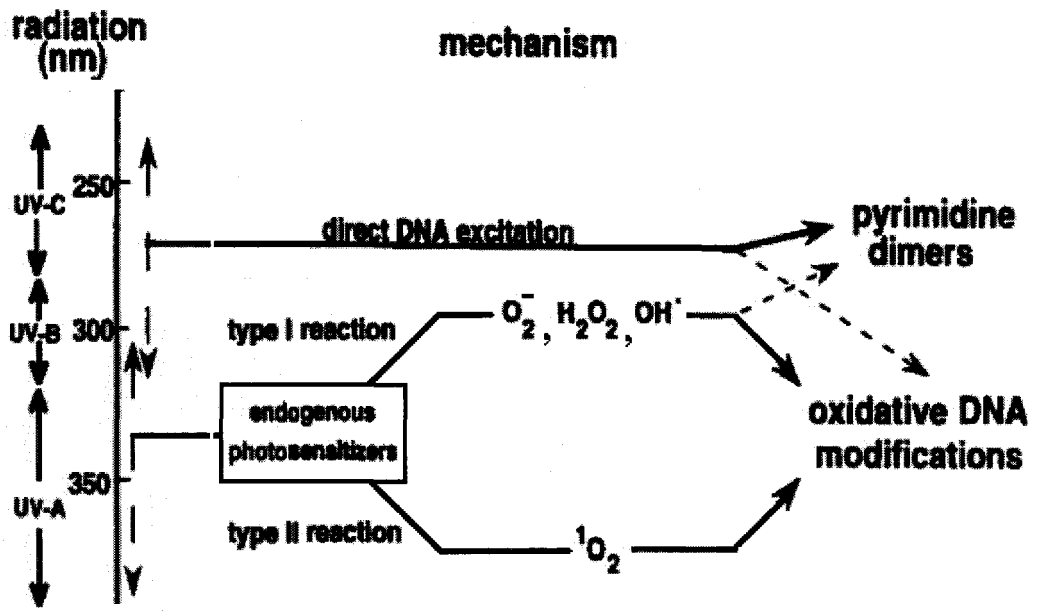

Figure 1-1: Direct and indirect mechanisms for the induction of pyrimidine dimers and oxidative DNA modifications upon exposure to UV radiation of different wavelengths. Solid lines represent major mechanistic pathways; dashed lines represent minor pathways (Adapted from Kielbassa *et al*, 1997).

Although it has been widely believed that UVA induces predominantly oxidative DNA lesions, recent studies into the spectrum of lesions produced by UVA has shown that even UVA is capable of producing significant levels of CPDs as well, even at levels greater than 8-oxoG production (Douki *et al*, 1999; Douki *et al*, 2003).

(2) Hydrogen Peroxide

2.1 Reactive Oxygen Species: Generation and Removal

Over the course of evolutionary history, species have adapted to utilize molecular O_2 in aerobic respiration, which has served as a powerful method to harness energy from various food sources. One serious side effect with exposure to an aerobic environment is

the constant production of reactive oxygen species (ROS) within cells, including hydrogen peroxide (H_2O_2) (see Table 1-1, Halliwell and Aruoma, 1991). To minimize the effects of oxidative stress, aerobic organisms have evolved various defenses that include enzymes (superoxide dismutase, catalase, and peroxidase) (Madigan *et al*, 2000) as well as various antioxidants such as amino acids, vitamins, tea polyphenols, and enzyme-bound minerals (Slupphaug *et al*, 2003).

Table 1-1: Mechanisms for the Generation and Deactivation of Reactive Oxygen Species (from Madigan *et al*, 2000; Slupphaug *et al*, 2003; Kehrer, 2000).

Generation of ROS:
$H_2O \rightarrow H_2O^+ + e^-$
$H_2O^+ + H_2O \rightarrow H_3O + OH^\bullet$ (hydroxyl radical)
$OH^\bullet + OH^\bullet \rightarrow H_2O_2$ (hydrogen peroxide)
$e_{aq} + O_2 \rightarrow O_2^-$ (superoxide anion)
$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet$ (Fenton reaction)
$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH^\bullet$ (Haber-Weiss reaction)
Deactivation of ROS:
$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (superoxide dismutase)
$2H_2O_2 \rightarrow 2H_2O + O_2$ (catalase)
$H_2O_2 + NADPH + H^+ \rightarrow 2H_2O + NADP^+$ (glutathione peroxidase)

2.2 Exogenous and Endogenous Sources of H_2O_2

The formation of H_2O_2 can be derived from numerous sources (see Fig 2). Two of the most common exogenous sources are ultraviolet and ionizing radiation. Exposure of cells and living organisms to background levels of ionizing radiation leads to the radiolytic fission of the oxygen-hydrogen bonds in water to produce $OH^\bullet$, of which approximately 40% of the $OH^\bullet$ radicals proceed to form H_2O_2 (LaVerne, 2000). With

respect to ultraviolet radiation, it was found that exposure of cell culture media containing tryptophan and riboflavin (photosensitizers commonly found *in vivo*) to UVA generated significant amounts of H_2O_2 (Mahns *et al*, 2003). Additionally, intracellular H_2O_2 production was found to be a contributing factor in mediating UVA and UVB-induced formation of 8-hydroxyguanosine, a precursor of DNA base damage (Zhang *et al*, 1997).

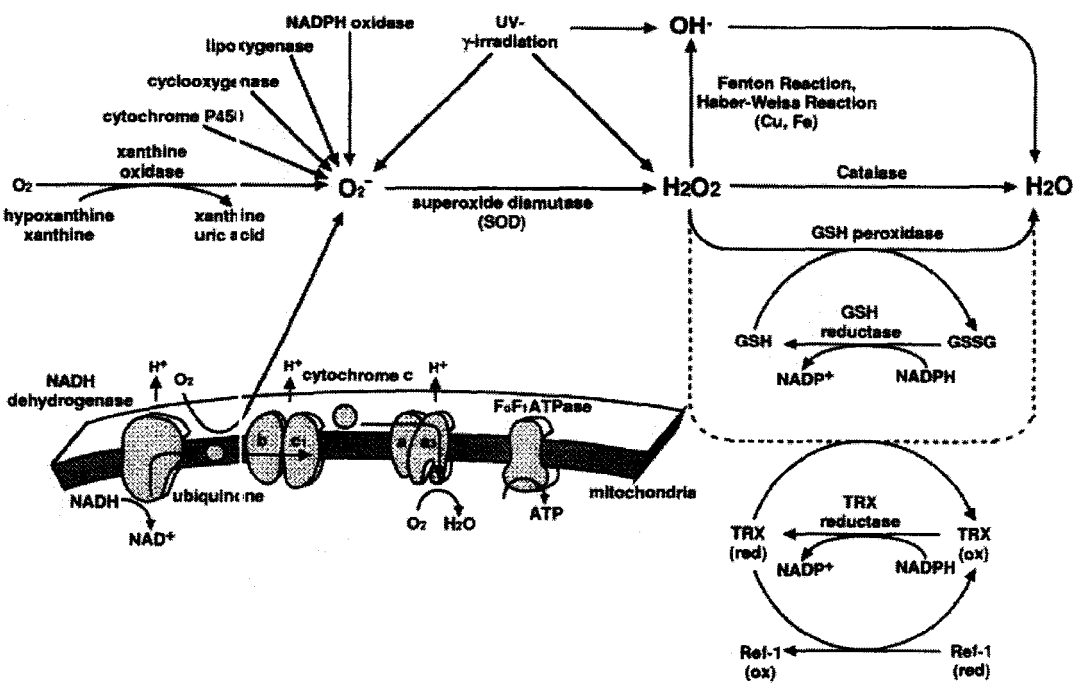

Figure 1-2: Metabolic pathways of reactive oxygen species (ROS). Two main exogenous sources of H_2O_2 are UV and γ-radiation. The major intracellular source of H_2O_2 is the production of the superoxide anion (O_2^-) as a byproduct of the electron transport chain in mitochondria; alternatively O_2^- is also produced in smaller amounts by various enzymes within the cell. Superoxide dismutase converts O_2^- to H_2O_2, which in turn can form the $OH^\bullet$ radical or be reduced to H_2O (From Kamata and Hirata, 1999).

By far the major source of H_2O_2 within the cell is the electron transport chain located in mitochondria. Even though almost all of the O_2 is efficiently consumed in this process, about 1 to 2% of the elections are leaked to generate O_2^-, which is subsequently converted into H_2O_2 by superoxide dismutase (Kamata and Hirata, 1999). Indeed, steady state levels of 8-oxoG have been measured in mitochondrial DNA at several folds higher than in nuclear DNA (Wiseman and Halliwell, 1996). Alternatively, O_2^- (and thus H_2O_2) is produced at lower levels by numerous enzymes *in vivo*, such as lipoxygenase, cyclooxygenase, and cytochrome P450 (see Fig. 2). NADPH oxidase has been shown to play a significant role in H_2O_2 production by phagocytic cells (Rhee, 1999).

2.3 Role in Cell Signaling

In addition to it's role as a member of *in vivo* ROSs, there has been increasing evidence that has revealed H_2O_2 as an important player in cellular regulation through redox signaling and in various signal transduction pathways. The notion that an ROS can play a role in cell signaling isn't that novel, since nitric oxide (NO), another ROS, has been well characterized as having an important role in cell signaling; indeed the 1998 Nobel Prize for Medicine and Physiology was awarded to Robert Furchgott, Louis Ignarro, and Ferid Murad for their discovery of NO as an important cell-signaling molecule (Williams, 1998). There have been suggestions that H_2O_2 may play a role as an intracellular second messenger, since it is small, diffusible, ubiquitous, and it can be synthesized and destroyed rapidly in response to external stimuli (Rhee *et al,* 2003).

The production of H_2O_2 from cell receptor activities has been extensively studied in phagocytic cells presumably due to their ability to produce high amounts of H_2O_2 to protect against invading organisms. However, recent evidence has suggested similar H_2O_2-producing mechanisms also exist in non-phagocytic cells (Lambeth, 2002). As previously mentioned, this mechanism has been found to be mediated by NADPH oxidase, which in turn is activated by various cell-surface receptors such as TNFR, GPCR, and RTKs (see Fig 3, below).

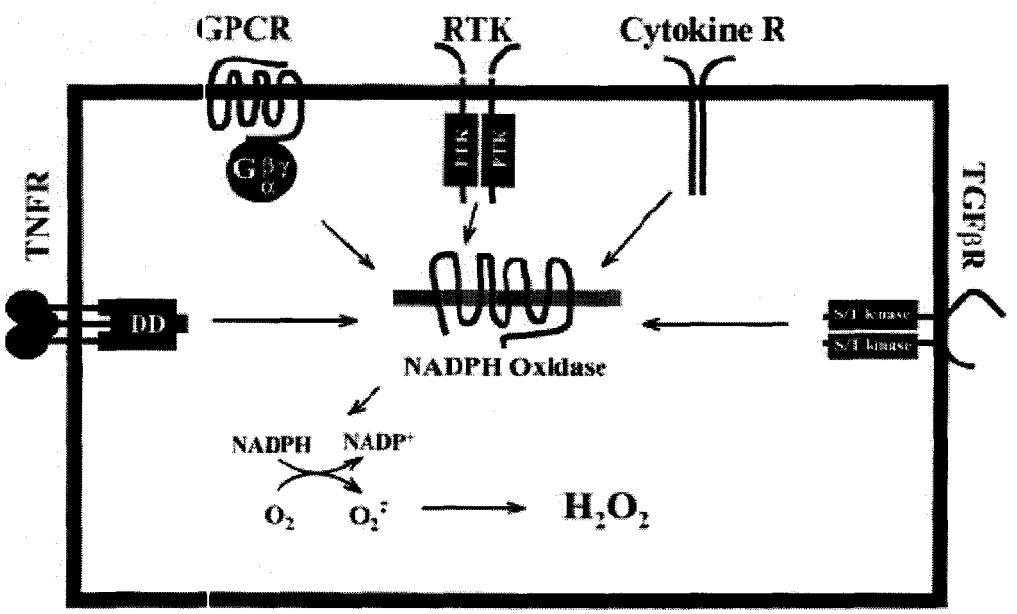

Figure 1-3: Various cell surface receptors that have been found to produce intracellular H_2O_2 through the activation of NADPH oxidase (From Rhee *et al*, 2003).

H_2O_2 (as well as other ROSs) have been found to cause both positive and negative effects on various protein targets involved in growth responses, cellular proliferation, transcriptional activation, signal transduction, and apoptosis (Kehrer, 2000). Due to its

simple structure, it is unlikely that H_2O_2 exhibits its messenger effects via specific recognition and binding by target proteins. Rather H_2O_2 may exert its effect in a redox regulation mechanism, since it is a mild oxidant that can oxidize cysteine residues in proteins (Kamata and Hirata, 1999), resulting in the formation of disulfide bridges between proteins, or more generally alter protein-protein interactions (Kehrer, 2000).

2.4 Effects on DNA

By far the most important (and consequently the most studied) cellular target of H_2O_2 (and other ROSs) is DNA because of the sheer number of different oxidized lesions that are produced and their implications in mutagenesis and carcinogenesis. However, H_2O_2 isn't likely to directly cause the formation of oxidative DNA damage, since H_2O_2 itself (as well as O_2^-) is relatively unreactive with DNA (Wiseman and Halliwell, 1996), but rather it is the more reactive $OH^\bullet$ radical arising from the breakdown of H_2O_2. Since DNA is a strong chelator of Cu^+ and Fe^{2+} ions, the breakdown of H_2O_2 is likely to occur through a Fenton reaction mechanism (Rodriguez *et al*, 1997). Due to the highly reactive nature of the $OH^\bullet$ radical, a large number of DNA lesions have been associated with H_2O_2-induced oxidative stress, including single-strand breaks, deoxyribose fragmentation, and a large number of DNA base modifications (Halliwell and Aruoma, 1991).

Studies into the oxidation of DNA bases by H_2O_2 have revealed a complex system of the H_2O_2-induced effects on DNA with respect to modes and spectrum of oxidized lesions produced. An initial study dealing with the chemical nature of base damage by H_2O_2 detected 10 different base products that were all attributable to $OH^\bullet$ induction, with

8-hydroxyguanine (8-oxoG) and 8-hydroxyadenine (8-oxoA) being the most common lesions produced (Dizdaroglu *et al*, 1991). There are numerous reports that have also examined the induction of thymine glycols (Tg) by H_2O_2, which is likely due to the fact that Tg is the most common thymine lesion generated by exogenous sources (Wilson III *et al*, 2003). In addition, the nature of local DNA sequences appears to mediate the frequency of base oxidation by H_2O_2 at "hot spots" (Rodriguez *et al*, 1995; Rodriguez *et al*, 1997). Furthermore, a "bimodal" dose-response relationship for H_2O_2-induced cytotoxicity and DNA damage has been reported for *Escherichia Coli*, (Imlay *et al*, 1988), Chinese hamster ovary cells (Kaneko *et al* 1994), and human cells (Nakamura *et al*, 2003). "Mode I" of the bimodal killing effect occurs at low to moderate H_2O_2 concentrations with the rate of killing/DNA oxidation is first order with respect to exposure time, whereas "mode II" takes place at higher H_2O_2 concentrations and is characterized by an increase in cell survival and a reduced rate in DNA oxidation (Imlay *et al*, 1987). The nature of mode II may be due to the *quenching* of $OH^{\bullet}$ by high concentrations of H_2O_2 according to the reaction: $OH^{\bullet} + H_2O_2 \rightarrow HO_2^{\bullet} + H_2O$ (Nakamura *et al*, 2003).

(3) DNA Mismatch Repair (MMR)

DNA mismatch repair (MMR) constitutes an important DNA repair pathway that plays a vital role in maintaining genomic stability. MMR has been shown to be relatively conserved in a number of species including bacteria, yeast, and mammalian cells, and it is responsible for the recognition and repair of bases incorrectly incorporated during DNA

replication. Additionally, other components of the MMR system are also involved in meiotic recombination (Harfe and Jinks-Robertson, 2000). In human cells, this repair process involves two principal repair proteins: (i) hMLH1 (human mutL homologue 1) and (ii) hMSH2 (human mutS homologue 2). As their names suggest, these two proteins are functional homologues of the mutL and mutS bacterial proteins respectively. The *hMLH1* gene is found on chromosome 3p21-23 and the *hMSH2* gene on chromosome 2p21, both of which are areas initially identified as important candidate regions for genes involved in hereditary non-polyposis colorectal cancer (HNPCC) (Mitchell *et al*, 2002). Indeed cell lines derived from HNPCC patients that are deficient in *hMLH1* and *hMSH2* have been characterized as mutator strains and exhibit high levels of microsatellite instability (Umar *et al*, 1994; Papadopoulos *et al* 1994).

hMLH1 can dimerize with other MMR proteins such as hPMS2 (to form the hMutLα heterodimer), hPMS1 (hMutLβ), and hMLH3, whereas hMSH2 dimerizes with hMSH6 or hMSH3 to form the hMutSα and hMutSβ heterodimers respectively (Friedberg, 2001; see Fig 1-4A). The human mutS heterodimers recognize and bind to all 8 base-base mismatch combinations made during DNA replication as well as insertion/deletion loops. The human mutL heterodimers binds to mutS heterodimers and serve as molecular matchmakers in recruiting the rest of the MMR machinery to excise and repair the errors (Mitchell *et al,* 2002). This is accomplished by introducing a nick up to 1 to 2 kilobases from the mismatch in the corresponding strand by an endonuclease, followed by degradation of the nicked strand past the mismatched base by exonuclease

activity. The appropriate bases are then filled by DNA polymerase δ and the nick sealed by DNA ligase (See fig. 4B, Sancar, 1999).

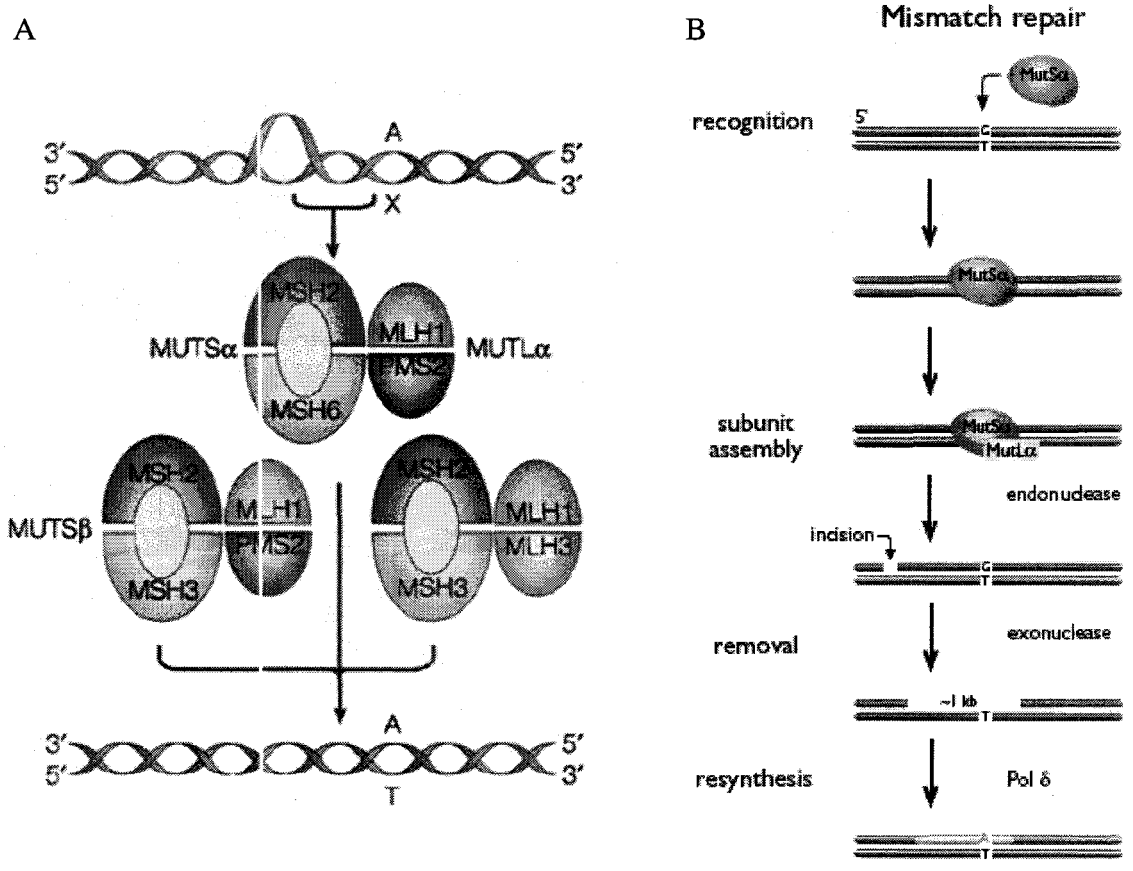

Figure 1-4: The Human DNA Mismatch Repair Pathway. **A**: MMR proteins involved in mismatch recognition by the heterodimers hMutSα (hMSH2 and hMSH6) or hMutSβ (hMSH2 and hMSH3). These proteins then recruit other heterodimers constituted by hMLH1 associated with hPMS2 (hMutLα), hMLH3, or hPMS1 (hMutLβ, not shown). **B**: Overview of the steps involved in removing base-base mismatches, which involves lesion recognition by hMutSα, recruitment of hMutLα and endonucleases (currently not well characterized), removal of the mismatched base, and resynthesis of the appropriate bases by DNA polymerase (From Friedberg, 2001, and Sancar, 1999).

One of the questions that still remains unanswered about MMR in eukaryotes is how does DNA strand discrimination take place, or in other words, how does the MMR system know which base in a mismatch is the incorrect one, since it is presumed that the mismatch occurs on the strand that was the most recently synthesized? In *E. coli*, methylation of adenine residues at GATC sequences serves as a marker for strand discrimination. The DNA becomes temporarily *hemimethylated* immediately following replication, with the newly replicated strand containing unmethylated adenines. The bacterial MutH protein (which contains endonuclease activity) is directed to the unmethylated strand at the base mismatch and forms a MMR promoting nick in that strand (Drummond and Bellacosa, 2001). Since there are no MutH homologues in eukaryotes, a number of proposals have been made in an effort to explain how MMR strand discrimination can occur in humans. It has been suggested that this may be accomplished through the methylation status of CpG islands, but an *in vitro* study has shown that human MMR operates independently of this system (Drummond and Bellacosa, 2001). Since MutH induces a nick effecting strand-specific repair, there have also been reports demonstrating that the presence of nicks in DNA *in vitro* plays a role in targeting the human MMR system to corresponding strands (Iams *et al*, 2002). This may have implications in the lagging strand where nicks between Okazaki fragments are common, but how this might apply to the more continuous leading strand requires further study. Alternatively, strand discrimination may also be accomplished through the interaction of human MMR proteins with the proliferating cell nuclear antigen (PCNA), a processivity factor associated with the DNA polymerase complex (Gu *et al*, 1998).

(4) Nucleotide Excision Repair

Nucleotide excision repair (NER) is a pathway that removes bulky lesions from the genome that distort the conformation of the DNA double helix, including UV-induced DNA lesions such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4 PP). NER has also been shown to act upon various chemical agents such as benzo(a)pyrene diol epoxide, aflatoxin B1, and psoralen, all which induce DNA adduct formation (van Hoffen *et al*, 1995). Its clinical importance is especially evident in human skin cells, which are exposed to the omnipresence of UVA and UVB radiation in the environment, potentially damaging the DNA in these cells.

Defects in NER have been linked to 3 different rare autosomal recessive diseases in humans, namely xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD) (reviewed in Friedberg, 2001). As previously mentioned, the occurrence of XP is rare with an average incidence of 1 in 10^6 to 1 in 10^5 individuals (Stary and Sarasin, 2002). XP is clinically characterized by sun-hypersensitivity, excessive freckling, rapid skin aging, and a high predisposition to multiple skin cancers, where the average age of onset is 8 years old. A small number of XP patients also exhibit neurological disorders. Individuals afflicted with Cockayne syndrome exhibit many of the XP-type symptoms, except that CS patients are not as cancer prone and in addition they are characterized by small size at birth, short stature, and early death with the mean life expectancy of 12 years (Le Page *et al*, 2000; Slupphaug *et al*, 2003). The clinical manifestations of TTD are also distinct from the other two disorders in that although sensitive to sunlight, TTD patients do not show the severe skin symptoms associated with

XP nor do they exhibit typical CS features. Rather, individuals with TTD have brittle hair and nails due to a deficiency of sulfur-rich proteins normally found in these tissues; physical and mental retardation as well as ichthyosis (scaly skin) are also common TTD characteristics (Friedberg, 2001).

The NER process requires the products of at least 30 genes in eukaryotes and these proteins have been highly conserved over the course of evolutionary history (Stary and Sarasin, 2002), demonstrating the essential nature of NER. Many of these proteins were discovered through complementation studies involving NER-deficient cells from XP and CS individuals. Generally, the process of NER in mammalian cells can be described in the following steps: (i) specific recognition of the DNA lesion and the recruitment and assembly of the complete NER machinery, (ii) incisions made at 5' and 3' on either side of the lesion, forming an oligonucleotide around 25-30 bases long, (iii) removal of the lesion-containing oligonucleotide fragment from the genome, (iv) repair synthesis by DNA polymerase δ/ε to fill in the gap using the complementary strand as the template, and (v) seal of the nicks by DNA ligase, restoring the covalent integrity of the newly repaired strand (Friedberg, 2001).

Further elucidation that NER of CPDs occurs at different rates throughout the genome has revealed two related but distinct sub-pathways. Transcription-coupled repair (TCR) is characterized by the rapid repair of the transcribed strand of active genes, whereas global genome repair (GGR) involves slower removal of UV-induced lesions in the non-transcribed strand of active genes (Mellon *et al*, 1987) as well as transcriptionally inactive regions of the genome (Bohr *et al*, 1985). The importance of these two NER

sub-pathways becomes more obvious when considering individuals afflicted with the autosomal recessive disorders XP or CS. There are eight XP complementation groups (XP-A to XP-G and XP-V) most of which are deficient in both TCR and GGR (except XP-C and XP-E, which retains proficient TCR but deficient GGR). XP-V cell lines (which stands for xeroderma pigmentosum variant) are actually proficient in NER, but code for a defective DNA polymerase η (Friedberg, 2001). This particular DNA polymerase is responsible for *translesion synthesis*, which is the ability to efficiently replicate past TT photoproducts by incorporating a pair of adenines in the newly synthesized DNA strand (Stary and Sarasin, 2002).

In contrast to the XP groups, both CS complementation groups (CS-A and CS-B) exhibit deficiencies in the TCR sub-pathway of NER, but retain proficient GGR (van Hoffen *et al*, 1993). Additionally, there are also XP/CS complexes, in which mutations in the XP genes (specifically XP-B, XP-D, and XP-G) confer clinical symptoms that combine the skin abnormalities of XP with the neurological deficiencies of CS patients (Rapin *et al*, 2000). Cell lines derived from individuals with XP-B/CS, XP-D/CS, and XP-G/CS are all deficient in both TCR and GGR (see Table 2).

Aside from the differential involvement of the CS and XP complementation proteins in the two NER sub-pathways, TCR and GGR also differ in their lesion recognition step (see Fig. 5). In TCR, RNA polymerase II detects the lesion during transcription (compared to XP-C/hHR23B complex or the XP-E protein in GGR), which is stalled at the lesion during transcription of an active gene. Following the lesion recognition step in both TCR and GGR, the XP-G protein is recruited to stabilize the

open DNA complex. The TFIIH protein complex then binds to the open complex and the XP-B and XP-D protein components exercise their helicase activities to maintain promoter clearance. Other proteins such as XP-F, XP-A and the replication protein A (RPA) are also recruited to this site, where the XP-F and XP-G produces incisions at 5' and 3' of the lesion respectively. The resulting 25-30 base oligonucleotide (containing the lesion) is removed, the gap is filled in by DNA polymerase δ or ε, and the nicks are then sealed by DNA ligase (reviewed in Friedberg, 2001; Stary and Sarasin, 2002).

