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By

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ABSTRACT

MECHANISMS OF MUTAGENESIS AND DAMAGE AVOIDANCE IN HUMAN FIBROBLASTS

By

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The main focus of this research concerns the mechanisms by which human cells tolerate spontaneous or induced DNA damage that blocks the replication fork. Damage tolerance consists of two sub-pathways, i.e., translesion synthesis and damage avoidance. Translesion synthesis involves a series of newly discovered specialized DNA polymerases that, in contrast to the major DNA replication polymerases, can use damaged DNA as a template. It can be error-prone or error-free depending on the nature of the lesion and the availability of a particular DNA polymerase or set of DNA polymerases. The alternative pathway for cells to deal with fork-blocking lesions is to use an undamaged homologous copy of the sequence, i.e., the newly replicated daughter strand of the sister duplex or an allelic copy, as a template temporarily to circumvent the lesion. This error-free pathway is referred to as damage avoidance. To determine whether hMms2 protein is involved in the damage avoidance pathway, an antisense strategy was used to eliminate hMms2 protein in the target cells. The results demonstrated that hMms2 is essential for damage avoidance in human cells dealing with UV- or BPDE-induced replication fork-blocking lesions (providing direct evidence for use of the allelic copy and indirect evidence for use of the newly synthesized

daughter strand of sister duplex). Loss of hMms2 protein eliminated the ability of human cells to use the undamaged copy as the template dealing with fork-blocking lesions and significantly increased the frequency of UV- or BPPDE-induced mutants. Analysis of the damage avoidance products revealed that they are predominantly caused by copying a homologous sequence using gene conversion not associated with crossing-over. Furthermore, the results indicated that damage avoidance and translesion synthesis are used almost equally for tolerating BPDE-induced replication fork-blocking lesions. In addition, the role of *hREV3* in error-prone translesion synthesis, i.e., carcinogen-induced mutagenesis, was also determined. Compared to their parental cell strain, the strains expressing high levels of *hREV3* antisense exhibited a significantly reduced UV- and BPDE-induced mutation frequency (up to 6 fold). These data indicate that hRev3 protein, the catalytic subunit of DNA polymerase zeta, plays a causal role in generating mutations during translesion synthesis.

This work is dedicated to:

My wife, Min Zhuang

My son, Jack Hang Li

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LIST OF ABBREVIATIONS

AP:	apurinic/apyrimidinic
AAF:	*N*-2-acetyl-2-aminofluorene
AAF-G:	AAF-adducted guanine
BPDE:	(±