4.1 Role for MMR in NER (TCR)

There appears to be evidence for the role of the MMR genes *hMLH1* and *hMSH2* in the TCR pathway of NER, although the results of the studies in this area are conflicting. This notion was suggested first by Mellon and Champe when they showed that the protein products of the bacterial genes *mutS* and *mutL* were required for the TCR of the *E. coli* lactose operon (Mellon and Champe, 1996). Additional studies involving other species have lent support to this idea, including a report that in using a yeast two-hybrid system, it was found that yeast MSH2 physically interacts with various RAD proteins involved in NER in *Saccharomyces cerevisiae* (Bertrand *et al*, 1998). Furthermore, MSH2 was also able to suppress UVC-induced mutations in Chinese hamster ovary cells (Nara *et al*, 2001) and UVB-induced skin tumorigenesis in mice (Yoshino *et al*, 2002, Peters *et al*, 2003). By contrast, Sonneveld *et al.* reported that *MSH2*-deficient murine embryonic fibroblasts (MEFs) were able to remove CPDs much more rapidly from the transcribed compared to the non-transcribed strand of active genes suggesting proficient TCR in MEFs (Sonneveld *et al*, 2001).

Likewise, this controversy extends into studies involving human cell lines. Using a strand-specific Southern blot-based assay, Mellon *et al* reported that the MMR deficient HCT116 and LoVo human adenocarcinoma cells (carrying mutations in the *hMLH1* and *hMSH2* genes respectively) were defective in TCR of CPDs at the active *dihydrofolate reductase* gene locus (Mellon *et al*, 1996). Indeed, a possible role of human MMR in at least NER lies in the ability of hMutSα to bind to CPDs and 6-4 PPs situated opposite adenine and guanine residues (Wang *et al*, 1999). In contrast, using a ligation-mediated polymerase chain reaction that measures CPD removal at nucleotide resolution, Rochette *et al* have recently reported that these same MMR deficient adenocarcinoma cells are fully proficient in TCR (Rochette *et al*, 2002). Adimoolam *et al* have also reported that *hMLH1*-deficient HCT116 cells are fully competent in TCR at the *DHFR* locus (Adimoolam *et al*, 2001). Furthermore, Kobayashi *et al* claim that XP-C lymphoblasts deficient in hMutSα are also proficient in TCR of CPDs in the transcribed strand of the p53 gene (Kobayashi *et al*, 2004). In any case, additional studies are required in order to elucidate if or how MMR is involved in TCR, or at least NER.

Table 1-2: Functior of NER Proteins Derived from NER Deficient Complementation Groups (Adapted fro m Francis, 2000; Stary and Sarasin, 2002).

Group	NER			BER		Function
	6-4 PP	CPD				
		TCR	GGR	TCR	GGR	
XP-A	-	-	-	+/-	+	damage recognition interacts with TFIIH, XP-F
XP-B	-	-	-	+	+	3'→5' helicase in TFIIH promoter clearance
XP-C	-	+	-	+	+	damage recognition (GGR)
XP-D	-	-	-	+	+	5'→3' helicase in TFIIH promoter clearance
XP-E	slow	+	-	+	+	damage recognition (GGR)
XP-F	-	-	-	+	+	5' incision endonuclease damage recognition?
XP-G	-	-	-	+/-	+	3' incision endonuclease open complex stabilizer
XP-V	+	+	+	+	+	Error-free translesion synthesis
CS-A	+	-	+	-	+	RNA synthesis recovery
CS-B	+	-	+	-	+	RNA synthesis recovery Transcript elongation
XP-B/CS	-	-	-	-	+	same as XP-B
XP-D/CS	-	-	-	-	+	same as XP-D
XP-G/CS	-	-	-	-	+	same as XP-G
RPA	-	+	-	+	+	damage recognition
TTD-I	+	+	+	+	+	unknown
TTD-II	+	-	-	?	+	same as XP-B or XP-D
TTD-III	+/-	-	-	?	+	same as XP-B or XP-D

+ = no evidence of repair defect ? = has not been examined
- = significantly impaired repair capacity +/- = inconclusive results

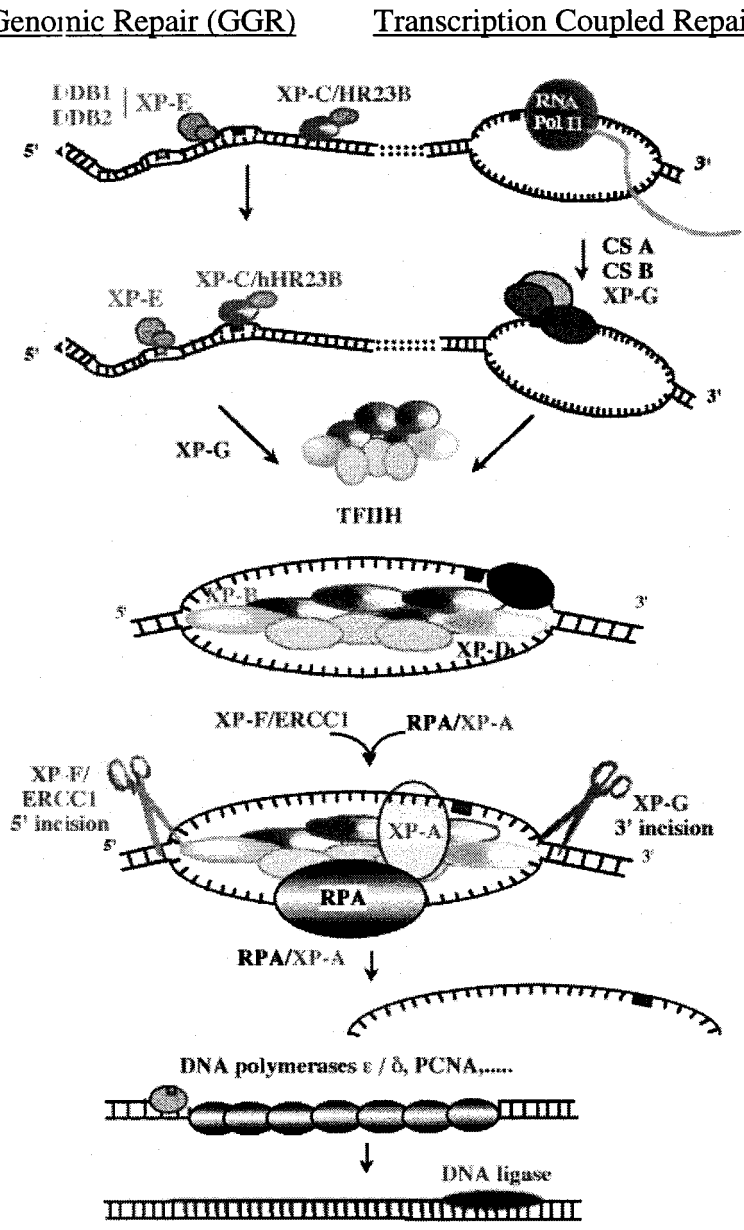

Figure 1-5: Schematic of the nucleotide excision repair (NER) pathway in human cells. Initial lesion recognition steps for global genomic repair (GGR) are shown on the left and for transcription-coupled repair (TCR) on the right (From Stary and Sarasin, 2002).

(5) Base Excision Repair

Base excision repair (BER) comprises yet another DNA repair pathway that represents the most essential and diverse pathway in correcting base damage caused by oxidizing and alkylating agents. Alkylating agents have important implications in cancer treatment since they are routinely used in the treatment of brain tumors, ovarian cancer, malignant melanomas, and various hematological tumors (Liu *et al*, 2002). The formation of DNA lesions caused by various oxidizing agents such as H_2O_2, UVA, and ionizing radiation is mediated by the formation of ROS that form within the cell upon exposure to these various agents (Kielbassa *et al*, 1997). These in turn react with cellular DNA to produce oxidative DNA modifications, which constitutes some of the most commonly induced DNA lesions present in a wide range of organisms such as bacteria, yeast, and mammals. Indeed in humans, clinical implications of oxidative damage become evident from studies that have investigated the role of ROS in cardiovascular disease, immune system decline, brain dysfunction (such as Alzheimer's disease), accelerated aging, and cancer development (Wiseman *et al,* 1996; Gu *et al*, 2002). It has been proposed that the full spectrum of oxidative lesions in endogenous mammalian DNA exceeds 100 different types (Croteau *et al*, 1997). Currently, two of the most studied lesions are 8-hydroxyguanine (8-oxoG) and thymine glycols (Tg), which represent the most common forms of base damage to purines and pyrimidines respectively (Slupphaug *et al*, 2003).

Due to the constant exposure to ROSs resulting in such a wide range of oxidative DNA lesions, many species have evolved a network of complex and redundant repair

mechanisms that has generally been labeled as BER. Much of this redundancy is due to the incorporation of a large number of DNA glycosylases needed to recognize a variety of base modifications. At least 12 different human DNA glycosylases have been identified (such as hOGG1 and hNTH1) (Slupphaug *et al*, 2003) with several functional homologues present in bacteria and yeast. In spite of comprehensive research into the nature of BER, there are no known naturally occurring human mutations that specifically confer defects in BER (unlike XP and CS mutations in NER and *hMSH2* and *hMLH1* mutants in MMR) (Le Page *et al*, 2002). Conversely, no distinct human disease has been associated with defective BER (Slupphaug *et al*, 2003).

Emerging evidence has also shown that the removal of oxidative damage occurs at a more rapid rate in actively transcribing regions of the genome, suggesting the existence of transcription-coupled BER mechanism (TCBER) (see Fig 1-6; Slupphaug *et al*, 2003, Le Page *et al*, 2002). In like manner with the TCR and GGR sub-pathways of NER, the initial lesion recognition step in TCBER is likely to involve a stalled RNA polymerase II, whereas specific DNA glycosylases fulfill this function in the non-transcribing regions of the genome (Le Page *et al*, 2000). Information mainly derived from *in vitro* reconstitution experiments have elucidated common aspects between the different sub-pathways of BER, namely specific recognition of the damaged base and cleavage of the N-glycosylic bond by the DNA glycosylase, cleavage of the DNA-phosphate backbone by AP lyase, and the removal of the 3'- deoxyribose moiety by an AP endonuclease. DNA polymerase I then fills the missing bases and DNA ligase seals the free DNA ends (Crouteau *et al*, 1997).

5.1 Role for NER in BER

Studies into the determination of the role of NER genes in BER usually involve the examination of the rate of removal of various oxidative DNA such as 8-oxoG and Tg, whose formation is induced by a variety of oxidizing agents. One of the first reports in this particular area describes that human lymphoblast cell extracts from XP-A, XP-B, XP-C, XP-D, and XP-G were all deficient in the repair of plasmids treated with H_2O_2 or γ-radiation. However, the exact nature of the lesions produced was not known, although it was suggested that they might be purine dimers (Satoh *et al*, 1993). Leadon and Cooper also found that cell lines deficient in CS-A or CS-B were defective in the preferential repair of the transcribed strand of the γ-irradiated *metallothionein* locus, whereas XP-A cells, which are hypersensitive to UV, were proficient in this aspect of repair (Leadon and Cooper, 1993). Interestingly, an editorial expression of concern has recently been issued regarding the report by Leadon and Cooper, calling into question the validity of the results (Cozzarelli, 2003).

Nevertheless, additional studies have suggested the involvement of other NER-associated factors including XP-G, TFIIH, XP/CS (including XP-B/CS, XP-D/CS, and XP-G/CS), and CS-B in BER (more specifically TCBER) (Cooper *et al*, 1997; Balajee *et al*, 1999; Le Page *et al*, 2000; Tuo *et al*, 2003). Surprisingly, XP-A lymphoblasts were also found to be deficient of oxidative lesions induced by fluorescent light, γ-radiation, and OsO_4 (which induces Tg formation in single-stranded DNA) (Lipinski *et al*, 1999). The involvement of CS proteins in the repair of oxidative DNA damage may help explain the cause of the neurological disorders found in CS patients. These symptoms are not

likely to be solely attributed to the persistence of CPDs and 6-4 PPs in CS patients, since individuals in whom there is a greater degree of NER compromise (i.e. XP-A patients) do not exhibit CS-type characteristics. The neurological abnormalities common to CS patients are rather likely to be due to oxidative stress in the brain, which is believed to be relatively high (Le Page *et al*, 2000).

5.2 Role for MMR in BER

Although their primary role is to repair single base-base mismatches and insertion/deletion loops, several lines of evidence have demonstrated roles for MMR factors, most notably *hMLH1* and *hMSH2* and their functional homologues, in BER. Many research groups have indicated an involvement of *hMSH2* in BER due to its ability to recognize and bind to 8-oxoG, a lesion that often mispairs with adenine (Ni *et al*, 1999) and is often a target of BER. In one report, mouse embryonic fibroblasts and human colon tumour lines that were $Msh2^{-/-}$ and $hMLH1^{-}$ respectively exhibited increased 8-oxoG levels before and after H_2O_2 treatment compared to wildtype cells (Colussi *et al*, 2002). Using radiolabeled DNA in gel-mobility shift assays, it was found that the *MSH2-MSH6* heterodimer in *S. cerevisiae* forms complexes around 8-oxoG:A mispairs and is required for the removal of adenine misincorporated opposite 8-oxoG (Ni *et al*, 1999). Furthermore, various mispairs of 8-oxoG were found to activate hMutSα through binding affinity experiments and its associated increases in ATPase and ADP → ATP exchange activities (Mazurek *et al*, 2002).

There have also been reports suggesting additional roles in BER that are outside the context of MMR protein interactions with 8-oxoG. The DNA glycosylase activities of hMYH were increased through the physical interaction with hMutSα (Gu *et al*, 2002). Likewise, MED1, a glycosylase that recognizes U/G and T/G mismatches, has been identified as a protein that interacts with *hMLH1*, suggesting a coordinated mutation avoidance mechanism by MED1 and MMR (Marti *et al*, 2002). Finally, *MSH2* and *MLH1*-nullizygous mouse fibroblasts were found to exhibit higher levels of clonogenic survival following exposures to ionizing radiation, presumably due to an absence of abortive MMR and cell death that may have otherwise occurred (Fritzell *et al*, 1997)

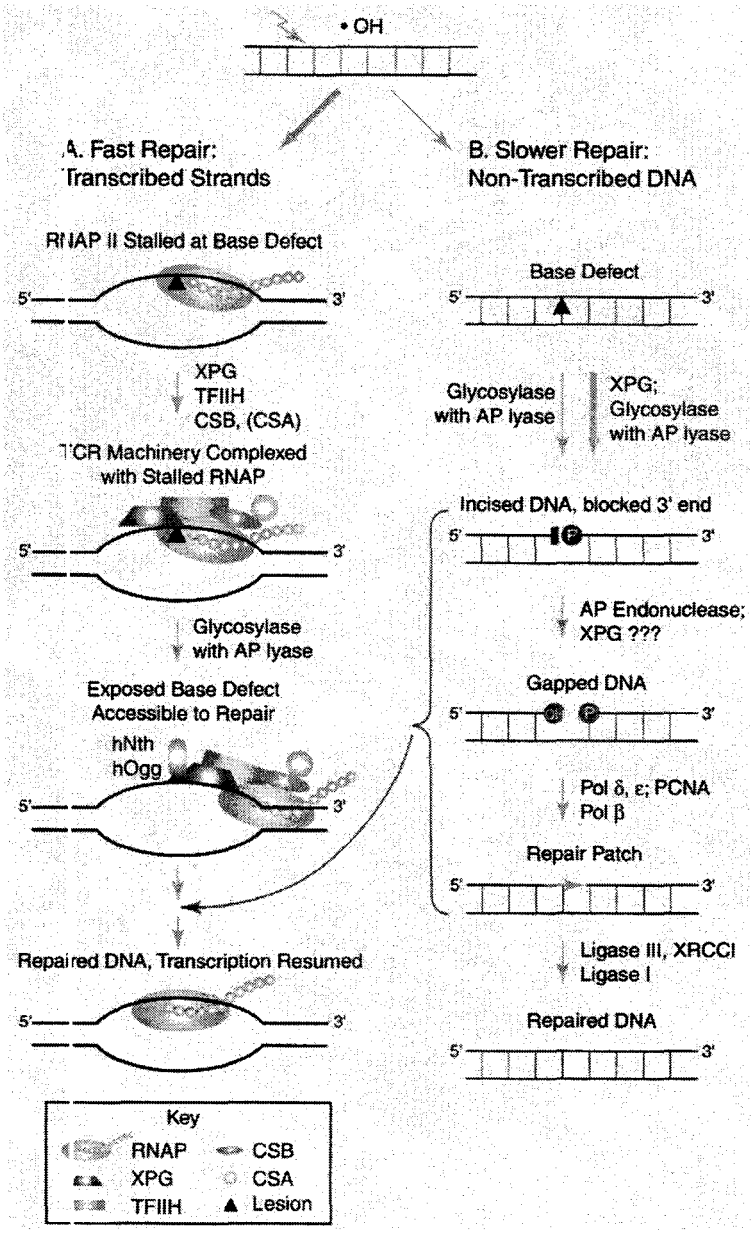

Figure 1-6: Models for base excision repair in human cells. In a similar manner to the TCR and GGR sub-pathways in NER (see Fig. 1-5), the initial recognition step of TCBER (left pathway) involves a stalled RNA polymerase II, where DNA glycosylases specific for the detection of the oxidized base performs this function on non-transcribing DNA strands (right pathway). Subsequent steps (i.e. removal 3'-deoxyribose moiety by AP endonuclease, gap-filling by DNA polymerase I and nick-sealing by DNA ligase) are the same in both pathways (From Le Page *et al*, 2000).

(6) Involvement of p53 in DNA Repair

The p53 tumor suppressor has been known as an important protein associated with cancer prevention, considering that it is the most commonly altered gene in cancer (Hollstein *et al*, 1991). This particular protein is intimately involved in multiple biochemical pathways aimed at preventing neoplastic transformation in mammalian cells. This is generally accomplished through enhancing DNA repair, arrest in cell cycle progression, or the induction of apoptosis (Smith and Seo, 2002). Increased expression and stabilization of the p53 gene product typically occurs after DNA damage by chemical mutagens or by radiation such as UV exposure, suggesting that p53 plays a role in a cellular system involved in genome surveillance.

6.1 Role of p53 in Nucleotide Excision Repair

Several lines of evidence have clearly shown a functional role for p53 in NER, especially in GGR (Ford and Hanawalt, 1997), whereas its role in TCR has been more controversial. However, there is evidence that suggests a p53 involvement in the preferential repair of actively transcribing genes in mammalian cells. Cells from individuals afflicted with Li-Fraumeni syndrome (LFS), which have a mutant p53 allele (and consequently a pre-disposition to cancer), had significantly reduced capacities to support the host cell reactivation (HCR) of a UV-damaged reporter gene in adenoviruses (McKay *et al*, 1997). It has also been recently shown that p53 assumes a protective role against UV-induced apoptosis in human fibroblasts proficient in TCR (McKay *et al*, 2001). Using the highly sensitive ligated-mediated PCR method, it was also shown that

LFS and other p53-compromised human fibroblasts exhibited defective GGR and TCR of CPDs induced by UVB (Therrien *et al*, 1999). Conversely, there are also a number of reports that claim otherwise, showing that cells from LFS patients (Ford and Hanawalt, 1995; Ford and Hanawalt 1997) as well as human fibroblasts transformed with the human papillomavirus E6 gene that abrogates p53 (Ford *et al*, 1998) exhibit proficient TCR despite having a deficiency in GGR. Insight into the nature of this controversy may come from the finding that p53-deficient lymphoblasts exhibit deficiencies (in comparison to their p53-proficeint counterparts) in the TCR of CPDs induced by UVB, but not UVC radiation, suggesting a wavelength dependence of p53 in TCR (Mathonnet *et al*, 2003).

Nevertheless, it has been suggested that p53 may regulate NER through protein-protein interactions (such as TFIIH; see Fig 1-7a), cell-cycle checkpoint functions, and transcriptional control of downstream NER genes (Adimoolam *et al*, 2001). Indeed, studies into the latter role have revealed at least three genes that are up-regulated by p53. Two of them are p48XP-E and XP-C (which are derived from XP complementation groups) and the third being Gadd45 (which binds to *in vitro* DNA damage) (Smith and Seo, 2002).

6.2 Role of p53 in Base Excision Repair

Studies into this particular research area of p53 have been relatively new with the initial discovery of p53-enhanced BER of AP sites on plasmid DNA (Offer *et al*, 1999). Further elucidation of p53 in BER has revealed the requirement of the core and C-terminal domains whereas the N-terminus domain (which mediates transcriptional

activity) is dispensable, suggesting a direct involvement of p53 (Offer *et al*, 2001). Indeed this is reflected in additional reports showing that p53 interacts and directly enhances the DNA polymerase β subunit (responsible for repair synthesis in BER, see Fig 1-7b) in the repair of abasic sites (Zhou *et al*, 2001) and alkylated bases induced by methyl methanesulfonate (Seo *et al*, 2002). Recently, p53 was also found to modulate the DNA glycosylase 3-MeAde in response to oxidative stress caused by ionizing radiation and H_2O_2 (Zurer *et al*, 2004). Due to the recent nature of these studies, future experiments will most likely reveal additional p53 functions in BER.

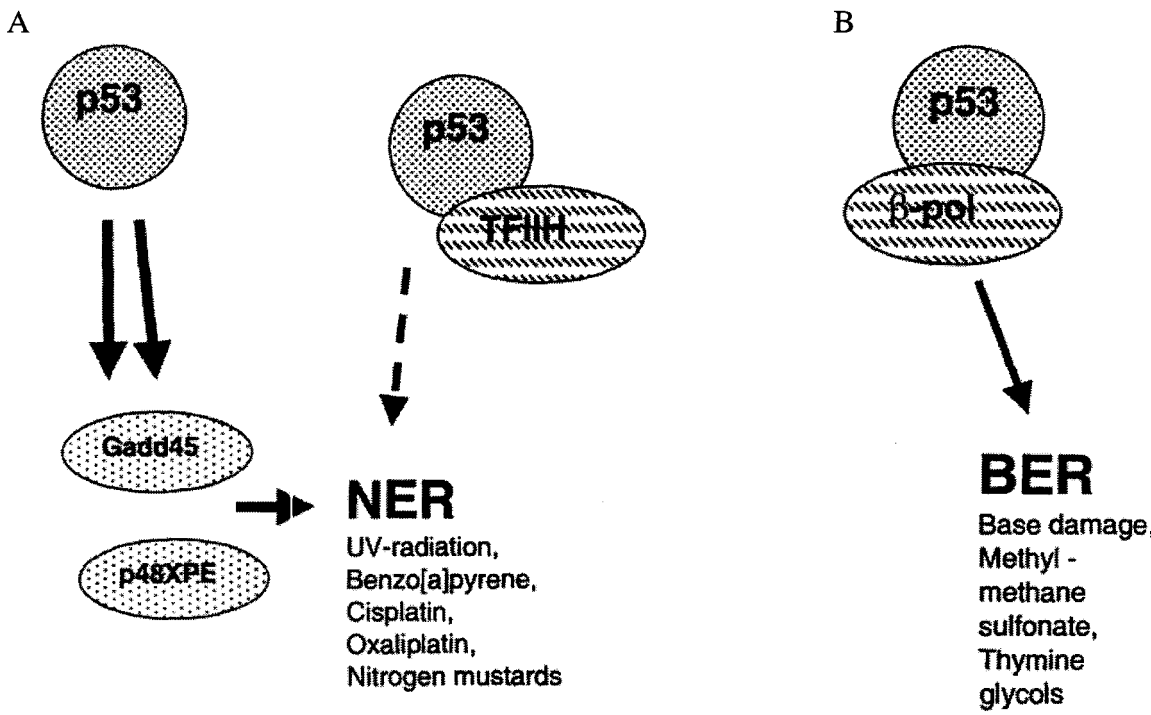

Figure 1-7: Roles for p53 in DNA Repair. A: The involvement of p53 in NER may arise through the transcriptional activation of NER associated genes such as Gadd45, p48XPE, or XPC (not shown), as well as direct interactions with proteins such as the TFIIH complex. B: Recent evidence has shown an interaction of p53 with DNA polymerase β in enhancing the BER of various base lesions (From Smith and Seo, 2002).

(7) The Use of Recombinant Adenoviruses to Examine DNA Repair

7.1 Recombinant Adenovirus Vectors

Adenoviruses are non-enveloped viruses whose genomes consist of approximately 36 kilobase pairs of double-stranded DNA (Reviewed in Madigan *et al*, p. 280, 2000). These viruses have been shown to cause mild respiratory infections in humans and were first isolated from adenoid glands and tonsils from infected individuals (hence the Latin term *adeno* for "gland"). Adenoviruses have been widely used as efficient vectors for gene therapy and recombinant DNA delivery in transfections, since they are easy to propagate in high titers, they can infect a wide variety of human and other mammalian cell types, and they are capable of accommodating large DNA inserts (Imperiale and Kochanek, 2004). Adenoviruses are also being developed and tested in vaccine therapy (Glick and Pasternak, p 271, 1998).

Non-replicating, recombinant adenoviruses are normally constructed by replacing the deleted early 1 (E1) region (required for adenoviral replication) with the gene of interest (Bett *et al*, 1994). A number of adenovirus vectors have been constructed for use in reporter gene assays, where the E1 region has been replaced with the *lacZ* gene (coding for β-galactosidase) or genes coding for luciferase (see Fig. 1-8) (Addison *et al*, 1997). The expression of these genes is under the control of the human cytomegalovirus immediate-early (HCMV-IE) promoter, which has been shown to drive high levels of gene expression in human cells (Boshart *et al*, 1985).

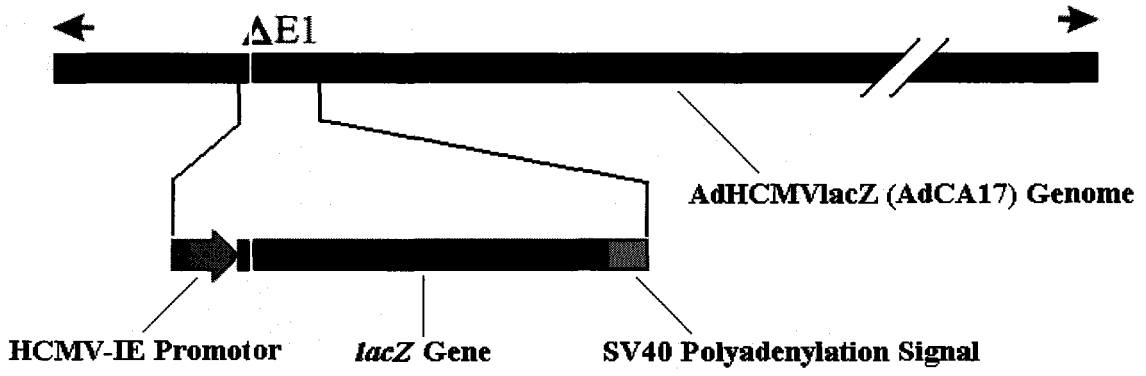

Figure 1-8: Diagram of the recombinant AdHCMV*lacZ* (AdCA17) virus genome. The AdHCMV*lacZ* vector is a non-replicating adenovirus that expresses the β-galactosidase (β-gal) reporter gene under the control of the human cytomegalovirus immediate-early (HCMV-IE) promoter in place of the deleted E1 region, which is responsible for adenoviral replication. Solid arrows represent the inverted terminal repeats of the adenoviral genome. (Adapted from *Addison et al,* 1997).

7.2 Host Cell Reactivation

Due to the complex nature of NER in mammalian cells, further characterization of both TCR and GGR pathways has proven to be a difficult one. This is reflected in attempts to reconstruct *in vitro* models for NER, which have posed significant issues regarding the determination of experimental results (Friedberg, 2001). Since the NER system is activated in response to DNA damage resulting from UV irradiation, it would stand to reason that one could UV-irradiate cells and assay for NER activity. However, it would be difficult to determine if the repair pathways are constitutively active or induced by the damaging agent (Francis and Rainbow, 1999). It would also be difficult to ensure that cellular DNA would be the only target, since other cellular components such as cell signaling and stress pathways may also be affected by UV irradiation. In order to circumvent these obstacles, we have developed an *in vitro* experimental model using non-

replicating recombinant adenoviruses as probes for NER. Specifically these were AdHCMV*lacZ* (AdCA17), which is unable to replicate in human fibroblasts but can efficiently infect and express the β-galactosidase reporter gene in these cells (Francis and Rainbow, 1999). UV-irradiation of these viruses produces lesions in viral DNA similar to that produced in mammalian cells, and a single pyrimidine dimer in the *lacZ* reporter gene is thought to be sufficient to inhibit its expression (McKay *et al*, 1999). These UV-irradiated adenovirus constructs are used to infect various cell lines deficient in NER in a host cell reactivation assay (HCR), which involves the host cell removing the transcription-blocking lesions from the reporter gene in the viral DNA (see Fig 1-9 (1)). Since proper β-galactosidase expression in mammalian cells is expected to only occur from transcription of a lesion-free *lacZ* gene, HCR of the reporter gene is thought to be reflective of the TCR capacity in mammalian cells (McKay *et al*, 1999).

7.3 Enhanced Expression of an Undamaged Reporter Gene

We have also developed another reporter gene assay examining NER status in cells, which involves infecting UVC-pretreated cells with the undamaged AdHCMV*lacZ* vector (see Fig 1-9 (2)). To this effect, we have previously reported that UV-enhanced expression of the undamaged *lacZ* reporter gene is induced at lower UV fluences to cells and at higher levels in TCR deficient fibroblasts (such as CS and XP-A), but not GGR deficient-only fibroblasts (such as XP-C) compared to normal human fibroblasts. This indicates that persistent damage in active genes triggers increased activity from the HCMV driven reporter construct (Francis and Rainbow 2000). Current models of NER

suggest that it is the persistent stalling of RNA polymerase II at sites of DNA damage that are not repaired by the TCR pathway which acts as a trigger for this response. Additionally, it was found that this enhanced expression effect was absent in mouse fibroblasts containing mutations in pRb and p107 genes, suggesting a role for retinoblastoma family members (Francis and Rainbow 2000). Furthermore, the HCMV-IE promoter contains various binding sites for NF-κB as well as consensus sequences for transcriptional activation such as AP1, CRE/ATF, and SP1 (Francis and Rainbow 2000), suggesting possible roles for these factors in mediating the enhanced expression of the undamaged reporter gene.

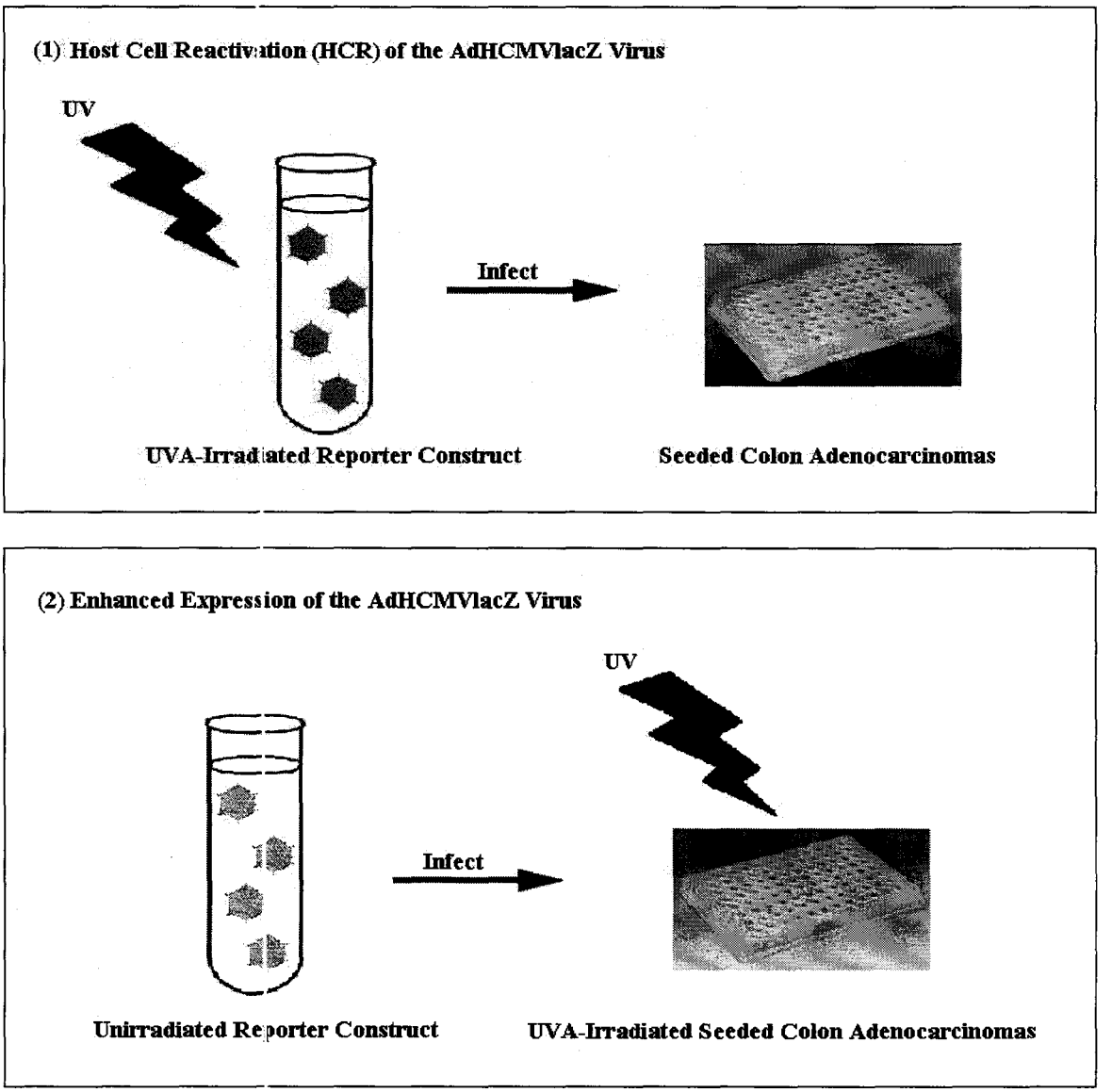

Figure 1-9: **(1)** Schematic of the host cell reactivation (HCR) assay of the AdHCMV*lacZ* virus. UV-irradiation of the virus probe produces lesions in the viral DNA similar to that produced in mammalian cells. The irradiated adenovirus construct is then used to infect the cell line of interest (Francis and Rainbow, 1999). **(2)** Schematic of the enhanced expression assay of the unirradiated AdHCMV*lacZ* virus. We have reported previously that UV-enhanced expression of the undamaged reporter gene is induced at lower UV fluences to cells and at higher levels in TCR-deficient but not GGR-deficient fibroblasts compared to normal human fibroblasts, indicating that persistent damage in active cellular genes triggers increased activity from the HCMV driven reporter construct (Francis and Rainbow, 2000).

(8) Project Introduction

The primary goal of this research project was to examine whether or not the MMR genes *hMLH1* and *hMSH2* have a role in NER and BER. In light of the numerous conflicting reports surrounding the possible involvement of these two particular genes in NER (more specifically TCR), we were very much interested in attempting to reveal additional insights into this controversy. Using the recombinant AdHCMV*lacZ* (AdCA17) adenovirus (which has previously been used as a probe for NER), we have utilized the HCR and enhanced expression assays, as well colony survival assays, in an effort to determine if indeed *hMLH1* and *hMSH2* are involved in TCR or possibly even figure out the nature of this controversy.

The role of *hMLH1* and *hMSH2* in at least some aspect of BER has been more generally accepted. Thus it was not so much the goal in determining if these two genes have a role in BER, but rather if we were able to modify the HCR and enhanced expression assays to examine BER, since they have been primarily used in experiments concerning NER up to this point. Here we describe the use of two oxidizing agents commonly employed in BER experiments, namely UVA and H_2O_2, and their effects in these particular reporter gene assays. Additionally, we have also looked at human fibroblasts derived from various CS and XP complementation groups, since their putative roles in BER are a little more controversial.

Finally, yet another controversy surrounds the role of p53 in the TCR sub-pathway of NER, although its role in GGR has been well established. We have previously utilized the AdHCMV*lacZ* virus in examining the NER status of various cell

lines. In this project, we were also interested in determining if wildtype p53 activity is enhanced as a result of the HCR of the UVC-irradiated reporter construct.

Chapter 2

Detection of an Involvement of the Human Mismatch Repair Genes *hMLH1* and *hMSH2* in Nucleotide Excision Repair is Dependent on UVC Fluence to Cells

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Detection of an involvement of the human mismatch repair genes *hMLH1* and *hMSH2* in nucleotide excision repair is dependent on UVC fluence to cells[1]

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Running title: Role of the mismatch repair genes in NER

Abstract

There is conflicting evidence for the role of the mismatch repair (MMR) genes *hMLH1* and *hMSH2* in the transcription-coupled repair (TCR) pathway of nucleotide excision repair (NER). In the present work we have examined the role of these MMR genes in TCR using two reporter gene assays. AdHCMV*lacZ* is a replication deficient recombinant adenovirus that expresses the β-galactosidase (β-gal) reporter gene under the control of the human cytomegalovirus (HCMV) immediate early promoter. We have previously reported a reduced host cell reactivation (HCR) for β-gal expression of UVC-irradiated AdHCMV*lacZ* in TCR deficient Cockayne syndrome (CS) fibroblasts compared to normal fibroblasts indicating that HCR depends, in part at least, on TCR. In addition we have reported that UVC-enhanced expression of the undamaged reporter gene is induced at lower UVC fluences to cells and at higher levels following low UVC fluences in TCR deficient compared to normal human fibroblasts suggesting that persistent damage in active genes triggers increased activity from the HCMV driven reporter construct. We have examined HCR and UV-enhanced expression of the reporter gene in *hMLH1*-deficient HCT116 human colon adenocarcinoma cells, and HCT116-chr3 cells (the MMR-proficient counterpart of HCT116) as well as *hMSH2*-deficient LoVo human colon adenocarcinoma cells and their *hMSH2*-proficient counterpart SW480 cells. We show a greater UV-enhanced expression of the undamaged reporter gene following low UVC exposure in HCT116 compared to HCT116-chr3 cells and in LoVo compared to SW480 cells. We show also a reduced HCR in HCT116 compared to HCT116-chr3 cells and in LoVo compared to SW480 cells. However, the reduction in HCR was less or

absent when cells were pretreated with UVC. These results suggest that detection of an involvement of *hMLH1* and *hMSH2* in TCR is dependent on UVC (254 nm) fluence to cells.

Introduction

Nucleotide excision repair (NER) is a highly conserved DNA repair pathway that removes lesions from the genome induced by UVC exposure, such as cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs). Its clinical importance is especially evident in human skin cells, which are exposed to the omnipresence of UV radiation in the environment, potentially damaging the DNA in these cells. Further elucidation that NER of CPDs occurs at different rates throughout the genome has revealed two related but distinct sub-pathways: transcription-coupled repair (TCR), which is characterized by the rapid repair of transcribed strands of active genes, and global genome repair (GGR), which involves slower removal of UV-induced lesions in the non-transcribed strand of active genes as well as transcriptionally-inactive regions of the genome (genomic heterogeneity of NER reviewed in Ref. 1).

It has been reported that the significance of TCR for the removal of UVC-induced DNA lesions depends not only on the type of lesion but also the UVC exposure employed. After a UVC exposure of 10 J/m^2, repair of CPDs in normal human fibroblasts is accelerated in the transcribed compared to the non-transcribed strand of active genes. In contrast, normal fibroblasts exposed to 30 J/m^2 lack strand specific repair of both 6-4PPs and CPDs, suggesting that TCR is overruled by GGR at this higher dose (2). In addition,

Li and Ho (3) reported that normal human fibroblasts respond differently to low compared with high exposures of UVB radiation. Pretreatment of human fibroblasts with 50 J/m^2 of UVB resulted in enhanced NER of a UVC-damaged reporter gene, whereas pretreatment with higher exposures of 100 and 200 J/m^2 did not result in enhanced NER. Taken together these studies suggest that the relative contribution of TCR to NER of UVC-induced DNA lesions is dependent on the magnitude of the UV exposure employed.

Due to the complex nature of both GGR and TCR in mammalian cells, examination of the role of TCR and GGR can be a difficult one. Since many experiments investigating cellular repair involve damaging the cell in some manner, it is difficult to ensure that cellular DNA is the only target, since other cellular components such as membranes and signal transduction pathways may also be affected by UVC irradiation. To address this problem, we have employed two reporter gene assays involving the recombinant adenovirus vector AdHCMV*lacZ*, which is a non-replicating virus that expresses the β-galactosidase (β-gal) reporter gene under the control of the human cytomegalovirus (HCMV) immediate early promoter (4). The first assay is a host cell reactivation (HCR) assay, in which UVC irradiation of the virus probe produces lesions in the viral DNA similar to that produced in mammalian cells. The irradiated adenovirus construct is then used to infect the cell line of interest. Since proper β-galactosidase expression in mammalian cells is expected to occur only from transcription of a lesion-free *lacZ* gene, HCR of the reporter gene is thought to be reflective of the NER capacity of the infected cell. We have previously reported a reduced host cell reactivation (HCR) for β-gal expression of UVC-irradiated AdHCMV*lacZ* in TCR deficient Cockayne

syndrome (CS) cells compared to normal human fibroblasts indicating that HCR depends, in part at least, on TCR (5). The second reporter gene assay involves infecting UVC pretreated cells with the undamaged AdHCMV*lacZ* vector. We have reported previously that UV-enhanced expression of the undamaged reporter gene is induced at lower UV fluences to cells and at higher levels for low UV fluence in TCR deficient fibroblasts, but not in GGR deficient fibroblasts, compared to normal human fibroblasts, indicating that persistent damage in active genes triggers increased activity from the HCMV driven reporter construct (6,7).

DNA mismatch repair (MMR) constitutes another important, relatively conserved DNA repair pathway found in mammalian cells involving the recognition and repair of bases incorrectly incorporated during DNA replication. In human cells, this repair process involves the expression of two principal repair genes, namely *hMLH1* (human mutL homologue 1) and *hMSH2* (human mutS homologue 2). As their names suggest, these two proteins are functional homologues of the mutL and mutS bacterial proteins respectively. At the present time, there appears to be conflicting evidence for the role of these MMR genes in the TCR pathway of NER in human cells. Using a strand-specific Southern blot-based assay Mellon et al. 1996 reported that the MMR deficient HCT116 and LoVo human adenocarcinoma cells (carrying mutations in the *hMLH1* and *hMSH2* genes respectively) were defective in TCR of CPDs at the active *dihydrofolate reductase* gene locus (8). In contrast, using a ligation-mediated polymerase chain reaction that measures CPD removal at nucleotide resolution, Rochette et al. have recently reported that these same MMR deficient adenocarcinoma cells are fully proficient in TCR (9). In

addition Sonneveld et al. reported that *hMSH2*-deficient murine embryonic fibroblasts (MEFs) are able to remove CPDs much more rapidly from the transcribed compared to the non-transcribed strand of active genes suggesting proficient TCR in MEFs (10) and Adimoolam et al reported that *hMLH1*-deficient HCT116 cells are fully competent in TCR at the *DHFR* locus (11). In the present work we have examined HCR of a UVC-damaged reporter gene and UVC-enhanced expression of the undamaged reporter gene in *hMLH1*-deficient HCT116 human colon adenocarcinoma cells, and HCT116-chr3 cells (a derivative of HCT116 wherein wild-type expression of *hMLH1* expression has been restored via chromosome 3 transfer) as well as *hMSH2*-deficient LoVo human colon adenocarcinoma cells and their *hMSH2*-proficient counterpart SW480 cells.

Materials and Methods

Cell Lines and Virus Strains. SW480 and LoVo colon adenocarcinoma cells were purchased from the American Type Culture Collection. HCT116-Chr3 and HCT116 human colon adenocarcinoma cell lines were kindly provided by Dr. Thomas Kunkel (National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA). The repair proficient human fibroblast GM 9503, as well as the deficient cell line GM 739A (CS-B) were obtained from NIGMS (Camden, NJ). All cell cultures were grown in a humidified incubator at 37 °C in 5% CO_2. Fibroblast cell lines were cultured in Eagle's α-minimal essential medium (α-MEM); SW480, HCT116-Chr3, and HCT116 carcinomas were cultured in McCoy's modified medium; LoVo's were cultured in a 1:1 mixture of D-MEM and F-12 media (with 4500 mg/L glucose). All cell culture media

was supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. AdHCMV*lacZ* (also called AdCA17*lacZ* (12)) is a non-replicating, recombinant adenovirus containing the *lacZ* gene under the control of the HCMV-IE promoter inserted in the deleted E1 virus gene. Virus stocks were prepared as described previously (13).

UV-C Irradiation of Cells and Virus. UVC irradiation used a General Electric germicidal lamp (model G8T5) emitting predominantly at 254 nm. Irradiation of cells has been reported previously (4). Fibroblasts were seeded at a density of 2.0 x 10^4 cells/well in 96-well plates (Falcon, Lincoln Park, NJ); carcinoma cell lines were seeded at 4.0 x 10^4 cells/well. Between 18-24 hours after seeding, media was replaced with 40 µl of PBS and UVC irradiated at a fluence rate of 1 J/m^2/s and then re-fed with appropriate supplemented growth medium. Irradiation of the virus by UVC has been described previously also (14). Briefly, viral suspensions were prepared in 1.8 ml phosphate buffered saline (PBS) in 35 mm petri dishes on ice. With continuous stirring, virus suspensions were irradiated with UVC fluences up to 600 J/m^2 at a fluence rate of 1 J/m^2/s. Aliquots of 200 µl were removed following each exposure to the virus and diluted appropriately in unsupplemented α-MEM.

HCR of the AdHCMVlacZ Reporter Gene. For HCR experiments, untreated and UVC-treated cells were immediately (within 30 minutes) infected with a 40 µl volume of irradiated or non-irradiated virus at a multiplicity of infection (MOI) of 20 pfu/cell. Following viral absorption for 90 minutes, cells were re-fed with the appropriate supplemented medium and allowed to incubate at 37 °C for 40-44 hours prior to harvesting. At harvesting the infected cell monolayers were incubated with 1 mM

chlorophenolred-β-D-galactopyranoside (CPRG, Boehringer-Mannheim, Indianapolis, IN) in 0.01% Triton X100, 1 mM MgCl2, 100 mM phospate buffer at pH 8.3. Absorbance readings at 570 nm were taken several times after the addition of CPRG solution using a 96-well plate reader (Bio-Tek Instruments EL340 Bio Kinetics Reader).

Enhanced Expression of the AdHCMVlacZ Reporter Gene. Cells were UVC irradiated or left untreated and subsequently infected with non-irradiated AdHCMV*lacZ* at an MOI of 20 pfu's/cell. LoVo, SW480, HCT116, and HCT116-chr3 cell lines were infected 24 hours following UVC irradiation and harvested 24 hours after viral infection; alternatively these cell lines were also infected 6 hours following UVC irradiation and harvested 12-14 hours after infection.

Results

HCR of reporter gene expression of UVC-irradiated AdHCMVlacZ in MMR proficient and deficient cell lines. Typical survival curves of β-gal activity for UV-irradiated AdHCMV*lacZ* in untreated (left panel) and UVC-treated (right panel) cells are shown in Figure 2-1 for TCR-deficient CS-B and normal human fibroblasts. It can be seen than HCR of the UV-damaged reporter gene in untreated cells is significantly reduced in the CS-B strain compared to normal as reported previously (5), indicating that HCR depends, in part at least, on TCR. It can be seen also that UVC-pretreatment of cells results in an enhancement of HCR in both normal and CS-B cells, consistent with an upregulation of GGR. The UVC-enhancement of HCR is greater in the CS-B compared

to the normal fibroblasts, such that the difference in HCR between the TCR deficient CS-B strain and the normal strain is not significant in the pre-UVC-treated cells.

Survival curves of β-gal activity for UVC-irradiated AdHCMV*lacZ* in MMR proficient and MMR deficient colon carcinoma cells are presented in Figure 2-2. In experiments involving no UVC-pretreatment of cells (left two panels), HCR of β-gal activity for UVC-irradiated AdHCMV*lacZ* was significantly reduced in the MMR deficient LoVo and HCT116 cells compared to their MMR proficient SW480 and HCT116-chr3 cell counterparts respectively. These results indicate some NER deficiency in the MMR deficient cells compared to their MMR proficient counterparts.

In contrast there was no significant difference in HCR for the MMR deficient cells compared to their MMR proficient counterparts for cells pretreated with 12 J/m^2 UVC (right two panels). Additional survival curves of β-gal activity for UVC-irradiated AdHCMV*lacZ* were obtained using other pre-UVC fluences to cells. The UV fluence to virus required to reduce β-gal activity to 37% of that for non-irradiated virus (D_{37}) was calculated for each survival curve (Table 2-1). Relative D_{37} values in MMR proficient compared to MMR deficient cells are plotted as a function of UVC fluence to cells in Figure 2-3. It can be seen that the difference in HCR for the MMR deficient and MMR proficient cell lines detected in untreated cells was reduced or absent when cells were pretreated with UVC.

It can also be seen that 12 J/m^2 UVC-pretreatment of cells resulted in a significant enhancement of HCR for LoVo cells, but not for SW480, such that there was no significant difference in the HCR curves for LoVo and SW480 cells pretreated with 12

J/m^2 (Fig.2-2, Table 2-1). In contrast, pre-UVC-irradiation actually reduced HCR for SW480, HCT116 and HCT116-chr3 cells, although this reduction was only significant for HCT116 and HCT116-chr3 cells and the reduction was greater in the MMR-proficient HCT116-chr3 cells, such that there was no significant difference in the HCR curves for HCT116 and HCT116-chr3 cells pretreated with 12 J/m^2. It appears that pre-UVC-treatment of cells actually inhibits repair in the transcribed strand of the reporter gene in HCT116 and HCT116-chr3 cells and that this inhibition is greater for the MMR proficient HCT116-chr3 cells.

Enhanced expression of the undamaged reporter gene in MMR proficient and deficient cell lines. We have previously reported that UVC-enhanced expression of the undamaged reporter gene is an indirect measurement of lesion removal by TCR in the transcribed strand of cellular genes (6,7). UVC-enhanced expression of the undamaged reporter gene is induced at lower UVC fluences to cells and at higher levels following low UVC fluence to cells in TCR deficient CS fibroblasts, but not in XP-C and XP-E fibroblasts which are deficient only in GGR, compared to normal human fibroblasts, indicating that persistent damage in active genes, that is not repaired by TCR, triggers increased activity from the HCMV driven reporter construct (6,7). The expression of β-gal following infection of non-irradiated AdHCMV*lacZ* was examined in pre-UVC-treated MMR proficient and MMR deficient colon carcinoma cells. Results for normal and CS-B fibroblasts carried out under identical conditions are shown for comparison purposes. Mellon et al. 1996 have reported a difference in repair of the transcribed strand of the active *dihydrofolate reductase* gene in HCT-116 compared to HCT116-chr3 cells

and in LoVo compared to SW480 cells (8). On the basis on this data, we chose carefully the time between UV exposure to cells and infection as well and the time between infection and scoring for β-gal in order to maximize the difference in repair of CPDs from the transcribed strand of active cellular genes in the MMR-deficient compared to the MMR-proficient cells.

Figure 2-4 shows results for CS-B fibroblasts, normal fibroblasts, LoVo, SW480, HCT116 and HCT116-chr3 cells infected 6 hours after UVC irradiation and subsequently scored for β-gal activity 12-14 hours after infection. It can be seen that persistent damage in the active genes of the TCR deficient CS-B cells resulted in UVC-enhanced expression of the undamaged reporter gene at lower UVC fluences to cells and at higher levels compared to that in normal human fibroblasts as reported previously (6,7). In addition, UVC-enhanced expression of the reporter gene was also observed at higher levels in LoVo compared to SW480 cells and in HCT116 compared to HCT116-chr3 at equivalent low UVC fluences to cells, suggesting a TCR deficiency in the MMR- compared to the MMR+ cells. Figure 2-5, shows results for CS-B fibroblasts, normal fibroblasts, LoVo, SW480, HCT116 and HCT116-chr3 cells infected 24 h after UVC irradiation and subsequently harvested 24 h after infection. It can be seen that under these conditions, higher levels of UVC-enhanced expression of the reporter gene were detected in CS-B compared to normal fibroblasts and in LoVo compared to SW480, but not in HCT116 compared to HCT116-chr3 cells at equivalent low UVC fluences to cells.

These results suggests a similar level of repair in the transcribed strand of active genes in HCT116 compared to HCT116-chr3 cells by 24 h after UVC to cells and a

reduced rate of repair in HCT116 compared to HCT116-chr3 cells at earlier times after UVC, since a difference in UV-enhanced expression was detected for cells infected at 6 h after UVC treatment (Fig. 2-4). In contrast, an increased UVC-enhanced expression of the unirradiated reporter gene was detected in LoVo compared to SW480 cells for infection at both 6 h and 24 h after infection, suggesting the TCR deficiency in LoVo cells is not just a deficiency in repair rate. These results are consistent with the difference in repair reported by Melon et al. for the transcribed strand of the active *dihydrofolate reductase* gene in HCT-116 compared to HCT116-chr3 cells and in LoVo compared to SW480 cells (8).

Discussion

UV-induced CPDs are removed more rapidly from the transcribed strand compared to the non-transcribed strand of actively transcribing cellular genes. Although both GGR and TCR contribute to NER in the transcribed strand of active cellular genes, the TCR pathway acts only on transcription blocking CPDs in the transcribed strand resulting in more rapid removal from the transcribed compared to the non-transcribed strand. However, the relative contribution of TCR and GGR to repair in the transcribed strand of an active gene appears to be dependent on the magnitude of the UV exposure to the cells (2).

Assessment of TCR in cellular DNA generally involves a comparison of lesion removal from the transcribed compared to the non-transcribed strand of an active gene or a comparison of lesion removal in an active compared to an inactive gene (15, 16, 17). In

contrast, the assessment of TCR in the current work involved an indirect measurement of lesion removal by NER from the transcribed strand of the reporter gene in the HCR assay, and an indirect measurement of lesion removal by TCR in the transcribed strand of cellular genes in the assay for enhanced expression of the undamaged reporter gene (4).

We have reported previously that HCR of the UVC damaged reporter gene in unirradiated cells is significantly reduced in TCR deficient CS fibroblasts as well as in GGR deficient XP-C and XP-E fibroblasts compared to that in normal human fibroblasts (5), indicating that both TCR and GGR contribute to expression of the UVC-damaged reporter gene in untreated human cells. Using a quantitative PCR technique, we have reported previously a significant removal of UVC-induced photoproducts from the reporter gene in normal human fibroblasts, but a reduced removal in TCR-deficient CS-B and GGR deficient XP-C fibroblasts (18). These previous reports suggest that differences in HCR for expression of the UVC-damaged reporter gene reflect more likely differences in the removal of UVC-induced photoproducts by NER rather than differences in the rate of transcription and/or lesion bypass by polymerase II. The reduced HCR of the UVC-damaged reporter gene in the untreated MMR deficient cells compared to their MMR proficient counterparts reported here (Fig. 2-2, left panels and Table 2-1) indicates that these human colon adenocarcinoma cells bearing homozygous mutations in hMLH1 or *hMSH2* are deficient in the TCR and/or GGR pathway of NER.

We have reported previously that UVC-enhanced expression of the undamaged reporter gene is induced at lower UV fluences to cells and at higher levels following low UV fluence in TCR deficient fibroblasts, but not GGR deficient fibroblasts, compared to

normal human fibroblasts, suggesting that persistent damage in active genes that is not repaired by TCR, triggers increased activity from the HCMV-driven reporter construct (4, 6). Current models of NER suggest that it is the persistent stalling of RNA polymerase II at sites of DNA damage that are not repaired by the TCR pathway which acts as a trigger for this response. We have reported also that p53 does not play an essential role in mediating UVC-induced expression from the CMV-IE driven reporter gene, such that the UVC-enhanced expression is independent of the p53 status of the cell (7). Enhanced expression of β-gal for untreated AdHCMV*lacZ* virus was significantly greater at lower UVC fluences to cells in LoVo cells compared to SW480 cells; and in HCT116 cells compared to HCT116-chr3 cells (Fig. 4). These results indicate that the efficiency of removal of UV lesions in the transcribed strand of active cellular genes by the TCR pathway is less in the MMR-deficient compared to the MMR-proficient cells.

There is evidence that some of the p53 regulated gene products are involved in inducible NER, including the p53-mediated and DNA damaged induced GADD45 gene (19, 20), the p48XPE gene (21, 22) and the XPC gene (23, 24). In particular, transcription from the p48 gene, which is mutated in GGR-deficient, damage-specific DNA binding (DDB) protein deficient, XP-E cells (20), is up regulated (in a p53-dependent manner) in response to UV treatment in human cells. This is consistent with a p53 dependent upregulation of GGR in cellular DNA reported for human cells (21). We have reported previously that wild type p53 is required for the expression of enhanced HCR of a UVC-damaged reporter gene in UVC-pretreated human cells (25). In addition, using a quantitative PCR technique, we have reported an enhanced removal of UVC-

induced photolesions from the reporter gene in pre-UVC-treated human fibroblasts (18). In the present work, we show that UVC-pretreatment of cells results in an enhancement of HCR for the UV-damaged reporter gene in TCR deficient CS-B cells, consistent with an enhanced removal of lesions from the transcribed strand due to a p53 dependent upregulation of GGR. UV induced DNA lesions efficiently block transcript elongation and induce the p53 response, indicating that persistent UV lesions in the transcribed strand of active genes trigger the p53 response (26, 27, 28). Therefore cells with impaired TCR induce the p53 response at lower UVC fluences compared to normal cells with functional TCR (27, 28, 29). This suggests that the p53 dependent upregulation of GGR would occur at lower UVC exposures and to a greater extent in TCR deficient compared to TCR proficient cells. Consistent with this suggestion, we found that the UVC-enhancement of HCR was greater in the CS-B compared to the normal fibroblasts for pre-treatment of cells with 12 J/m^2. The net result was that HCR of the UVC-damaged reporter gene in the TCR deficient CS-B strain compared to the normal strain, although substantially different in untreated cells (Figure 2-1A), was not significantly different in cells pretreated with 12 J/m^2 of UVC (Figure 2-1B).

UVC-pretreatment of cells with 12 J/m^2 resulted in enhanced HCR for the MMR-LoVo, similar to that observed for the TCR deficient CS cells, consistent with an up regulation of GGR and the p53 wild type (+/+) status of LoVo cells (30). In contrast, no UVC-enhanced HCR was detected in SW480 cells consistent with their mutant p53 (-/0) status (30) or in HCT116 and HCT116-chr3 cells (Fig. 2-2, Table 2-1). Although, HCT116 cells have a p53 wild type (+/+) status (30), we have reported previously a lack

of both heat-shock and UVC-enhanced HCR in this human colon cancer cell line (31) indicating that genetic alteration in tumor cells, other than inactivation of p53 can inhibit enhanced HCR.

It has been reported that normal fibroblasts exposed to high UVC exposure (30 J/m^2) lack strand specific repair of both 6-4PPs and CPDs, suggesting that TCR is overruled by GGR following high UVC exposure to cells (2). The reduction in TCR could result from a severe inhibition of transcription and/or the induction or inhibition of others factors affecting the TCR pathway following high UVC exposure. This suggests that there would be a greater reduction in the repair of the transcribed strand by this mechanism in TCR proficient cells (compared to TCR deficient cells) following high UVC exposure, which is consistent with the greater reduction in HCR for HCT116-chr3 cells compared to HCT116 cells. It appears that the level of repair in the transcribed strand of the reporter gene in pre-UVC-treated cells results from the net effect of the p53 dependent upregulation of GGR (which would result in a greater enhancement in repair for TCR deficient cells) together with a reduced contribution of TCR at high exposures (which would tend to result in a greater reduction in repair for TCR proficient cells).

Our results are consistent with a role for both *hMSH2* and *hMLH1* in the TCR of UVC-induced lesions in human colon adenocarcinoma cells. We suggest that their role in TCR is not detectable in cells pretreated with 12 J/m^2 UVC and greater, due in part to a p53 dependent upregulation of GGR together with an inhibition of TCR in the transcribed stand of the reporter gene at this high UV fluence to cells.

For no UVC exposure to cells, HCR of β-gal activity for UVC-irradiated AdHCMV*lacZ* virus was significantly reduced in LoVo cells compared to SW480 cells, and in HCT116 cells compared to HCT116-chr3 cells. This indicates an NER deficiency in the MMR deficient LoVo and HCT116 cells compared to their MMR proficient counterparts and is consistent with a TCR deficiency in these same MMR deficient cells as reported by Mellon et al. for repair in the active *dihydrofolate reductase* gene following a UVC exposure of 10 J/m^2 to cells. (8). The reduction in HCR was greater for the *hMSH2*-deficient LoVo cells compared to the *hMSH2*-proficient SW480 cells than for the *hMLH1*-deficient HCT116 cells compared to the *hMLH1*-proficent HCT-chr3 cells, indicating a greater effect of hMSH2 deficiency compared to *hMLH1* deficiency on NER in the transcribed strand of the reporter gene. These results for HCR in non-treated cells are consistent with the greater effect of hMSH2 deficiency compared to *hMLH1* deficiency on removal of CPDs from the transcribed strand of the active *dihydrofolate reductase* gene following a UVC exposure of 10 J/m^2 to these cells (8). In contrast, Adimoolam et al. (11) report that *hMLH1*-deficient HCT116 cells are fully competent in TCR at the DHFR locus following a similar UVC exposure of 10 J/m^2 to cells. The reason for the contradictory TCR results with the HCT116 cells remains unclear, although it has been suggested that the cell clones used by Adimoolam et al. may have acquired a secondary mutation that either restores TCR or relieves the inhibition of TCR due to the primary *hMLH1* mutation (11). It is also possible that the contradictory TCR results for HCT 116 cells arise from differences in UV dosimetry and/or differences in the UV spectrum employed, since the results of the present work suggest that relatively

small differences in UVC exposure to cells can affect the relative contribution of TCR and GGR.

The difference in HCR of β-gal activity for UVC-irradiated AdHCMV*lacZ* virus detected in untreated cells for the MMR deficient compared to the MMR proficient cell lines, was reduced or absent when cells were pretreated with UVC. For pre-UVC-treatment of cells with 12 and 18 J/m^2, there was no significant difference between the HCR in the MMR deficient compared to the MMR proficient cell lines (Figure 2-3). This is consistent with the results of Rochette et al. (9) showing a similar removal of CPDs from the transcribed strand of the *c-jun* and/or *p53* gene in the MMR deficient compared to the MMR proficient cell lines following a UVC exposure of 15 J/m^2 to these same cell lines.

Taken together the HCR and UVC-enhanced expression results of the present study indicate that human colon adenocarcinoma cells bearing homozygous mutations in *hMLH1* or hMSH2 are deficient in TCR of UVC-induced DNA lesions and that the ability to detect the involvement of these MMR genes in TCR is dependent on UVC fluence to cells. We suggest that the apparent discrepancy in previously published results regarding the role of *hMLH1* and *hMSH2* in TCR results, in part at least, from differences in the UVC fluence to cells used to examine TCR.

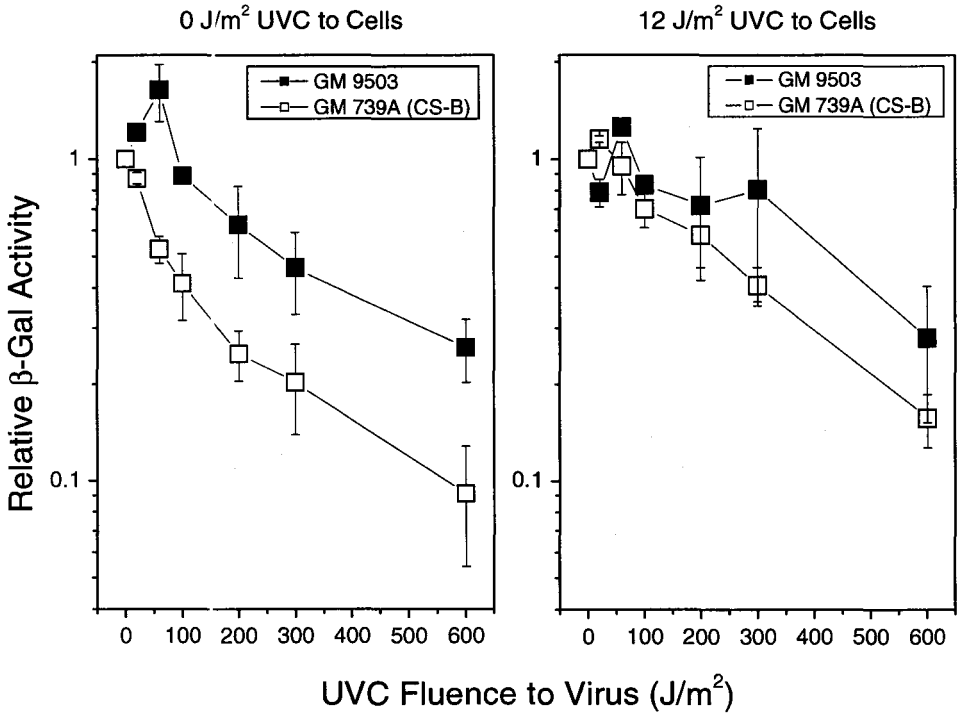

Figure 2-1: HCR cf β-galactosidase activity for UVC-irradiated AdHCMV*lacZ* virus in non-pretreated cells (0 J/m^2 UVC to cells) or cells irradiated with UVC (12 J/m^2 UVC to cells). Results are shown for GM 9503 (!) and GM 739A (■). Each point is the average ± SE of two to three independent experiments, each performed in triplicate.

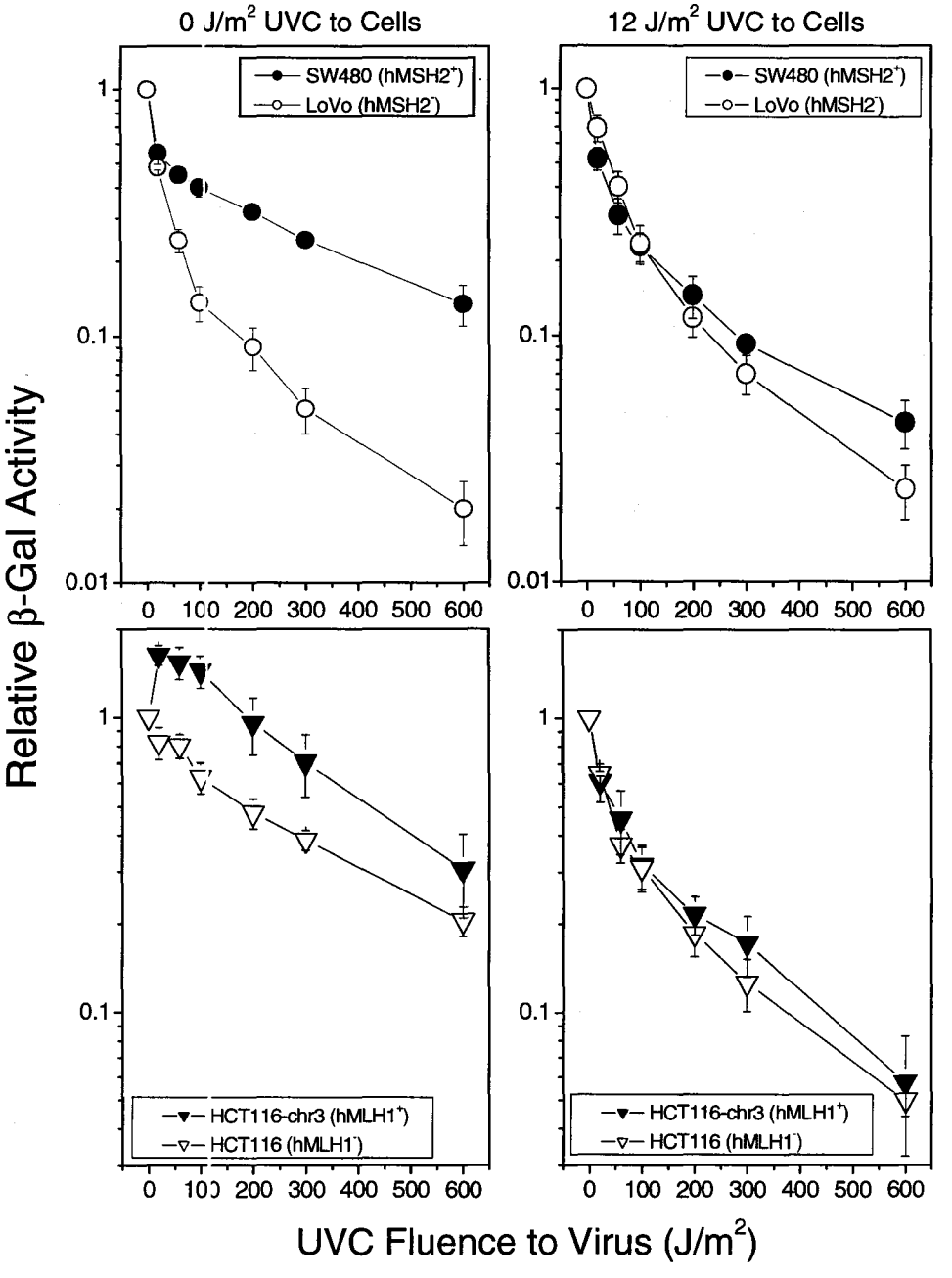

Figure 2-2: HCR of β-galactosidase activity for UVC-irradiated AdHCMV*lacZ* virus in non-pretreated cells (0 J/m^2 UVC to cells) or cells irradiated with UVC (12 J/m^2 UVC to cells). Results are shown for SW480 (,), LoVo (–), HCT116-chr3 (B), and HCT116 (X). Each point is the average ± SE of three independent experiments, each performed in triplicate.

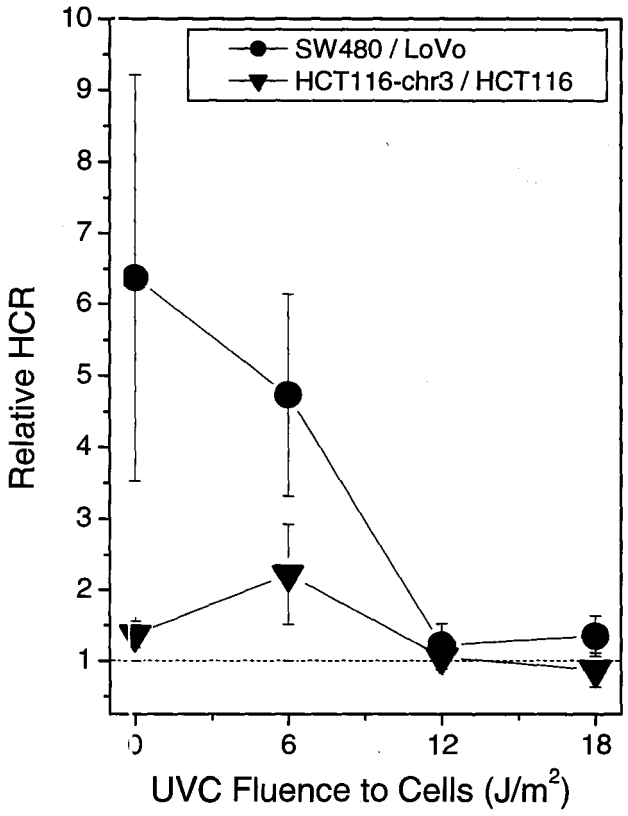

Figure 2-3: Relative HCR of the reporter gene of UVC-irradiated AdHCMV*lacZ* virus of MMR proficient compared to MMR deficient cell lines. Ratios of average D_{37} values for SW480 compared to LoVo cell lines (,) and HCT116-chr3 compared to HCT116 (B) were used to obtain relative HCR points. Each point is the average ± SE of two to seven independent experiments, each performed in triplicate.

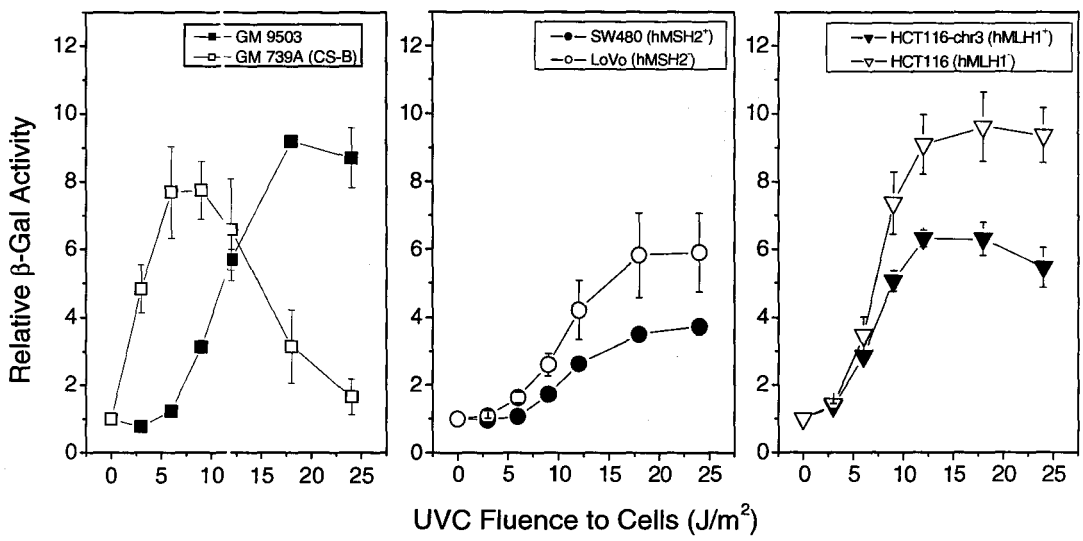

Figure 2-4: Enhanced expression of β-galactosidase in UVC-irradiated human fibroblasts and colcn carcinoma cell lines following infection with unirradiated AdHCMV*lacZ* virus. Cells were infected 6 hours after UVC irradiation and subsequently harvested 12-14 hours after infection. Results are shown for GM 9503 (!), GM 739A (□), SW480 (,), LoVo (–), HCT116-chr3 (B), and HCT116 (X). Each point is the average ± SE of two to five independent experiments, each performed in triplicate.

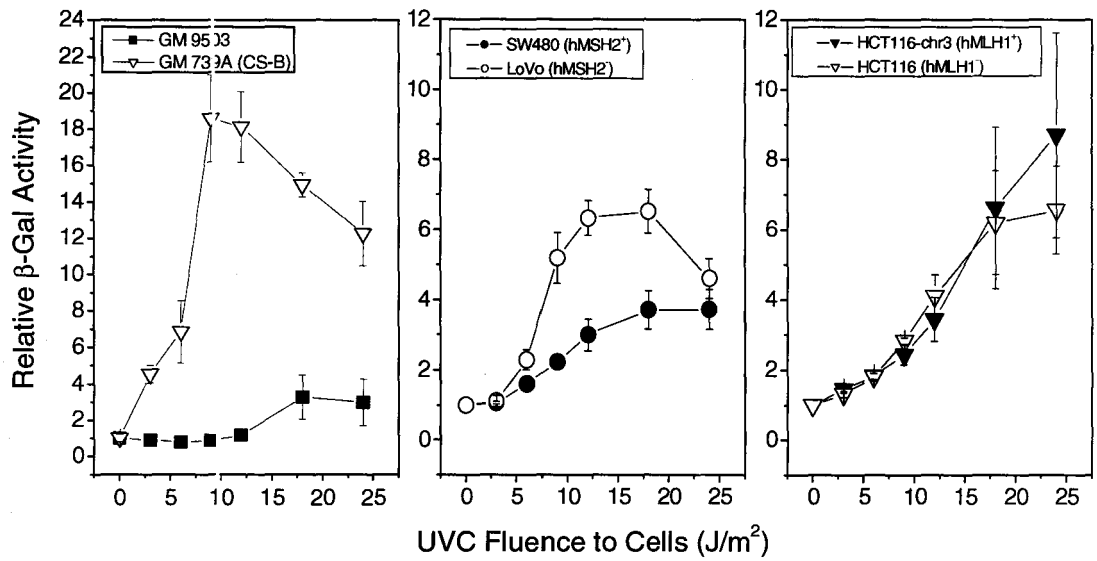

Figure 2-5: Enhanced expression of β-galactosidase in UVC-irradiated human fibroblasts and colon carcinoma cell lines following infection with unirradiated AdHCMV*lacZ* virus. Cells were infected 24 hours after UVC irradiation and subsequently harvested 24 hours after infection. Results are shown for GM 9503 (!), GM 739A (X), SW480 (,), LoVo (–), HCT116-chr3 (B), and HCT116 (X). Each point is the average ± SE of three to four independent experiments, each performed in triplicate.

Table 2-1: HCR of β-galactosidase activity for UVC-irradiated AdHCMV*lacZ* virus in MMR proficient and MMR deficient colon carcinoma cells pretreated with increasing UVC exposures

Cell Line	D37 (UVC Dose to Virus in J/m^2)			
	0 J/m^2	6 J/m^2	12 J/m^2	18 J/m^2
SW480	227.75 ± 99.58	218.96 ± 61.21	94.29 ± 24.35	123.52 ± 26.59
LoVo	35.76 ± 2.29	46.60 ± 0.965	81.11 ± 17.09	97.47 ± 27.80
HCT116-chr3	415.03 ± 76.03	376.24 ± 76.12	145.44 ± 35.35	180.28 ± 59.56
HCT116	298.09 ± 27.17	230.37 ± 77.33	149.12 ± 37.27	208.02 ± 45.97
Relative HCR				
SW480 / LoVo	6.37 ± 2.84 *	4.73 ± 1.41	1.22 ± 0.30	1.34 ± 0.28
HCT116-chr3 / HCT116	1.37 ± 0.18 *	2.22 ± 0.71	1.05 ± 0.17	0.87 ± 0.24

* Relative HCR significantly greater than 1 by one-tailed *t*-test ($P < 0.05$).

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Appendix: UVC Colony Survival of MMR-Proficient and Deficient Carcinomas

Introduction

There has been conflicting evidence for the role of the mismatch repair (MMR) genes *hMLH1* and *hMSH2* in the transcription-coupled repair (TCR) pathway of nucleotide excision repair (NER) (Mellon *et al*, 1996; Rochette *et al*, 2002). Indeed, UVC colony survival assays conducted by Mellon et al have reported that the *hMSH2*-deficient LoVo and *hMLH1*-deficient HCT116 adenocolon carcinomas were substantially more sensitive to UVC exposure than their *hMSH2*-proficient SW480 and *hMLH1*-proficient HCT116-chr3 counterparts respectively (see Fig. 2-6A, Mellon *et al*, 1996). By contrast, another UVC colony survival assay conducted by Rochette et al revealed no significant differences in surviving fractions between SW480s and LoVos, as well as HCT116-chr3 and HCT116 cell lines (see Fig. 2-6B, Rochette *et al,* 2002). We have recently reported that this apparent controversy that exists regarding the detection of the involvement of *hMLH1* and *hMSH2* in TCR is dependent on the UVC fluence to cells (Lee *et al*, 2004). Thus it was of interest to conduct our own UVC colony survival assay with these carcinomas lines in order to potentially shed some light on this issue.

Materials and Methods: UVC Colony Survival Assay

SW480 and LoVo cells were seeded in a 6-well plate (Falcon, Franklin Lakes, NJ) at a density of 400 cells/well. Between 6-8 hours after seeding, media was replaced with 1 ml of PBS and then UVC-irradiated at a fluence rate of 1 $J/m^2/s$ (or left untreated) and then re-fed with 2 ml of the appropriate medium. Cells were then allowed to

incubate at 37 °C, 5% CO_2, in a humidified incubator for 9-10 days. After the incubation period, colonies were stained with crystal violet prepared in 10% methanol and 63% ethanol (v/v). Colonies that contained more than 20 cells were counted and analyzed.

Results and Discussion

In agreement with the results reported by Rochette *et al*, we also show that there are no significant differences in surviving fraction between the respective MMR-proficient and deficient cell lines (Fig. 2-7). It is unclear as to why our results differ from those presented by Mellon *et al.*, since they have also reported a role for *hMSH2* and *hMLH1* in TCR. However, this may be due to the absence of error bars in their corresponding colony survival graph, which would make it difficult to discern whether or not the differences in colony survival between the respective MMR-proficient and deficient cell lines are indeed significant. Even though a role for MMR does exist in TCR, it appears that and MMR-deficiency doesn't confer any increased sensitivity to UVC radiation. Rather, this particular effect is likely due to defects in other repair genes such as those from the various Cockayne syndrome and xeroderma pigmentosum complementation groups, which have been traditionally associated as essential elements in NER.

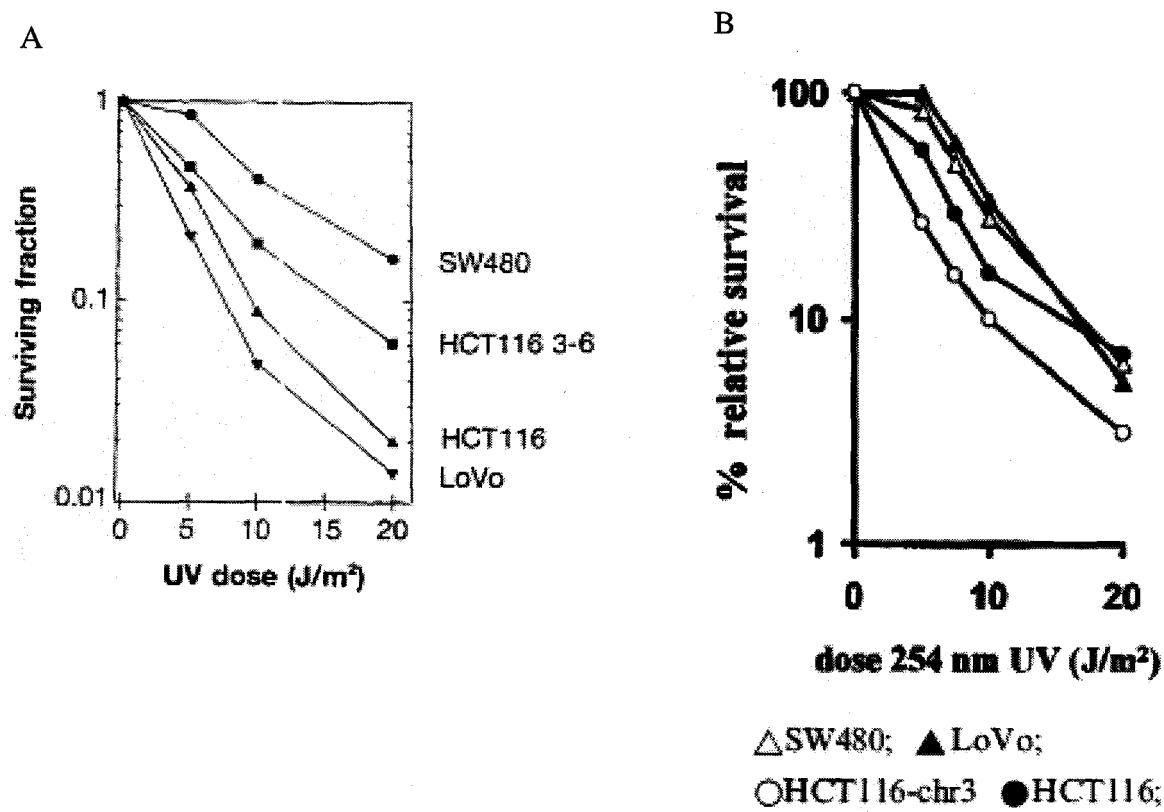

Figure 2-6: Results of colony survival assays of SW480, LoVo, HCT116-chr3, and HCT116 carcinomas in response to increasing levels of UVC exposure. **A**: Performed by Mellon *et al* (1996). Each point is an average of 3 experiments, each performed in duplicate. **B**: Performed by Rochette *et al* (2002). Each point is an average of at least 3 experiments.

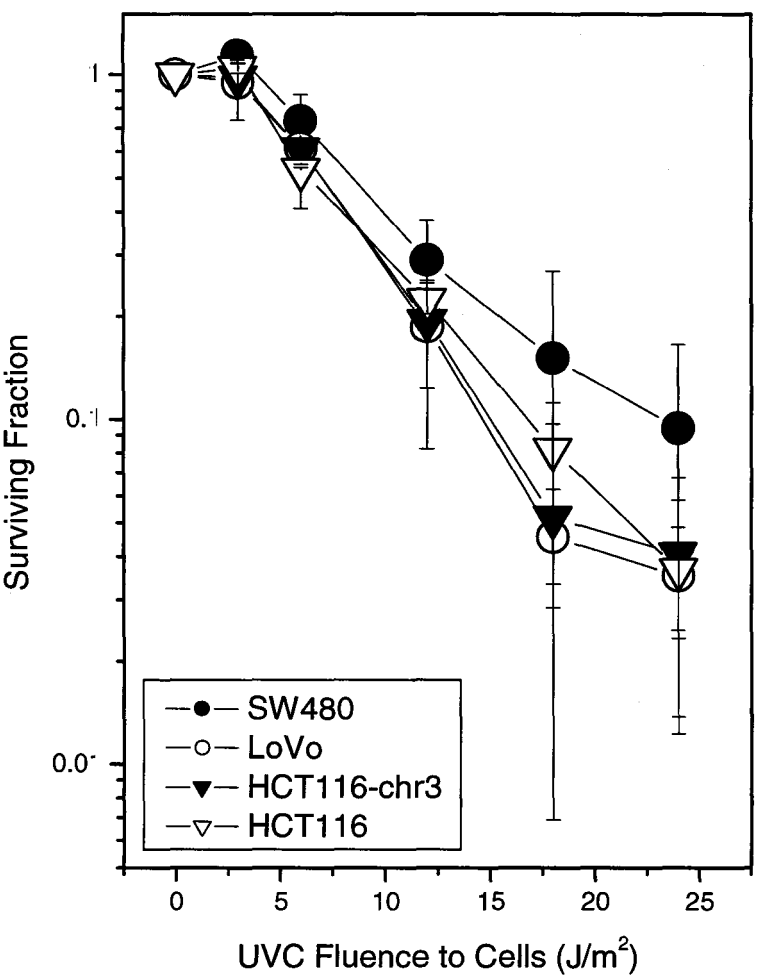

Figure 2-7: Clonogenic survival of SW480 (●), LoVo (○), HCT116-chr3 (▼), and HCT116 (▽) carcinomas in response to increasing levels of UVC exposure at a fluence rate of 1 J/m^2/s. Data is normalized to un-irradiated controls. Each point is the average ± SE of three to four independent experiments, each performed in triplicate.

Chapter 3

The Role of *hMLH1* and *hMSH2* in the Repair of UVA-Induced DNA Damage

Abstract

DNA mismatch repair (MMR) genes recognize and repair bases incorrectly incorporated during DNA replication. Germ line mutations in two MMR genes, namely *hMSH2* and *hMLH1*, account for approximately 98% of hereditary non-polyposis colorectal cancers. We and others have reported that cells defective in *hMSH2* or *hMLH1* show a deficiency in the transcription-coupled repair (TCR) for DNA damage induced by UVC, indicating their involvement in this pathway. In this report we have used two reporter gene assays to examine the role of *hMSH2* and *hMLH1* in the repair of oxidative DNA damage induced by UVA light. UVA produces predominantly 8-hydroxyguanines (8-oxoG) in DNA and is repaired by the base excision repair (BER) pathway. AdHCMV*lacZ* is a replication-deficient recombinant adenovirus that expresses the β-galactosidase (β-gal) reporter gene under the control of the human cytomegalovirus immediate-early promoter. We show a reduced host cell reactivation for β-gal expression of UVA-treated AdHCMV*lacZ* in *hMSH2*-deficient LoVo human colon adenocarcinoma cells compared to their *hMSH2*-proficient counterpart SW480 cells, but not in *hMLH1*-deficient HCT116 human colon adenocarcinoma cells compared to the *hMLH1*-proficient HCT116-chr3 cells. We have also reported previously that enhanced expression of the undamaged AdHCMV*lacZ* reporter gene is induced by the pre-treatment of cells with lower levels of the DNA-damaging agent and to higher expression levels in TCR-deficient compared to TCR-proficient cells. Here we show that pre-treatment of cells with UVA enhances reporter gene expression to higher levels and at lower UVA fluences in LoVo compared to SW480 cells but not in HCT116 compared to HCT116-chr3 cells.

These results suggest an involvement of *hMSH2* but not *hMLH1* in the repair of UVA-induced oxidative DNA damage.

Introduction

Oxidative damage constitutes some of the most commonly induced DNA lesions present in a wide range of organisms, such as bacteria, yeast, and mammals. Indeed in humans, a clinical aspect of the implications of oxidative damage becomes evident from studies that have investigated the role of reactive oxygen species (ROS) in cardiovascular disease, immune system decline, brain dysfunction (such as Alzheimer's disease), accelerated aging, and cancer development (Wiseman *et al*, 1996; Gu *et al*, 2002). ROS is a collective term to describe a group of oxygen radicals such as the superoxide anion ($O_2^{\cdot -}$) and the hydroxyl radical ($OH^{\cdot}$), or non-radicals that are either oxidizing agents or easily converted into radicals, namely singlet oxygen ($^1O_2$), ozone (O_3), or hydrogen peroxide (H_2O_2) (Wiseman *et al*, 1996). A significant amount of ROSs are formed as by-products of cellular metabolism in an aerobic environment. However, they may also be a result of exposure to exogenous sources such as ionizing radiation (DeWeese *et al*, 1998), UVA (Douki *et al*, 1999), and various other mutagenic and carcinogenic chemical sources. These in turn react with cellular DNA to produce modifications such as deaminated and oxidized bases; it has been proposed that the full spectrum of oxidative lesions in endogenous mammalian DNA exceeds 100 different types (Croteau *et al*, 1997). Currently, two of the most studied lesions are 8-oxoguanine (8-oxoG) and

thymine glycols, which represent the most common forms of base damage to purines and pyrimidines respectively (Slupphaug *et al*, 2003).

Due to the constant exposure to ROSs resulting in such a wide range of oxidative DNA lesions, many species have evolved a network of complex and redundant repair mechanisms that has generally been labeled as base excision repair (BER). BER is thought to represent the most essential and diverse pathway in correcting damage induced by oxidizing and alkylating agents, mainly due to the incorporation of a large number of DNA glycosylases needed to recognize a variety of base modifications. At least 12 different human DNA glycosylases have been identified (Slupphaug *et al*, 2003) with several functional homologues present in bacteria and yeast. In spite of comprehensive research into the nature of BER, there are no known naturally occurring human mutations that specifically confer defects in BER (unlike XP and CS in nucleotide excision repair and *hMSH2* and *hMLH1* mutants in mismatch repair) (Le Page *et al*, 2002). Conversely, no distinct human disease has been associated with defective BER (Slupphaug *et al*, 2003). However, emerging evidence has shown that the removal of oxidative damage occurs at a more rapid rate in actively transcribing regions of the genome, suggesting the existence of transcription-coupled BER mechanism (TCBER; Slupphaug *et al*, 2003, Le Page *et al*, 2002). Information mainly derived from *in vitro* reconstitution experiments have elucidated common aspects between the different sub-pathways of BER, namely specific recognition of the damaged base and cleavage of the N-glycosylic bond by the DNA glycosylase, cleavage of the DNA-phosphate backbone by AP lyase, and the removal of the 3'- deoxyribose moiety by an AP endonuclease. This is followed by the

filling in of the missing bases by DNA polymerase I and the rejoining of the free DNA ends by DNA ligase (Crouteau *et al*, 1997).

DNA mismatch repair (MMR) constitutes another important, relatively conserved DNA repair pathway found in bacteria, yeast, and mammalian cells involving the recognition and removal of bases incorrectly incorporated during DNA replication. In human cells, this repair process involves the expression of two principal repair genes, namely *hMLH1* (human mutL homologue 1) and *hMSH2* (human mutS homologue 2). As their names suggest, these two proteins are functional homologues of the mutL and mutS bacterial proteins respectively. Although their primary role is to repair single base-base mismatches and insertion/deletion loops, several lines of evidence have demonstrated roles for these two repair proteins that extend beyond their MMR capabilities. We and others have previously reported that cells defective in *hMSH2* or *hMLH1* show a deficiency in the transcription-coupled repair (TCR) for DNA damage induced by UVC, indicating their involvement in this pathway (Lee *et al*, 2004, Mellon *et al*, 1996). In addition, various research groups have suggested a role of *hMSH2* and its functional homologues in the recognition and removal of 8-oxoG, a lesion that often mispairs with adenine (Ni *et al*, 1999) and is often a target of BER. In another report, mouse embryonic fibroblasts and colon tumour lines that were *Msh2*$^{-/-}$, as well as a human ovarian carcinoma line that was *hMLH1*$^{-}$, both exhibited increased 8-oxoG levels before and after H_2O_2 treatment compared to wildtype cells (Colussi *et al*, 2002). Using radiolabelled DNA in gel mobility shift assays, it was found that the *MSH2-MSH6* heterodimer in *S. cerevisiae* forms complexes around 8-oxoG:A mispairs and is required

for the removal of adenine misincorporated opposite 8-oxoG (Ni *et al*, 1999). Furthermore, the DNA glycosylase activities of hMYH were increased through the physical interaction with hMutSα (hMSH2-hMSH6 heterodimer) (Gu *et al*, 2002). Finally, various mispairs of 8-oxoG were found to activate hMutSα through binding affinity studies and its associated increases in ATPase and ADP → ATP exchange activites (Mazurek *et al*, 2002).

Taken together, we were interested in determining whether or not the MMR genes *hMSH2* as well as *hMLH1* were indeed involved in the repair of oxidative damage using the host-cell reactivation and enhanced expression assays as described previously (Francis and Rainbow, 1999; Francis and Rainbow, 2000). Here we describe the use of UVA (335-400 nm) to induce oxidative DNA damage in human fibroblasts and carcinoma cell lines due to the environmental relevance of UVA exposure from the sun as well as the ability of UVA to cause 8-oxoG lesions in DNA (Kiebassa *et al*, 1997; Douki *et* al, 1999).

Materials and Methods

Cell Lines and Virus Strains: SW480 and LoVo colon adenocarcinoma cells were purchased from the American Type Culture Collection. HCT116-Chr3, and HCT116 human colon adenocarcinoma cell lines were kindly provided by Dr. Thomas Kunkel (National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA). The repair proficient human fibroblast lines GM 9503 and GM 38A, the TCR-deficient cell line GM 739A (CS-B), the NER-deficient cell lines GM 3021A (XP-

G), GM 3615 (XP-D), GM5509 (XP-A), and the transformed XPD-SV40 and XPG-SV40 cell lines were obtained from NIGMS (Camden, NJ). All cell cultures were grown in a humidified incubator at 37 °C in 5% CO_2. Fibroblast and SV40-transformed cell lines were cultured in Eagle's α-minimal essential medium (α-MEM); SW480, HCT116-chr3, and HCT116 carcinomas were cultured in McCoy's modified medium; LoVos were cultured in a 1:1 mixture of D-MEM and F-12 media (with 4500 mg/L glucose). All cell culture media was supplemented with 10% fetal bovine serum and 1% penicillin/ streptomycin. AdHCMV*lacZ* (also called AdCA17*lacZ*, Addison *et al*, 1997) is a non-replicating, recombinant adenovirus containing the *lacZ* gene under the control of the HCMV-IE promoter inserted in the deleted E1 virus gene. Virus stocks were prepared as described previously (Graham and Prevec, 1991).

UVA/UVC Irradiation of Cells and Virus: UVA irradiation was performed using a 1 kW mercury-xenon arc lamp with a 335 nm cut-off filter (Sciencetech Inc., London, Ontario), which effectively reduced UVB transmittance (300-320 nm) to 0.14% (according to manufacturer's specifications). Pretreatment of cells with UVC was performed at a fluence rate of 1 J/m^2/s using a General Electric germicidal lamp (model G8T5) emitting predominantly at 254 nm. Fibroblasts were seeded at a density of 2.0 x 10^4 cells/well in 96-well plates (Falcon, Franklin Lakes, NJ); carcinoma and SV40-transformed cell lines were seeded at 4.0 x 10^4 cells/well. Between 18-24 hours after seeding, media was replaced with 40 µl of PBS. Cells were irradiated at a fluence rate of 135 J/m^2/s and then re-fed with appropriate supplemented growth medium. Irradiation of the virus has been described previously (Bennett and Rainbow, 1988). Briefly, viral

suspensions were prepared in 1.8 ml phosphate buffered saline (PBS) in 35 mm petri dishes on ice. With continuous stirring, virus suspensions were UVA-irradiated at a fluence rate of 110 J/m^2/s. Aliquots of 200 µl were removed following each exposure to the virus and diluted appropriately in unsupplemented α-MEM. UVC and UVA fluence rates were assessed with short-wavelength (model #J225) and long-wavelength meters (model #J221) respectively (Ultraviolet products, San Gabriel, Ca).

UVA HCR of the AdHCMV*lacZ* Reporter Gene: For HCR experiments; untreated and UVA-treated cells were immediately infected (within 30 minutes) with a 40 µl volume of irradiated or non-irradiated virus at a multiplicity of infection (MOI) of 20 pfu/cell. Following viral absorption for 90 mins, cells were re-fed with the appropriate supplemented medium and allowed to incubate at 37 °C for 44 hours prior to harvesting. Following the addition of 1 mM chlorophenolred β-D-galactopyranoside (CPRG; prepared in 0.01% Triton X-100, 1 mM $MgCl_2$, 100 mM phosphate buffer at pH 8.3; Boehringer-Mannheim, Indianapolis, IN) to the infected cells, absorbance readings were taken using a 96-well plate reader (Bio-Tek Instruments EL340 Bio Kinetics Reader) at several time intervals at 570 nm.

UVA-Enhanced Expression of the AdHCMV*lacZ* Reporter Gene: Cells were UVA-irradiated or left untreated (as a control) and subsequently immediately infected with non-irradiated AdHCMV*lacZ*. Following viral absorption for 90 mins, cells were re-fed with the appropriate supplemented medium and allowed to incubate at 37 °C for 44 hours prior to harvesting (as previously described for *UVA HCR* experiments).

UVA Colony Survival Assay: SW480 and LoVo cells were seeded in a 6-well plate (Falcon, Franklin Lakes, NJ) at a density of 400 cells/well. Between 6-8 hours after seeding, media was replaced with 1 ml of PBS and then UVA-irradiated at a fluence rate of 100 J/m^2/s (or left untreated) and then re-fed with 2 ml of the appropriate medium. Cells were then allowed to incubate at 37 °C, 5% CO_2, in a humidified incubator for 9-10 days. After the incubation period, colonies were stained with crystal violet prepared in 10% methanol and 63% ethanol (v/v). Colonies that contained more than 20 cells were counted and analyzed.

Results

HCR of reporter gene expression of UVA-irradiated AdHCMV*lacZ* in MMR and NER proficient and deficient cell lines: Typical survival curves of β-gal activity for UVA-irradiated AdHCMV*lacZ* in *hMSH2*-deficient LoVo cells and their SW480 *hMSH2*-proficient counterparts (left panel) *hMLH1*-deficient HCT116 cells and their HCT116-chr3 *hMLH1*-proficient counterparts (right panel) cells are shown in Fig. 3-1. These results show that only LoVo cells have a significantly reduced HCR compared to SW480 cells, whereas no significant differences were observed between HCT116 and HCT116-chr3 cell lines, suggesting a TCBER deficiency in *hMSH2* but not *hMLH1* cells.

We have previously reported that pretreatment of cells with 12 J/m^2 UVC eliminated any significant differences in HCR capacity between SW480 and LoVo, HCT116-chr3 and HCT116, and GM 9503 and GM 739A (CS-B) cell lines, which is consistent with an upregulation of GGR in the MMR-deficient and NER-deficient strains

(Lee *et al*, 2004). Preliminary HCR results for SW480 and LoVo cells pretreated with UVC or UVA prior to infection with UVA-irradiated AdHCMV*lacZ* are shown in Fig. 3-2. It can be seen that prior treatment of LoVo cells with UVC or UVA increased β-gal activity over non-pretreated controls, although not at levels that were significantly different. In contrast to previous UVC-HCR experiments, none of the pre-treatment conditions here eliminated any HCR differences observed between the SW480 and LoVo cells.

Conditions that were used in the UVA-HCR experiments with regards to the MMR-proficient and deficient colon carcinomas were also employed in various NER-proficient and deficient human fibroblasts (Figs. 3-3 and 3-4). HCR curves of β-gal activity for UVA-irradiated AdHCMV*lacZ* in the normal fibroblast line GM 9503 as well as GM 5509 XP-A fibroblasts were conducted using parameters that differed with respect to viral MOI and the time between viral infection and scoring (Fig. 3-3). It can be seen that at both the 24 and 44 hour time points, viral infections at 40 MOI produced significant decreases in HCR capacity in both cell lines tested. Furthermore, significant differences between the HCR activity of GM 9503 and GM 5509 cell lines was observed at a viral infection of 20 MOI and scoring at 24 hours, and at 40 MOI with viral scoring at 40 MOI.

Further UVA HCR experiments were conducted in additional NER-deficient fibroblasts harboring mutations in the CS-B, XP-G, and XP-D genes as controls, since it has been previously reported that CS-B and XP-G proteins play a role in the repair of oxidative DNA damage (Satoh *et al*, 1993; Le Page *et al*, 2000). Conversely, human

fibroblasts from XP-D patients are efficient in the repair of 8-oxoG (Capelli *et al*, 2000). HCR capacity of the UVA-irradiated virus in CS-B cells was just as efficient as GM 9503 and GM 38A fibroblasts, whereas XP-A and XP-G lines displayed slightly decreased levels of β-gal activity (Fig 3-4). By contrast, XP-D fibroblasts exhibited the lowest HCR capacity of all fibroblasts tested, with significant levels below normals at points corresponding to 132 and 264 kJ/m^2 UVA to virus.

UVA-enhanced expression of the undamaged reporter gene in MMR and NER proficient and deficient cell lines: Fig. 3-5 shows the results for MMR-proficient and deficient colon carcinomas for the UVA-enhanced expression of the undamaged AdHCMV*lacZ* virus in which cells were harvested at multiple time points after immediate infection. At the 12, 24, and 44 hour time points, expression levels were consistently observed at higher levels in LoVo compared to SW480 cells at UVA fluences to cells, once again suggesting a deficiency in the repair of oxidative DNA damage in LoVo compared to SW480 cells (top three panels). In contrast, no significant differences in β-gal expression were observed between HCT116 and its MMR-proficient HCT116-chr3 counterpart in any of the time points observed (bottom 3 panels) except at the point corresponding to the UVA fluence of 400 kJ/m^2 to cells at the 24 hour time point (denoted by *). Nevertheless this generally suggests no TCBER deficiency in the *hMLH1*-deficient HCT116 cell line.

Preliminary results for the enhanced expression assay were also applied to NER-proficient and deficient human fibroblasts are shown in Fig. 3-6. We have previously reported that expression of the undamaged reporter gene is induced at higher levels and at

lower UV fluences to cells in repair deficient cell lines as a result of stalling by RNA polymerase II caused by persistent lesions in active genes. GM 9503 and GM 38A normal fibroblasts exhibit basal levels of β-gal expression at all UVA fluences to cells. At 200 kJ/m^2 UVA however, moderate increases in β-gal activity is observed with CS-B and XP-A cells, where substantial levels of enhanced expression is shown for XP-G and XP-D cells. These findings correlate with the results from the HCR experiments that XP-D cells exhibit the greatest deficiency in the repair of DNA damage caused by UVA.

Clonogenic survival of SW480 and LoVo cells in response to UVA exposure: Survival curves for SW480 and LoVo carcinomas in response to increasing UVA fluences to cells are shown in Fig. 3-7. It can be seen that the presence of a mutant *hMSH2* gene in LoVo cells confers increased resistance to UVA compared to that of the *hMSH2*-proficient SW480 cell line, where significant differences in surviving fraction exist at UVA fluences ≥ 200 kJ/m^2.

Discussion

Cells of many different species have developed a complex network of repair systems in an effort to recognize and remove a wide range of lesions in DNA, which is susceptible to damage by a variety of endogenous and exogenous sources such as cellular metabolism, error-prone DNA replication, radiation, and various chemical agents. Detailed investigations into the elucidation of these pathways have revealed common elements and mechanisms between DNA repair systems that were once thought to be unique from one another. Indeed in the case of BER, there is a growing body of evidence

has shown that the removal of oxidative damage occurs at a more rapid rate in actively transcribing regions of the genome (a mechanism primarily associated with NER), suggesting the existence of transcription-coupled BER mechanism (TCBER; Slupphaug *et al*, 2003; Le Page *et al*, 2002). Furthermore, the involvement of MMR proteins in NER and BER has also been demonstrated in multiple studies (see *Introduction* for specific references).

We have previously reported the use of two reporter gene assays, namely the host-cell reactivation (HCR) and the enhanced expression assay, to show that the detection of the involvement of the human MMR genes *hMLH1* and *hMSH2* in NER is dependent on UVC-fluence to cells (Lee *et al*, 2004). We have also previously reported a reduced host cell reactivation (HCR) for β-gal expression of UVC-irradiated AdHCMV*lacZ* in TCR deficient Cockayne syndrome cells compared to normal human fibroblasts indicating that HCR depends, in part at least, on TCR (Francis and Rainbow, 1999). In addition we have reported that UVC-enhanced expression of the undamaged reporter gene is induced at lower UVC fluences to cells and at higher levels in TCR-deficient compared to normal human fibroblasts indicating that persistent damage in active genes triggers increased activity from the HCMV driven reporter construct (Francis and Rainbow, 2000). Using the same experimental protocols, we were interested in investigating whether or not *hMLH1* and *hMSH2* plays in the BER of oxidative DNA damage induced by UVA, which causes primarily 8-oxoG lesions in DNA (Kiebassa *et al*, 1997, Douki *et al*, 1999). Based on both the HCR and enhanced expression results (Figs. 3-1 and 3-5), it appears that the *hMSH2*-deficient LoVo cells exhibit a deficiency in the removal of oxidative

DNA damage as compared to its *hMSH2*-proficient SW480 counterpart. By contrast, no differences were generally observed in HCR or enhanced expression of the AdHCMV*lacZ* between the *hMLH1*-deficient HCT116 and *hMLH1*-proficient HCT116-chr3 counterpart. Taken together, these results suggest the involvement of *hMSH2* but not *hMLH1* in the removal of oxidative damage (predominantly 8-oxoG) induced by UVA.

This finding is in agreement with results previously published by other groups as well as our own studies where we show an involvement of *hMSH2* in the removal of 8-oxoG damage caused by methylene blue + visible light (Pitsikas and Rainbow, 2004). Since the function of the hMSH2 protein (in the form of the hMSH2-hMSH6 heterodimer hMutSα) is to recognize and bind to base-base mispairs as well as insertion/deletion loops (Friedberg, 2001), it is quite conceivable that other mispairs such 8-oxoG:A, 8-oxoG:G, and 8-oxoG:T serve as "mismatch" binding substrates for hMutSα. Indeed it has been reported that these particular mispairs activate hMutSα through increased binding affinity of the heterodimer for these lesions as well as increases in its associated ATPase and ADP → ATP exchange activities (Mazurek *et al*, 2002).

We have also incorporated the use of various fibroblast cell lines in an effort to correlate the results with the above-mentioned MMR-proficient and deficient carcinomas with cell lines derived from CS-B, XP-A, XP-D, and XP-G complementation groups. Initial HCR experiments with normal and XP-A fibroblasts have revealed an "MOI" effect in which cell lines from both strains and a both 24 and 44 hour time points exhibit relatively lower levels of β-gal activity when infected with UVA-irradiated virus at 40

MOI compared to 20 MOI (Fig. 3-3). This is consistent with the notion that with a higher MOI, the sheer number of UVA-induced lesions in the viral DNA being introduced into the cell is higher, which would result in the commitment of limited cell resources in the HCR of more damaged viral DNA. In addition, we have also conducted UVA-HCR experiments on XPD-SV40 and XPG-SV40 cells, which consistently shown significantly reduced HCR compared to their non-transformed counterparts (results not shown). This is likely due to the ability of the SV40-large T antigen to bind to and abrogate p53, which is another protein shown to be involved in BER (*see section 6.2 for a review*).

Previous reports have suggested that CS-B and XP-G proteins play a role in BER (Satoh *et al*, 1993; Le Page *et al*, 2000), whereas cell lines from XP-A and XP-D individuals exhibit proficient repair of oxidative damage (Adayabalam *et al*, 1999; Cappelli *et al*, 2000). HCR experiments conducted on these cell lines reveal that XP-A and XP-G fibroblasts have relatively lower (although marginal) levels of β-gal activity. Surprisingly, CS-B cells exhibit HCR profiles similar to those of normal fibroblasts, whereas XP-D cells have the lowest HCR activity of all fibroblasts tested (Fig. 3-4). These results are further supported by the preliminary findings of the enhanced expression experiment, in which XP-D cells exhibited substantially higher levels of β-gal expression over basal levels, whereas enhancement of β-gal activity in CS-B cells was only marginal (fig. 3-6). These somewhat unexpected results with regards to XP-D may be due to the differing nature of mutations in the XP-D cell lines used here and by others. Indeed, mutations in XP-D that confer a CS phenotype (XP-D/CS) have been found to be defective in the TCBER of 8-oxoG (LePage *et al*, 2000).

However, due to the nature of UVA and the conditions used in the HCR assay, the results presented from the HCR experiments should be taken in light of the following context. A discrepancy with respect to the actual lesions in the viral DNA produced by UVA exposure may lie in the fact that DNA itself is a weak absorber of UVA. Indeed, DNA absorbs light most efficiently at around 260 nm, whereas the UVA source used here emits wavelengths at 335-400 nm. Furthermore, the presence of photosensitizers is required to mediate UVA-induced DNA damage, since upon exposure to UVA, photosensitizers initiate type I or type II reactions, ultimately leading to the formation of oxidized base lesions such as 8-oxoG (*see Section 1.2 for a review*). Since the UVA-irradiation of the virus takes place in PBS and it is unlikely that the AdHCMV*lacZ* contains any endogenous photosensitizers, further study needs to be conducted on the nature of the UVA-induced lesions in the reporter construct. One possible solution to this is found in a study in which the authors irradiate plasmid DNA in complete RPMI cell culture media, which provided the necessary photosensitizers (Lipinski *et al*, 1999). However, the finding that the UVA-irradiation of cell culture media generates significant amounts of H_2O_2 may complicate matters even further (Mahns *et al*, 2003).

In light of these considerations, the UVA HCR results presents the possibility that the reduced HCR capacity of LoVo (Fig. 3-1), XP-A (Fig. 3-3), and XP-D (Fig. 3-4) may also be due to the formation of CPDs and 6-4PPs. Indeed XP-A cell lines demonstrate extreme sensitivity due to their deficiencies in GGR and TCR subpathways of NER (Crouteau *et al*, 1997), and we and others have previously reported a deficiency of TCR in LoVo carcinomas (Mellon *et al*, 1996; Lee *et al*, 2004). Indeed UVA light (320 – 400

nm) has been documented to induce the formation of CPDs in DNA (Kielbassa *et al*, 1997), perhaps at levels even higher than 8-oxoG (Douki *et al*, 1999; Douki *et al*, 2003). Nevertheless, the spectrum of lesions induced by UVA are significantly different than those produced by UVC, which primarily produces CPDs and 6-4 photoproducts. This is reflected in the differential involvement of *hMLH1* in the repair of UVC induced lesions (Lee *et al*, 2004; Mellon *et al*, 1996), but not in lesions induced by UVA (Figs. 3-1 and 3-5). This is further supported by the inability to increase the UVA-HCR of the β-gal reporter gene in LoVo cells to levels similar to SW480s by pretreatment with UVC (Fig. 3-2), where this effect was otherwise observed in previous UVC-HCR experiments.

Our desire to utilize UVA radiation as a source of producing oxidative DNA lesions, namely 8-oxoG, was due to the clinical implications of the omnipresence of UVA in our environment from the sun, since chronic sunlight exposure is unambiguously associated in increased cancer risk. Surprisingly as well, there are currently no published studies that discuss the involvement of the *hMLH1* and *hMSH2* genes specifically in the repair of UVA-induced DNA damage. In addition to the UVA-HCR and enhanced expression assays already mentioned, we also performed colony survival assays with the SW480 and LoVo cells to test their sensitivity to UVA (Fig. 3-7). The mutant form of the *hMSH2* gene appears to actually confer increased resistance to UVA over the wildtype form. Although initially somewhat counterintuitive, similar trends in colony survival were reported in which *Msh2*$^{-/-}$ mouse embryonic stem cells and fibroblasts exhibited increased survival in response to ionizing radiation compared to their wildtype counterparts, presumably due to a failure to execute apoptosis in response to radiation

exposure (DeWeese *et al*, 1998; Fritzell *et al*, 1997). Indeed the *Msh2* gene was implicated in the prevention of UVB-induced tumorigenesis by facilitating apoptosis and p53 activation (Peters *et al*, 2003). Additionally, there is evidence that hMSH2 protein levels are altered in human non-melanoma skin cancers (Liang *et al*, 2001). These effects may also be occurring in UVA-irradiated LoVo cells, which would potentially have clinical implications for individuals that are genetically deficient in *hMSH2*, resulting in their predisposition to cancer as a result of chronic UVA exposure.

In summary, HCR of β-gal activity for UVA-irradiated AdHCMV*lacZ* virus was significantly reduced in LoVo cells compared to SW480 cells, but not in HCT116 cells compared to HCT116-chr3 cells. As well, enhanced expression of β-gal for untreated AdHCMV*lacZ* virus was significantly greater at lower UVA fluences to cells in LoVo cells compared to SW480 cells but generally not in HCT116 cells compared to HCT116-chr3 cells at all time points tested. Furthermore, the loss of the wildtype *hMSH2* gene confers resistance to UVA exposure, which may be due to failure of the mutant *hMSH2* to execute apoptosis at high UVA fluences to cells. These results suggest an involvement of *hMSH2* but no or substantially less involvement of *hMLH1* in the repair of oxidative damage induced by UVA.

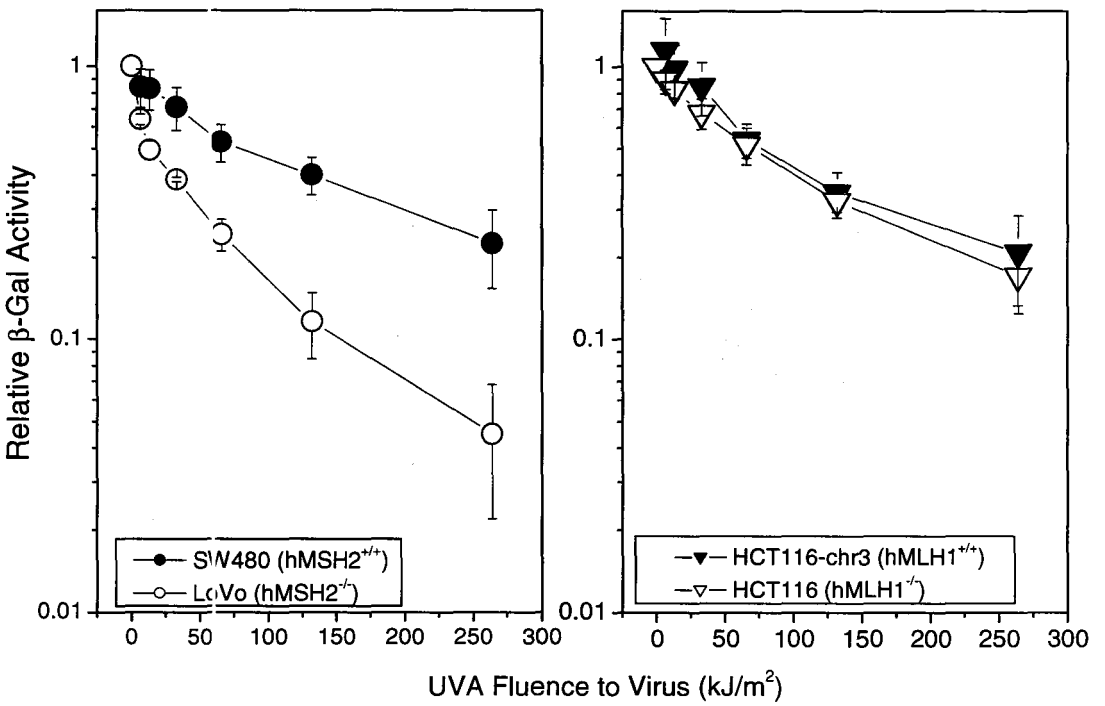

Figure 3-1: HCR of β-galactosidase activity for UVA-irradiated AdHCMV*lacZ* virus in MMR- proficient and deficient carcinomas. Cells were infected immediately after UVA exposure to virus and subsequently harvested 44 hours after infection. Results are shown for SW480 (●), LoVo (○), HCT116-chr3 (▼), and HCT116 (▽). Each point is the average ± SE of three independent experiments, each performed in triplicate.

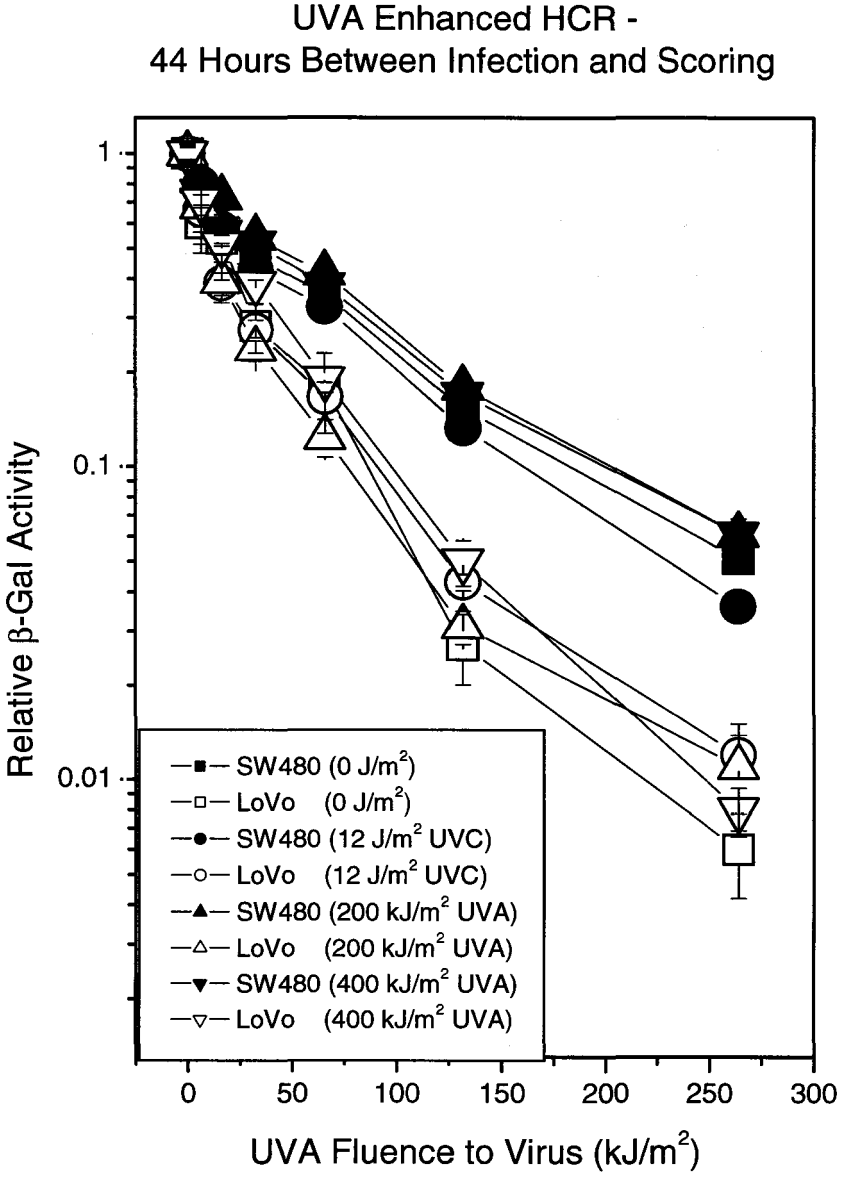

Figure 3-2: HCR of β-galactosidase activity for UVA-irradiated AdHCMV*lacZ* virus in non-pretreated cells (■, □) or cells pre-treated with UVC, 12 J/m^2 (●, ○), UVA, 200 kJ/m^2 (▲, △) or UVA, 400 kJ/m^2 (▼,▽). Filled symbols represent SW480 cells; open symbols represent LoVo cells. Each point is the average ± SE of a single experiment performed in triplicate.

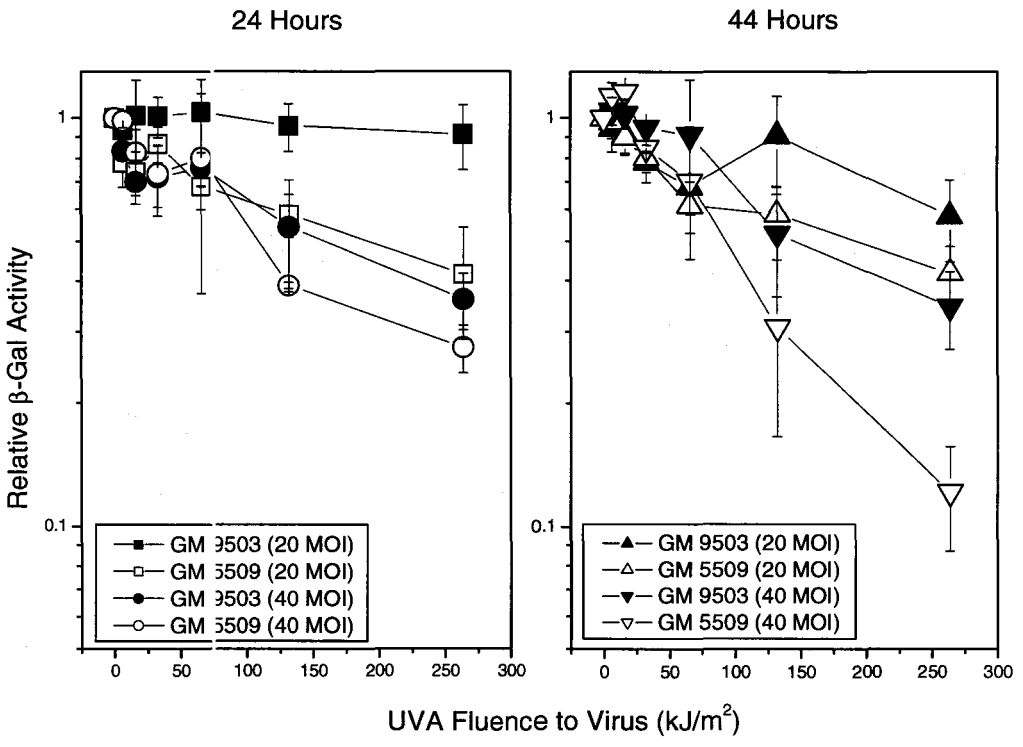

Figure 3-3: HCR of β-galactosidase activity for UVA-irradiated AdHCMV*lacZ* virus in human fibroblasts. Filled symbols represent GM 9503 cells; open symbols represent GM 5509 (XP-A) cells. Seeded fibroblasts were infected by UVA-irradiated virus at 20 or 40 MOI and subsequently harvested at 24 hours (left panel) or 44 hours (right panel) after infection. Each point is the average ± SE of two experiments performed in triplicate.

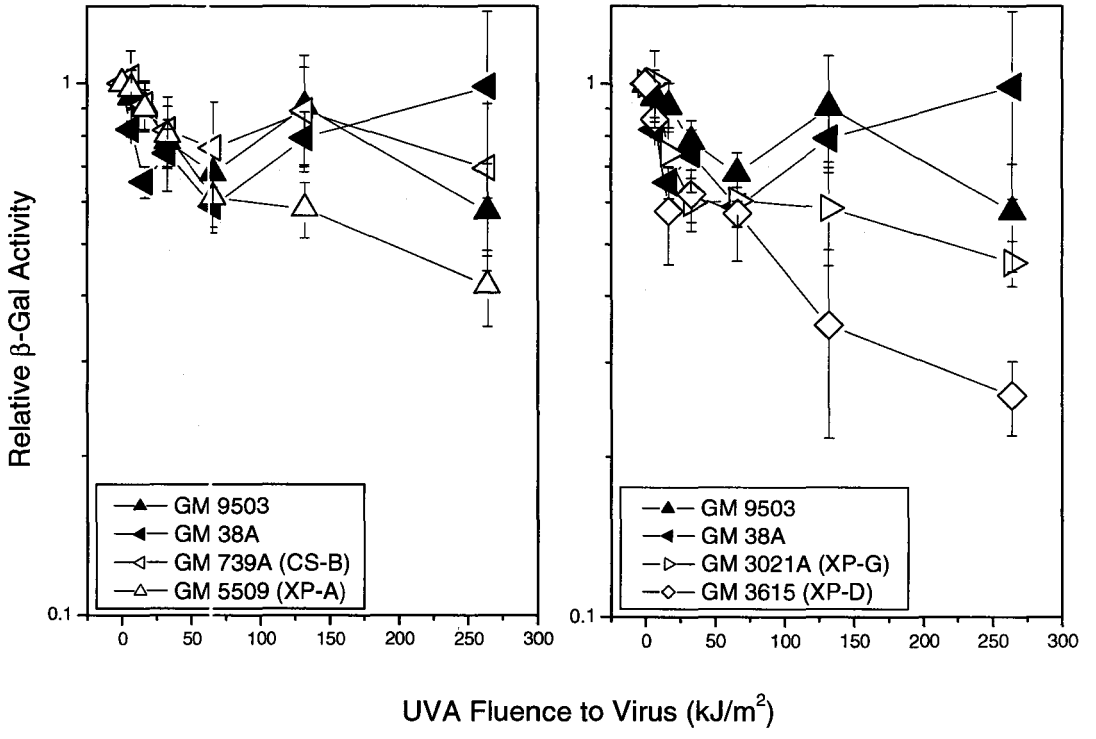

Figure 3-4: HCR of β-galactosidase activity for UVA-irradiated AdHCMV*lacZ* virus in human fibroblasts. Filled symbols represent repair proficient GM 9503 (▲) and GM 38A (◀) cells; open symbols represent NER-deficient GM 739A (◁), GM 5509 (△), GM 3021A (▷), and GM 3615 (◇). HCR curves for GM 9503 and GM 38A have been plotted in both panels for comparison. Seeded fibroblasts were infected by UVA-irradiated virus at 20 MOI and subsequently harvested at 44 hours after infection. Each point is the average ± SE of two to six experiments, each performed in triplicate.

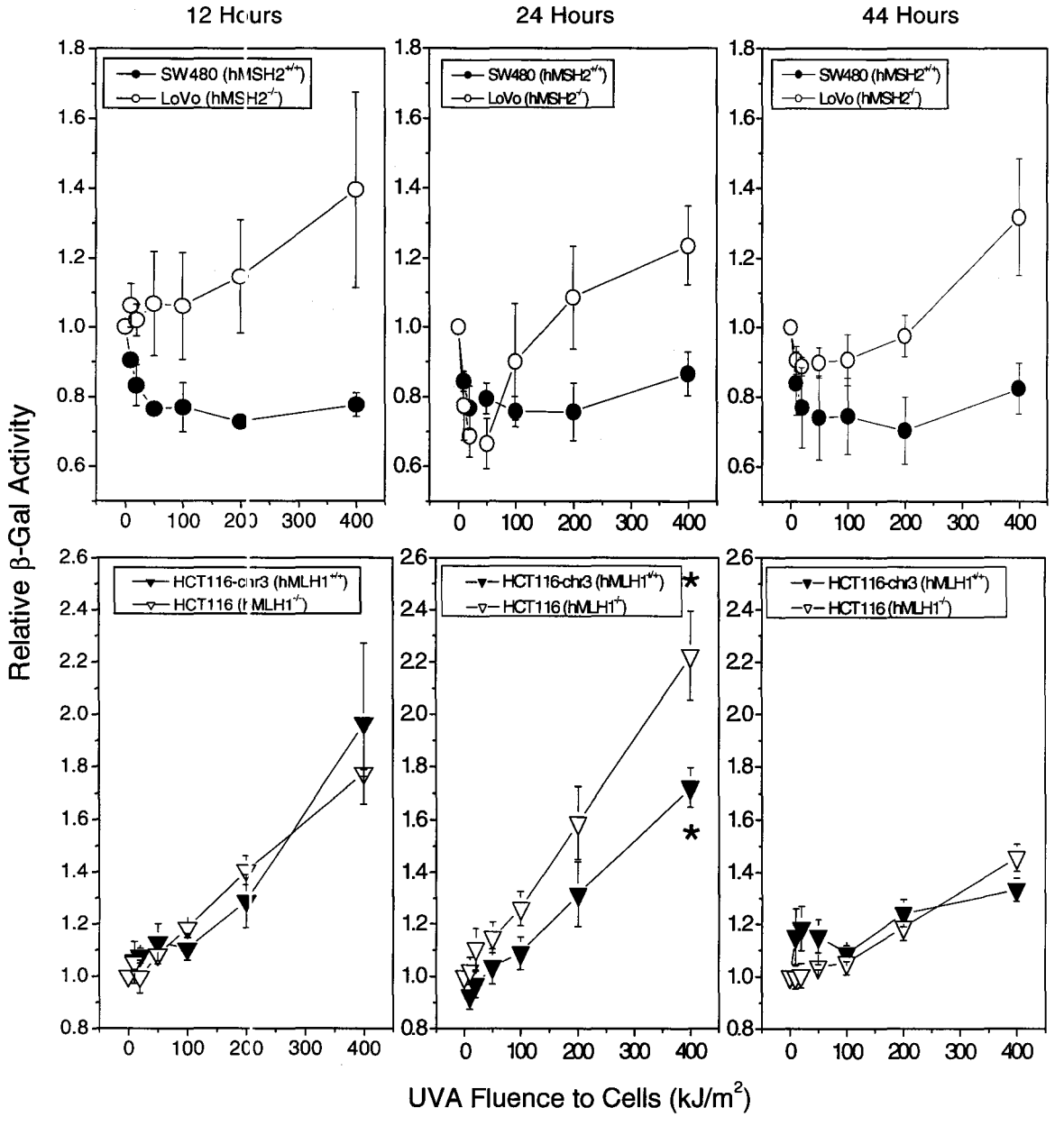

Figure 3-5: Enhanced expression of β-galactosidase in UVA-irradiated colon carcinoma cell lines following infection with un-irradiated AdHCMV*lacZ* virus. Cells were infected immediately after UVA irradiation and subsequently harvested at 12 hours (left panels), 24 hours (center panels), or 44 hours (right panels) after infection. Results are shown for SW480 (●), LoVo (○), HCT116-chr3 (▼), and HCT116 (▽). Each point is the average ± SE of two to four independent experiments, each performed in triplicate.

* significantly different by two sample independent *t*-test ($P < 0.05$).

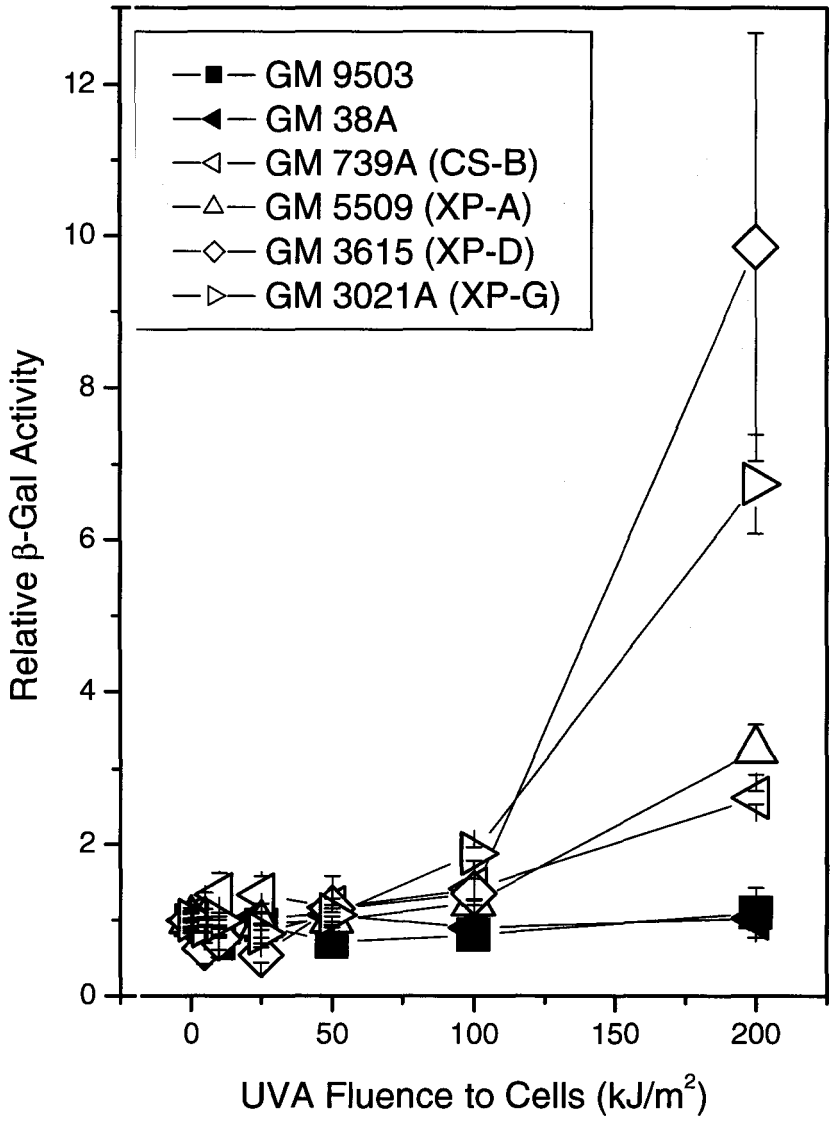

Figure 3-6: Enhanced expression of β-galactosidase in UVA-irradiated human fibroblasts following infection with un-irradiated AdHCMV*lacZ* virus. Cells were infected immediately after UVA irradiation and subsequently harvested 44 hours after infection. Results are shown for GM 9503 (▲), GM 38A (◀), GM 739A (◁), GM 5509 (△), GM 3021A (▷), and GM 3615 (◇). Each point is the average ± SE of a single experiment performed in triplicate.

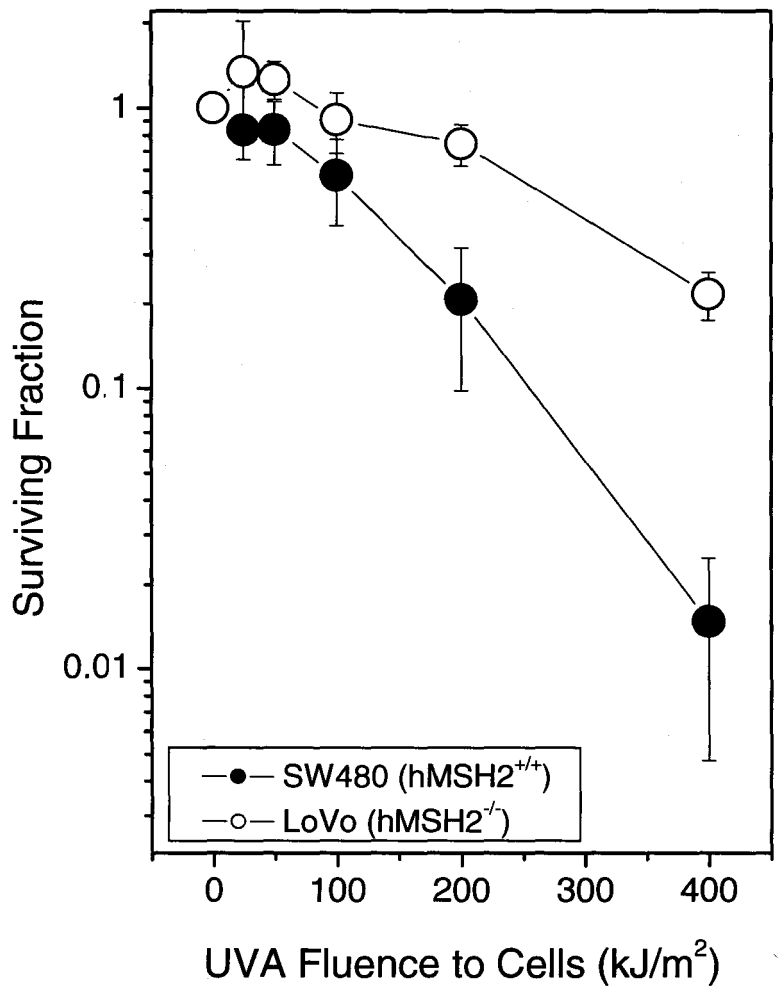

Figure 3-7: Clonogenic survival of SW480 (●) and LoVo (○) cells in response to increasing levels of UVA exposure at a fluence rate of 100 J/m^2/s. Data is normalized to non-irradiated controls. Each point is the average ± SE of three to four independent experiments, each performed in triplicate.

Chapter 4

The Role of *hMLH1* and *hMSH2* in the Repair of H_2O_2-Induced DNA Damage

Abstract

DNA mismatch repair (MMR) genes recognize and repair bases incorrectly incorporated during DNA replication. Germ line mutations in two MMR genes, namely *hMSH2* and *hMLH1*, account for approximately 98% of hereditary non-polyposis colorectal cancers. We and others have reported that cells defective in *hMSH2* or *hMLH1* show a deficiency in the transcription-coupled repair (TCR) for DNA damage induced by UVC, indicating their involvement in this pathway. Here we have used two reporter gene assays to examine the role of *hMSH2* and *hMLH1* in the repair of oxidative DNA damage induced by H_2O_2. H_2O_2 produces predominantly 8-hydroxyguanines (8-oxoG) and thymine glycols (Tg) in DNA via the Fenton reaction with transition metal ions; both lesions are repaired by the base excision repair (BER) pathway. AdHCMV*lacZ* is a replication-deficient recombinant adenovirus that expresses the β-galactosidase (β-gal) reporter gene under the control of the human cytomegalovirus immediate-early promoter. In contrast with previous experiments that used UVC or UVA as the DNA damaging agent, we show no detectable differences in HCR for β-gal expression of UVA-treated AdHCMV*lacZ* in *hMSH2*-deficient LoVo human colon adenocarcinoma cells compared to their *hMSH2*-proficient counterpart SW480 cells. We also do not observe any differences in HCR for *hMLH1*-deficient HCT116 human colon adenocarcinoma cells compared to the *hMLH1*-proficient HCT116-chr3 cells. Based on these results, the lack of significant differences between these cell lines are not likely a reflection of MMR gene involvement, but rather a result of H_2O_2 induced damage to the viral protein coat. We have also reported previously that enhanced expression of the undamaged AdHCMV*lacZ*

reporter gene is induced by the pre-treatment of cells with lower levels of the DNA-damaging agent and to higher expression levels in TCR-deficient compared to TCR-proficient cells. Likewise, no significant differences were observed between SW480 and LoVo cells, as well as HCT116-chr3 compared to HCT116 cells in the enhanced expression assays or in H_2O_2 clonogenic survival experiments. These findings suggest that neither *hMSH2* nor *hMLH1* play a significant role in the repair of oxidative damage induced by H_2O_2.

Introduction

Intracellular production of hydrogen peroxide (H_2O_2), as well as the superoxide anion ($O_2^{\cdot -}$) and the hydroxyl radical ($OH^{\cdot}$), arise as by-products during the reduction of O_2 to H_2O in aerobic respiration. Since these ROSs are capable of producing oxidized and deaminated bases in DNA, cells have developed specialized proteins to serve in three general lines of defense to counteract the constant induction of DNA base damage. The first is the removal of $O_2^{\cdot -}$ and H_2O_2 by superoxide dismutase and catalase respectively, which converts these molecules to O_2 and H_2O (Madigan *et al*, 2000). The second line of defense is the hydrolysis of oxidized dNTP's in the nucleotide pool, such 8-oxoGTP, to their corresponding dNMP's, thus preventing their incorporation into DNA during replication (Slupphaug *et al*, 2000). Finally, if oxidized bases are incorporated or induced in DNA, then the BER pathway (as well as components of NER and MMR) serves to remove the affected bases.

H_2O_2 represents one of the most stable ROSs within the cell, as it crosses cell and organelle membranes easily and it hardly reacts with DNA, if at all (Wiseman *et al*, 1999). Rather, it is the more reactive $OH^{\cdot}$ that arises from the breakdown of H_2O_2 by the Fenton reaction that causes oxidative damage in DNA, since $OH^{\cdot}$ reacts quickly and indiscriminately with almost anything (Demple and Harrison, 1994). One of the most commonly produced lesions in this process is the thymine glycol, which has been shown in NMR studies to induce significant localized distortions in the duplex DNA (Kao *et al*, 1993). This perturbation in the double helix is thought to serve as a replication block to DNA polymerases. Thus thymine glycols, although possessing low mutagenic potential (unlike 8-oxoG, which is highly mutagenic), is nevertheless cytotoxic because of it's ability to stall the DNA replication fork, resulting in lethal double strand breaks (Wilson *et al*, 2003).

There have been previous reports suggesting possible links between H_2O_2 exposure to cells and its effect on the various components on the MMR system. H_2O_2 was shown to induce a more prolonged G2/M arrest in MMR-deficient carcinomas compared to their MMR-proficient counterparts (Chang *et al*, 2003). It was also reported that low levels of oxidative stress induced by H_2O_2 exposure damaged the hMutSα, hMutSβ, and hMutLα heterodimers, resulting in their functional inactivation (Chang *et al*, 2002). Furthermore, it was found that the MLH1 protein was required for the induction of apoptosis following cytotoxic treatment by H_2O_2 (Yanamadala and Ljungman, 2003). In spite of all the studies mentioned here, there are presently no published reports dealing with the possible involvement of the MMR genes *hMLH1* or *hMSH2* in the detection or

repair of thymine glycols in cellular DNA, which is one of the principal targets of H_2O_2 (via $OH^{\cdot}$ production). Thus in utilizing the HCR and enhanced expression assays as previously mentioned, we were interested in investigating whether or not *hMLH1* or *hMSH2* were required in the repair of oxidative lesions produced by H_2O_2, which may shed additional light with respect to how MMR proteins mediate H_2O_2-induced cytotoxicity.

Materials and Methods

Cell Lines and Virus Strains: Cell lines used were the same and maintained in the same manner as described in *Materials and Methods* for UVA experiments (see page 75) In addition, SV40-transformed GM637F normal cells and CSB-SV40 cells were obtained from NIGMS (Camden, NJ). AdHCMV*lacZ* (also called AdCA17*lacZ*) virus stocks were prepared as described previously.

H_2O_2 treatment and HCR of the AdHCMV*lacZ* Reporter Gene: H_2O_2 solutions were made fresh from a 30% H_2O_2 stock (EM Biosciences). Briefly, a 3% diluted H_2O_2 solution (0.99235M) was prepared in sterile ddH_2O, filtered sterilized (0.2 µl filter), and serially diluted appropriately in sterile ddH_2O. For H_2O_2 treatment of the AdHCMV*lacZ* virus, 2.4 µl of the virus stock (at 10^{10} pfu/ml) was added to 17.6 µl of cold PBS and 20 µl of the appropriate H_2O_2 concentration. After incubation at 37 °C for 30 minutes (with gentle vortexing every 10 minutes), virus samples were diluted to a total volume of 1.2 ml with unsupplemented α-MEM. In some experiments, catalase (700 units ml, Sigma Aldrich) was added to virus samples in order to eliminate H_2O_2).

Fibroblasts were seeded at a density of 2.0 x 10^4 cells/well in 96-well plates (Falcon, Franklin Lakes, NJ); carcinoma cell lines were seeded at 4.0 x 10^4 cells/well. Between 18-24 hours after seeding, media was replaced with 40 µl of the diluted H_2O_2-treated virus at an MOI of 20 pfu's/cell. Following viral absorption for 90 mins, cells were re-fed with the appropriate supplemented medium and allowed to incubate at 37 °C for 24 or 44 hours prior to harvesting. Following the addition of CPRG to the infected cells, absorbance readings were taken using a 96-well plate reader (Bio-Tek Instruments EL340 Bio Kinetics Reader) at several time intervals at 570 nm.

H_2O_2 treatment of cells and Enhanced Expression of the Untreated AdHCMV*lacZ* Reporter Gene: Between 18-24 hours after seeding, media was replaced with 40 µl of various H_2O_2 concentrations (prepared in PBS) and was left for 30 min at 37 °C. An additional 200 µl of unsupplemented α-MEM was then added to each well (to dilute and rinse H_2O_2). The entire well contents were then aspirated and then re-fed with another 200 µl of the corresponding supplemented cell media. Cells were subsequently infected with non-treated AdHCMV*lacZ* virus immediately following H_2O_2 treatment to cells, and cells were harvested at 44 hours following viral infection.

H_2O_2 Colony Survival Assay: SW480, LoVo, HCT116-chr3, and HCT116, GM 637F, and CSB-SV40 cell cultures were trypsinized and counted using a hemocytometer. In sterile 15 ml tubes, suspended cell cultures were diluted to 5 x 10^5 cells/2 ml in unsupplemented α-MEM. Cells were treated with 2 ml of the appropriate H_2O_2 concentration (prepared in sterile ddH_2O) and were left to incubate at 37 °C for 30 minutes (with gentle vortexing every 10 minutes). Cells were then centrifuged at 1000

rpm for 10 minutes and the media was aspirated from the pellet in order to remove H_2O_2 from the cells. The cell pellet was re-suspended and diluted in appropriate supplemented media. Cells were then seeded in a 6-well plate (Falcon, Franklin Lakes, NJ) at a density of 400 cells/well and allowed to incubate at 37 °C, 5% CO_2, in a humidified incubator for 7 days. After incubation, colonies were stained with crystal violet prepared in 10% methanol and 63% ethanol (v/v). Colonies that contained more than 20 cells were counted and analyzed.

Results

HCR of reporter gene expression of H_2O_2-irradiated AdHCMV*lacZ* in MMR and NER-proficient and deficient cell lines: HCR survival curves of β-gal activity for AdHCMV*lacZ* treated with high doses of H_2O_2 for *hMSH2*-deficient LoVo cells and their SW480 *hMSH2*-proficient counterpart (left panels), as well as the normal GM 9503 and CS-B deficient GM 739A human fibroblasts (right panels) are shown in Fig. 4-1. There appears to be an increased HCR of β-gal activity in GM 9503 cells compared to GM 739A's when H_2O_2 doses to virus increased as a function of H_2O_2 concentration (Fig. 6, left panel). However no significant differences in HCR were observed between SW480 and LoVo cells when increased dose to virus was performed as a function of H_2O_2 concentration (Fig. 4-1A) or exposure time (Fig. 4-1B). Interestingly, the SW480, LoVo, and GM 739A cell lines all experienced a sharp decline in β-gal activity at lower H_2O_2 doses with subsequent recovery in HCR at moderate to higher doses.

Similar HCR experiments with lower doses of H_2O_2 were conducted on MMR-proficient and deficient carcinomas as well as NER-proficient and deficient fibroblasts (representative results shown in Fig. 4-2). It appears that treatment of the AdHCMV*lacZ* with lower H_2O_2 doses (max 200 μM H_2O_2 or 400 μM at a max. time of 60 mins) produced more appropriate HCR curves in all the cell lines tested. Nevertheless no significant differences in HCR activity were observed between the HCT116-chr3 and HCT116 cell lines, or between the SW480 and LoVo cell lines, although LoVo appears to exhibit significantly greater β-gal activity that SW480 at the 60-minute point. However, points corresponding to 60 minutes H_2O_2 exposure (denoted by *) have relative β-gal activities less than twice of that of background values. Thus their corresponding rate in absorbance increase is likely due in large part to the spontaneous breakdown of the CPRG substrate and thus would not be true indicators of actual viral reporter β-gal activity (Fig. 4-2A). Likewise, no substantial differences in HCR were observed in any of the fibroblast lines used (Fig. 4-2B).

H_2O_2-enhanced expression of the undamaged reporter gene in MMR- and TCR- proficient and deficient cell lines: Relative β-gal expression following infection of undamaged AdHCMV*lacZ* was examined in normal fibroblasts (GM 9503 and GM38A) as well as NER-deficient fibroblasts (Fig 4-3A). It can be seen that the XP-D and XP-G fibroblasts exhibit relatively low β-gal expression levels (right panel), whereas the CS-B, XP-A, and interestingly even the normal GM 9503 cell line all show substantial increases in β-gal activity at 200 μM H_2O_2 to cells. The GM 38A also experiences moderately higher β-gal expression over basal levels (~ 6 fold), but this peak

occurs at a higher H_2O_2 dose of 400 μM to cells. Figure 4-3B represents values extrapolated from graphs from individual enhanced expression experiments, corresponding to H_2O_2 doses required to illicit a 3-fold enhancement in β-gal expression in the respective fibroblast line. In agreement with the results in Fig. 4-3A, the normal fibroblast GM 9503 was able to enhance the expression of the reporter construct by 3 fold with only 75 μM H_2O_2, with XP-A and CS-B also requiring relatively low H_2O_2 doses to produce this particular effect. Similarly higher levels of required H_2O_2 concentrations were observed in the XP-G, XP-D, and the GM 38A normal fibroblast.

Equivalent enhanced expression assay conditions were also applied to the MMR-proficient SW480 and HCT116-chr3, and their MMR-deficient counterparts LoVo and HCT116 (Fig. 4-4). In contrast to the results derived from the fibroblasts, none of the carcinoma lines ever displayed any enhancement of β-gal expression that reached three fold over basal levels. Even though LoVo cells had slightly greater β-gal activity than SW480s at corresponding H_2O_2 doses to cells, these differences were not significant, nor were there any discernible differences in β-gal expression between HCT116-chr3 cells compared to their HCT116 counterpart.

Clonogenic survival of SV40 transformed and MMR-proficient and deficient carcinomas in response to H_2O_2 exposure: Survival curves in response to increasing H_2O_2 concentrations for GM 637F and CSB-SV40 transformed fibroblasts are shown in Fig. 4-5, and those for SW480, LoVo, HCT116-chr3, and HCT116 carcinomas are shown in Fig. 4-5. It can be seen that the TCR-deficient CSB-SV40 line is significantly more sensitive to H_2O_2 than it's GM 637F normal transformed counterpart at the

concentrations tested. By contrast, no significance difference in H_2O_2 clonogenic survival is observed between SW480 and LoVo cells or for HCT116-chr3 and HCT116 carcinomas.

Discussion

To test whether or not the role of *hMSH2* in BER was specific to UVA radiations (and thus 8-oxoG DNA lesions), we have employed the utilization of the HCR and enhanced expression assays using H_2O_2 as the damaging agent to virus and cells respectively, since H_2O_2 has been previously used to produce thymine glycols in cellular DNA (Kaneko *et al,* 1987; Cooper *et al*, 1997; Alanazi *et al*, 2002). However, a study using gas chromatography / mass spectrometry with selected-ion monitoring (GC / MS-SIM) found that after 2 mM of H_2O_2 exposure to mammalian cells, the yield of 8-oxoG was the highest of the 10 detectable lesions (~ 75 µmol lesion/mol of DNA). This value is significantly higher than that detected of Tg (~ 5 µmol lesion/mol of DNA) (Dizdaroglu *et al*, 1991). There are however likely to be differences in the spectrum of lesions produced by UVA and H_2O_2 in that UVA has a greater tendency to induce the formation of base lesions by a type I reaction, which would lead to the production of singlet oxygen species ($^1O_2$), which in turn reacts almost exclusively with guanine bases to produce 8-oxoG (Ravanat *et al*, 2001). H_2O_2, on the other hand, mimics a type II photoreaction through its decomposition into the more reactive $OH^{\bullet}$, which produces a wide range of oxidized lesions, including 8-oxoG and to a lesser extent Tg. Nevertheless, the differential involvement of *hMSH2* in response to oxidative stress induced by UVA

and H_2O_2 may not be exclusively due to the nature DNA lesions produced, since they both produce significant amounts of 8-oxoG. Rather, the MMR system as a whole may play other roles in mediating H_2O_2-induced oxidative stress, considering that H_2O_2 was found to induce a more prolonged G2/M cell cycle arrest in MMR-deficient compared to proficient cells (Chang *et al*, 2003). Additionally, p53 was shown to specifically interact with hMLH1 in mediating cytotoxicity and mutagenicity to H_2O_2 (Lin *et al*, 2000).

Initial experiments involving the HCR of the H_2O_2-damaged AdHCMV*lacZ* by MMR-proficient and deficient carcinomas, as well as TCR-proficient and deficient fibroblasts yielded unexpected HCR curves. The sharp decline in β-gal activity at lower H_2O_2 doses with subsequent recovery in HCR at moderate to higher doses was also a phenomenon first reported in a study where λ-bacteriophage was exposed to H_2O_2, allowed to infect plated *E. coli*, and monitored for plaque forming ability (Imlay *et al*, 1988). These authors have thus proposed a *two-mode H_2O_2 killing* model, in which the "mode-one" killing occurs at low/moderate H_2O_2 concentrations, and the rate of killing is essentially independent of H_2O_2 concentration but first-order with respect to exposure time (Imlay *et al*, 1987). This mode-one of H_2O_2 killing would likely correspond to the sharp decline in β-gal activity at lower H_2O_2 doses, where "mode-two" would be associated with the subsequent increase in HCR. Nevertheless, the lack of significant differences in any of the HCR curves in any of the MMR/NER-proficient and deficient cell lines may not actually be reflective of the ability of all of these cell lines to execute (equally) efficient removal of H_2O_2-induced lesions in DNA. Rather this is likely to be an indication that it is actually the viral protein coat that is being damaged upon H_2O_2

treatment of AdHCMV*lacZ*, since H_2O_2 (in the form of the hydroxyl radical) can cause protein fragmentation (Carmichael *et al*, 1992). This would effectively reduce adenoviral absorption (and thus infectivity) in a manner similar for all cell lines. Thus the HCR assay does not appear to be a viable method in testing gene involvement of H_2O_2-induced DNA damage.

By contrast, the enhanced expression of the undamaged reporter gene by the pretreatment of cells with H_2O_2 remains an effective assay in measuring BER deficiencies in cells, since H_2O_2 is able to cross cell membranes easily (Wiseman *et al*, 1996). Indeed a deficiency in the removal of H_2O_2 was observed in CS-B-deficient fibroblasts as indicated by its enhanced expression of β-gal levels compared to GM 38A normals (Fig. 4-3A), which isn't surprising since the CS-B protein has been shown to play a role in BER (Le Page *et al*, 2000). This notion is further supported by the finding that CSB-SV40 fibroblasts exhibit marked sensitivity to H_2O_2 compared to its normal counterpart GM 637F (Fig. 4-5). What is surprising however is the apparently significant deficiency of XP-A and the normal GM 9503 cell line in response to H_2O_2-induced DNA damage as determined by the enhanced expression experiments (Fig. 4-3) There is also the possibility the H_2O_2 may exert effects on cells not related to DNA damage, since H_2O_2 has been implicated as a redox messenger in various cell signaling pathways (*see Section 2.3 for a review*). Support for a possible role of XP-A in BER may come from a study that found a deficiency in XP-A lymphoblasts to repair cellular DNA damaged by UVA-containing fluorescent light (Lipinski *et al*, 1999). However, the fact that the GM 9503 required the least H_2O_2 concentration to induce a 3-fold expression of β-gal (Fig. 4-

3B) is somewhat counterintuitive, since this apparent deficiency in DNA repair defies the meaning of a "normal" cell line. Nevertheless, emerging evidence has revealed a growing number of polymorphisms in various DNA repair genes (Dusinska *et al*, 2004). Indeed even the "normal" population varies over about a 2-fold range of NER capacities (Smith and Seo, 2002).

Differences in enhanced β-galactosidase expression between respective MMR-proficient and deficient cell lines were undetectable. Coupled with the apparent absence of sensitivities to H_2O_2 between the respective MMR-proficient and deficient carcinomas (Fig. 4-6), there doesn't appear to be any role for *hMSH2* or *hMLH1* in mediating DNA damage or cytotoxicity as determined by the enhanced expression and H_2O_2 colony survival assays.

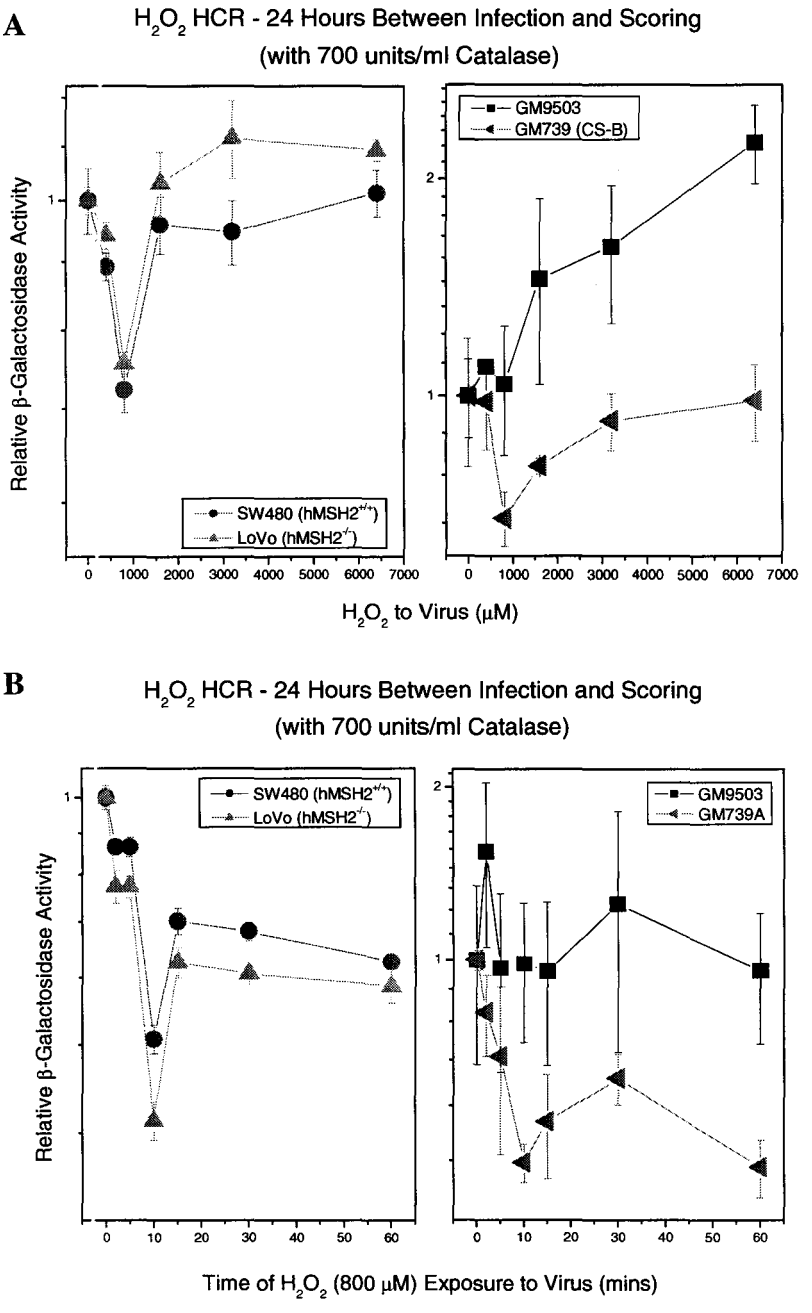

Figure 4-1: HCR of β-galactosidase activity for AdHCMV*lacZ* virus treated with high doses of H_2O_2. Relative β-gal activities are plotted as a function of increasing H_2O_2 concentrations to virus (**A**) and increasing virus exposure times to a fixed H_2O_2 concentration of 800 μM (**B**). Results are shown for SW480 (●), LoVo (▲), GM 9503 (■), and GM 739A (◀). Each point is the average ± SE of a single experiment performed in triplicate.

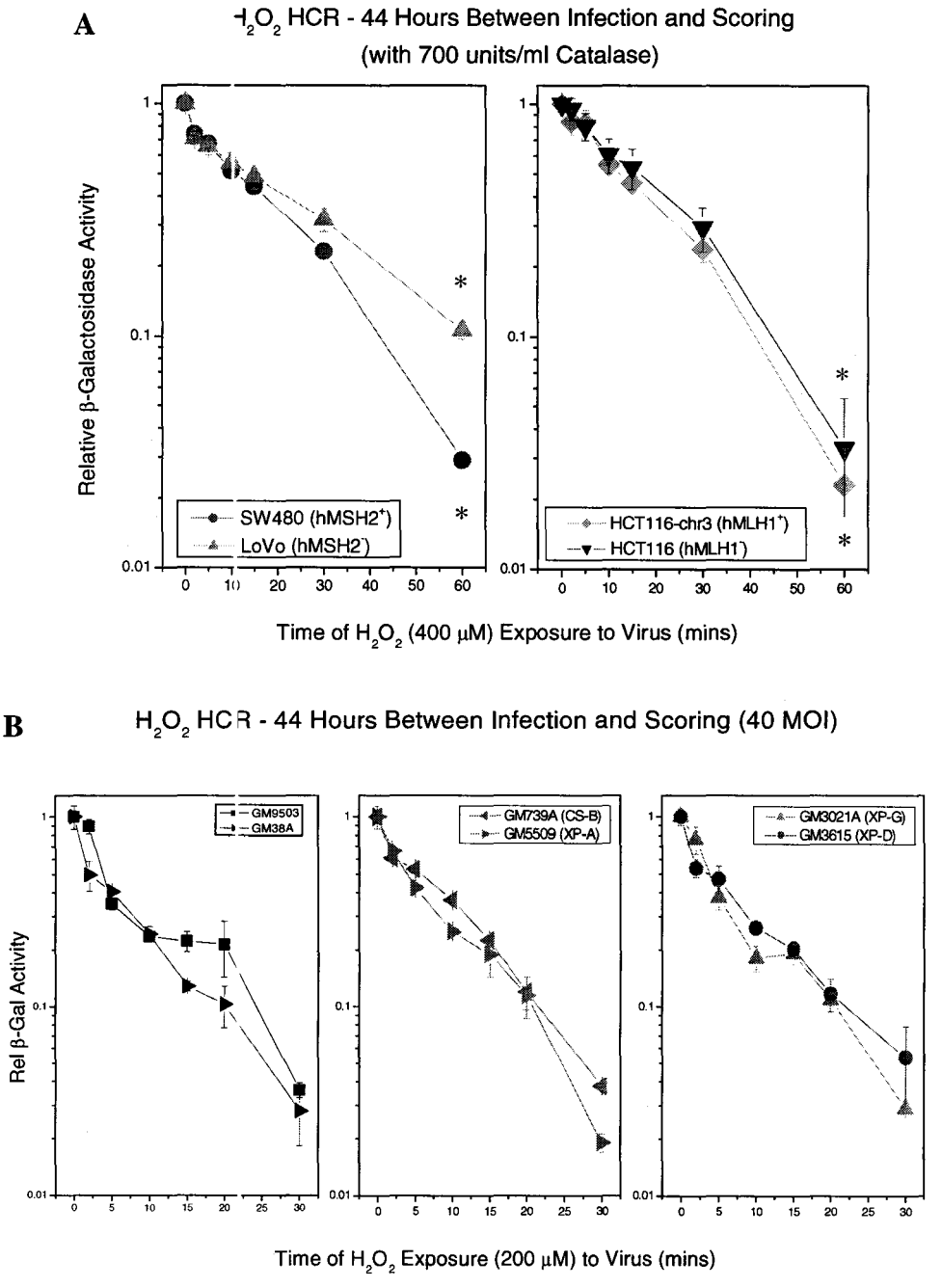

Figure 4-2: HCR of β-galactosidase activity for AdHCMV*lacZ* virus treated with low doses of H_2O_2. (**A**) HCR curves for MMR-proficient and deficient carcinomas. The points corresponding to 60 minutes H_2O_2 exposure (denoted by *) have relative β-gal activities less than twice of that of background values. (**B**) HCR curves for NER-proficient deficient fibroblasts. Each point is the average ± SE of a single experiment performed in triplicate.

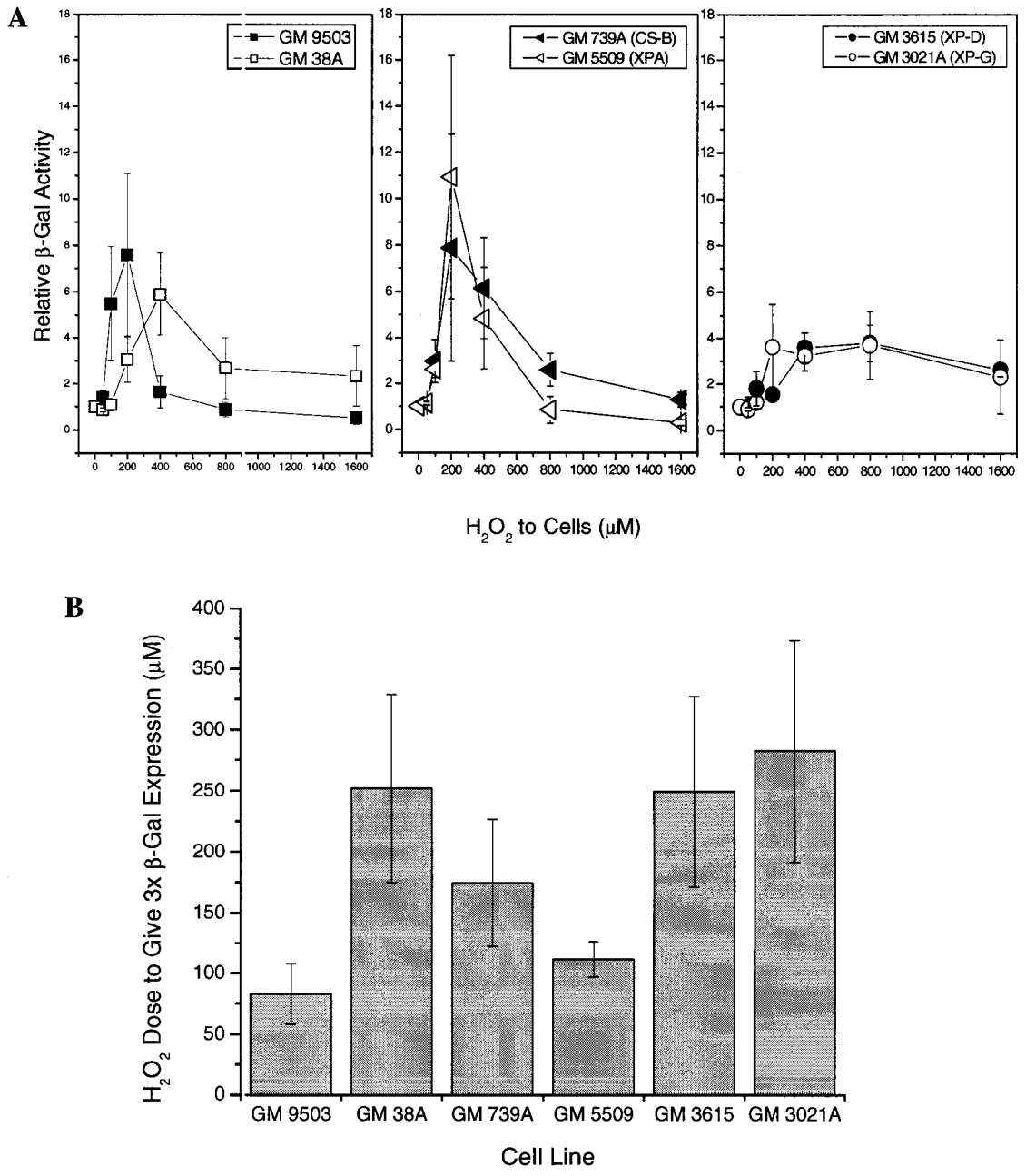

Figure 4-3: (**A**) Enhanced expression of β-galactosidase in H_2O_2-treated human fibroblasts following infection with untreated AdHCMV*lacZ* virus. Cells were immediately infected (within 30 mins) after cellular H_2O_2 treatment and subsequently harvested 44 hours after infection. Each point is the average ± SE of three to four independent experiments, each performed in triplicate. (**B**) H_2O_2 doses required to give 3-fold expression of β-Gal were extrapolated from enhanced expression curves and plotted as a function of the corresponding cell line. Error bars: ± SE.

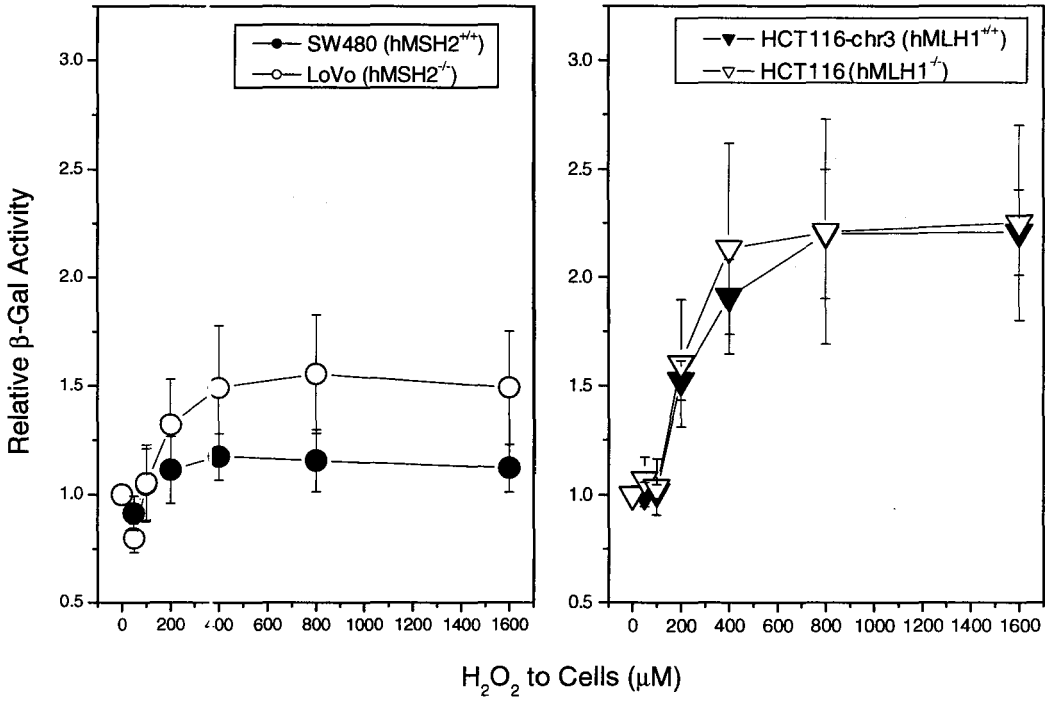

Figure 4-4: Enhanced expression of β-galactosidase in H_2O_2-treated MMR proficient and deficient colon adenocarcinoma cell lines following infection with untreated AdHCMV*lacZ* virus. Cells were immediately infected (within 30 mins) after cellular H_2O_2 treatment and subsequently harvested 44 hours after infection. Each point is the average ± SE of three to five independent experiments, each performed in triplicate.

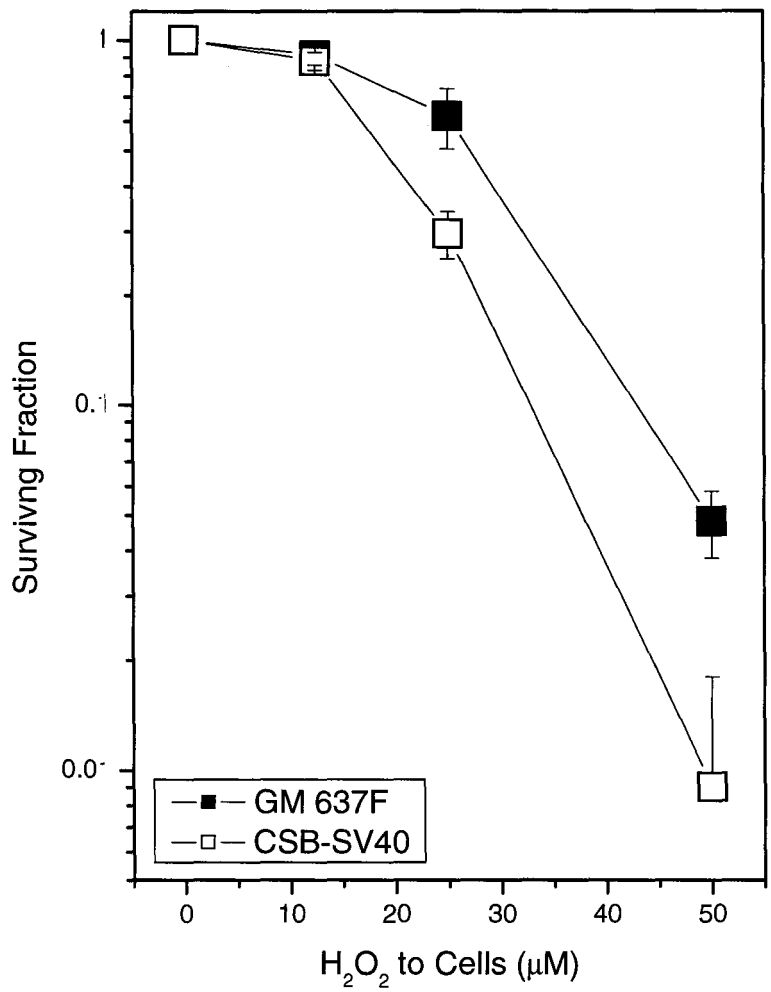

Figure 4-5: Clonogenic survival of GM 637F and CSB-SV40 transformed fibroblasts in response to increasing levels of H_2O_2 exposure for 30 mins at 37 °C. Cell suspensions were treated with H_2O_2 prior to seeding. Data is normalized to mock-treated controls. Each point is the average ± SE of three independent experiments, each performed in triplicate.

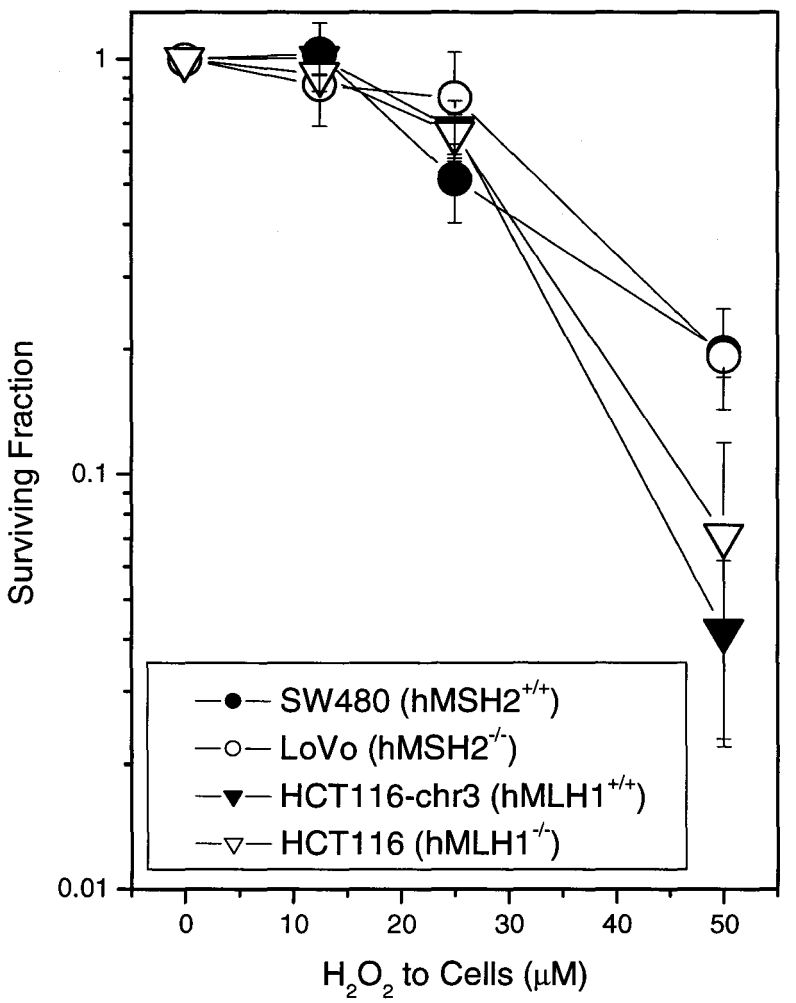

Figure 4-6: Clonogenic survival of MMR-proficient and deficient colon adenocarcinoma cell lines in response to increasing levels of H_2O_2 exposure for 30 mins at 37 °C. Cell suspensions were treated with H_2O_2 prior to seeding. Data is normalized to mock-treated controls. Each point is the average ± SE of three independent experiments, each performed in triplicate.

Chapter 5

Involvement of p53 in Host Cell Reactivation

Abstract

The involvement of the p53 tumor suppressor protein in NER, specifically the GGR sub-pathway, has been well established, whereas a controversy surrounds its role in TCR. Here, we examined the effects of the HCR of a UVC damaged-AdHCMV*lacZ* virus on p53 expression levels in HCT116 adenocarcinomas, which are deficient in *hMLH1* but wildtype for p53 ($p53^{+/+}$). Increases in p53 expression were observed when the virus were exposed to 300 and 600 J/m^2 of UVC, indicating that UVC-induced DNA lesions alone can up-regulate p53 expression. Additional experiments have also yielded increases in p53 expression in HCT116 cells upon infection of undamaged AdHCMV*lacZ* virus. However these results were not reproducible, suggesting the presence of high biological variation or the involvement of other factors that may mediate the response of p53 to AdHCMV*lacZ* infection.

Introduction

The p53 tumor suppressor has been known as an important protein associated with cancer prevention, considering that it is the most commonly altered gene in cancer (McKay *et al*, 1999). This particular protein is intimately involved in multiple biochemical pathways aimed at preventing neoplastic transformation in mammalian cells. This is generally accomplished through enhancing DNA repair, arrest in cell cycle progression, or the induction of apoptosis (Scherer *et al*, 2000). Increased expression and stabilization of the p53 gene product typically occurs after DNA damage by chemical mutagens or by radiation such as UV exposure, suggesting that p53 plays a role in a cellular system involved in genome surveillance. Evidence has also shown that p53 may also assume a functional role in NER, especially in GGR (Ford and Hanawalt, 1997), whereas its role in TCR is more controversial. However, there is evidence that suggests an involvement of p53 in the preferential repair of actively transcribing genes in mammalian cells. Cells from individuals afflicted with Li-Fraumeni syndrome (LFS), which have mutated p53 in both alleles (and consequently a pre-disposition to cancer), had significantly reduced capacities to support HCR of a UV-damaged reporter gene in adenoviruses (McKay *et al*, 1999). Also, it has been recently shown that p53 assumes a protective role against UV-induced apoptosis in human fibroblasts proficient in TCR (McKay *et al*, 2001). In an interesting recent development, it was also found that in p53-deficient lymphoblastoid cell lines, removal of CPD's by TCR and GGR was less efficient compared to their p53-proficient counterparts after exposure to UVB. However, only GGR of CPD's were less efficient, suggesting that TCR is dependent on p53 after

UVB but not UVC exposure; this may be due to the increase of reactive oxygen species within the cell after UVB exposure (Mathonnet *et al*, 2003). Here, we have examine the effects of the HCR of a UVC-damaged AdHCMV*lacZ* reporter gene in *hMLH1*-deficient HCT116 adenocarcinomas on wildtype-p53 expression levels in an effort to detect a possible involvement of p53 in HCR.

Materials and Methods

Western Blot Analysis of p53 Expression: HCT116 cells were seeded in 6-well plates (Falcon, Franklin Lakes, NJ) at a density of 1.285 x 10^6 cells/well. Between 18-24 hours after seeding, media was replaced with 1.285 ml of PBS and then UVC irradiated or left untreated and then re-fed with the appropriate medium. Cells were then immediately infected with UVC irradiated or non-irradiated AdHCMV*lacZ* (as described previously) and cells were suspended in lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP40) containing protease inhibitors. After centrifugation (1 min at 13000 rpm), the supernatants were isolated and protein concentrations were measured in duplicate using the Bio-Rad protein reagent in a Bradford assay (Bio-Rad, Richmond, CA). Protein aliquots were prepared and 100 µg of each protein sample was loaded and separated by SDS-PAGE (10%). Proteins were then transferred onto a nitrocellulose membrane and blocked overnight at 4 °C in 20% skim milk in TBS with 0.05% Tween 20. Blots were then probed with a mouse monoclonal antibody to p53 conjugated to HRP (Santa Cruz Biotechnology, Santa Cruz, CA). Protein loading was verified by monitoring actin levels in each lane. After the addition of ECL staining reagent (Western Lightning

Chemiluminescence Reagent, PerkinElmer Life Sciences), blots were visualized by exposure to Kodak X-Omat AR film or by chemiluminesence detection (Kodak Digital Science Image Station 440 CF). Relative quantitative levels of p53 expression were determined after correction for background signals and normalization to corresponding actin levels.

Results

Effect of AdHCMV*lacZ* infection on wildtype p53 expression levels: Induction of p53 expression was examined in HCT116 colon carcinoma cells that are wildtype for p53 ($p53^{+/+}$). These cells were initially UVC-irradiated with 20 and 30 J/m^2, which was shown to increase p53 expression over basal levels (Fig. 5-1). Cell lysates harvested 24 hours after infection with AdHCMV*lacZ* irradiated with 300 and 600 J/m^2 UVC also resulted in increased p53 expression, with 300 J/m^2 to virus resulting in greater p53 expression. This dose response effect of UVC-damaged viral DNA on p53 expression was not evident when cell lysates were harvested at a later time point of 36 hours after viral infection (Fig. 5-2). Interestingly, unirradiated AdHCMV*lacZ* resulted in the highest level of p53 expression as determined by chemiluminesence readings (panel B), although this effect was not as obvious from the corresponding western blot (panel A). Furthermore, attempts to reproduce the results from Fig. 5-2 have also proven unsuccessful (results not shown), which may suggest high biological variability based on the conditions used, such as cell passage number or confluency of culture flasks used.

Discussion

Effects of AdHCMV*lacZ* infection on wildtype p53 expression: In addition to its multiple roles in DNA repair and tumor suppression, evidence has also shown that p53 may also assume a functional role in NER, especially in GGR (Ford and Hanawalt, 1997), whereas its role in TCR is more controversial. However, several lines of evidence point to p53 involvement in TCR of bulky DNA lesions in its capacity to support HCR of a UV-damaged reporter gene in adenoviruses (McKay *et al*, 1999), to serve a protective role against UV-induced apoptosis in human fibroblasts proficient in TCR (McKay *et al*, 2001), and its involvement in TCR induced by UVB radiation (Mathonnet *et al*, 2003). Here, we have employed the HCR assay of a UVC-damaged AdHCMV*lacZ* virus to determine the effects of a UVC-irradiated reporter gene on wildtype p53 expression. It was found that UVC-damaged viral DNA alone triggered an increase in p53 expression (Fig. 5-1). Since HCT116 cells are deficient in *hMLH1* and we have assumed here that this MMR gene has an involvement in UVC HCR (and consequently TCR), it would be difficult to attribute the increased p53 expression levels observed here to its putative role in TCR, but perhaps through it's involvement in GGR upregulation. Additionally, it is possible that infection of the AdHCMV*lacZ* virus itself may serve as a trigger for this increase in p53 expression (Fig. 5-2B). Although not reproducible at the present time, these results may have important therapeutic implications, since many vectors used in gene therapy use recombinant adenoviruses similar to the AdHCMV*lacZ* used here (Glick et al. 1998). Indeed, activation of p53 and induction of its pro-apoptotic target genes in virally infected cells suggest that these events lead to an apoptotic response of

infected cells, which could be considered as "altruistic suicide" that limits virus replication (Takaoka *et al.* 2003). Due to the preliminary nature of the results presented here, further experiments are needed to ensure reproducibility.

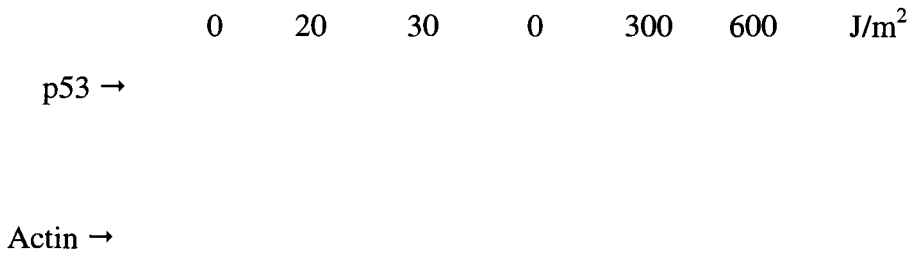

Figure 5-1: Induction of p53 by UVC irradiation to cells and by UVC-irradiated viral DNA. HCT116 colon carcinoma cells were either irradiated with UVC (left 3 lanes) or infected with UVC-irradiated AdHCMV*lacZ* virus (right 3 lanes). Cell lysates were harvested 24 hours after UVC-irradiation to cells or infection and lysates were separated by SDS-PAGE (10%). Proteins were then transferred to a nitrocellulose membrane and probed by antibodies for p53 or actin.

A No Virus

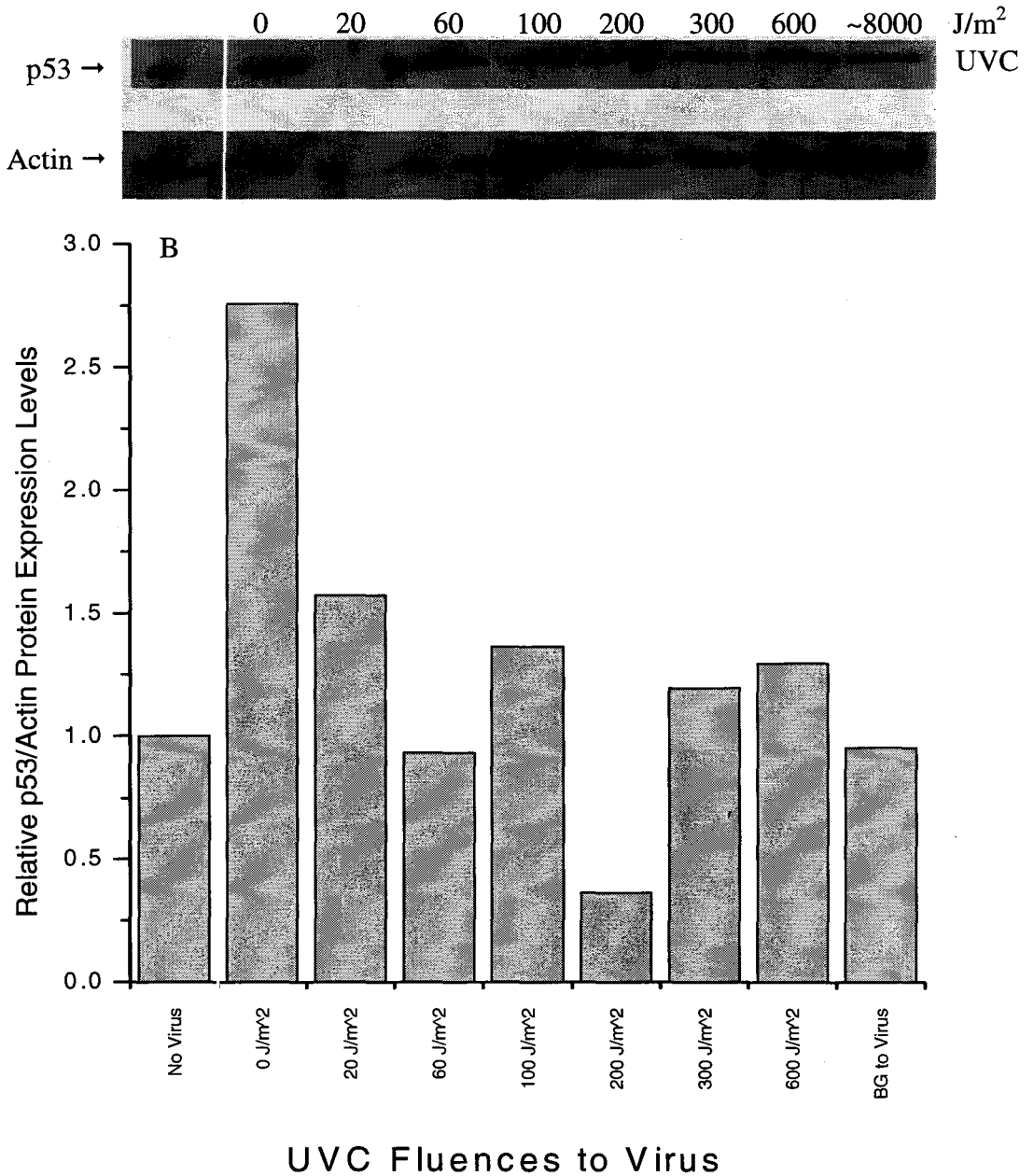

Figure 5-2: Induction of p53 by UVC-irradiated and non-irradiated viral DNA. HCT116 colon carcinoma cells were infected with AdHCMV*lacZ* virus irradiated with increasing UVC fluences. Cell lysates were harvested 36 hours after infection and lysates were separated by SDS-PAGE (10%). Proteins were then transferred to a nitrocellulose membrane and probed by antibodies for p53 or actin. **(A)** Western blots of p53 and actin levels. **(B)** Corresponding relative p53/actin levels as determined by chemiluminesence.

Summary and Future Directions

It has become clear that the unique, complex systems of human DNA repair pathways are actually quite interconnected. In spite of the fact that these three important DNA repair pathways studied here (NER, BER, and MMR) primarily target distinctly different lesions and abnormalities in the genome, multiple lines of evidence have reported the involvement of various DNA repair proteins in other repair systems in addition to their own. Given that this is a relatively new research area in the subject of DNA repair, there still exists considerable controversy regarding what specific roles (if any) do these repair proteins (i.e. hMSH2, hMLH1, p53) assume in mediating the effects of one or more of these DNA repair pathways.

Because of the many conflicting reports that have surrounded the roles of the human mismatch repair genes *hMSH2* and *hMLH1* in the TCR sub-pathway of NER, we were interested in attempting to shed some light in this controversial area. Using the AdHCMV*lacZ* recombinant adenovirus in our HCR and enhanced β-gal expression assays, we first found that both *hMSH2* and *hMLH1* were indeed involved in TCR. Furthermore, we also found that detection of their involvement is dependent on UVC fluence to cells (consistent with an up-regulation of p53 dependent GGR), since several research groups have used different UVC fluences in their experiments and have thus reported conflicting results. Colony survival experiments revealed however that deficiencies in these MMR genes do not actually confer increased sensitivity to UVC radiation, which indicates that this effect may be attributed to defects in the XP and CS gene traditionally associated with NER.

Using the AdHCMV*lacZ* virus, we were also interested in analyzing the potential involvement of *hMSH2* and *hMLH1* in BER using UVA and H_2O_2 as agents of inducing oxidized lesions in DNA. Initial UVA experiments revealed a significantly higher level of HCR in *hMSH2* proficient vs. deficient carcinomas, but not between *hMLH1* proficient and deficient lines, suggesting a role for *hMSH2* in BER. Additional HCR experiments also revealed a potential involvement of XP-A and XP-D proteins in BER. However, these corresponding cell lines may have experienced decreases in BER due to the likely formation of CPDs by UVA in the viral DNA. Furthermore, the ability to induce oxidized lesions in viral DNA by UVA needs further study, since irradiations may have taken place without the presence of necessary photosensitizers. Nevertheless, the enhanced expression assays further supported the involvement of *hMSH2*, but not *hMLH1* in BER of UVA-induce damage. Colony survival experiments also revealed that loss of wildtype *hMSH2* confers resistance to UVA radiation, which is consistent with previous reports that *hMSH2* is involved in an apoptotic response to genotoxic stress.

Experiments involving H_2O_2 as the DNA damaging agent proved to be substantially more complex. Initial HCR experiments that used high H_2O_2 concentrations to virus revealed a "bimodal" killing effect, whereas HCR studies that used low H_2O_2 doses yielded almost identical HCR profiles in all carcinoma and fibroblast lines tested. This latter effect is reflective of H_2O_2 damaging the viral protein coat, thus reducing viral infectivity in all cell lines. Nevertheless, the enhanced expression assay, as well as the H_2O_2 colony survival assay, detected no significant differences between the respective MMR-proficient and deficient cell lines, which suggests an unlikely involvement of

hMSH2 or *hMLH1* in the repair of DNA damage induced by H_2O_2. By contrast, the H_2O_2 enhanced expression assay was able to detect relative deficiencies in CS-B, XP-A, and even in GM 9503 normal fibroblasts, which may be due to polymorphisms in BER genes in the general population.

Finally, preliminary studies have showed that p53 may have a role in the HCR (and consequently TCR) of the UVC-irradiated AdHCMV*lacZ* virus. Interestingly as well is the finding that infection of unirradiated virus was able to enhance p53 levels, although additional experiments need to be conducted in order to confirm these results.

Due to the novel and controversial nature of many of the experiments presented in this project, there are many potential avenues for future research, some which include the following:

1. The incorporation of *Msh2* and *Mlh1* knockout mouse cell lines. Since these lines are isogenic, detection of their involvement in NER or BER could be solely attributed to the presence or absence of the MMR gene in these cell lines.
2. Repeated experiments with the p53 project, which are needed to confirm whether or not p53 is indeed required for the HCR response.
3. Additional studies investigating the involvement of various XP and CS cell lines in BER, which would prove beneficial in elucidating potential roles of these proteins in the repair of oxidative damage.
4. Additional experiments testing the responses of a variety of normal cell lines with respect to NER and BER, which may further reveal the presence of polymorphisms in the repair genes of these cell lines.

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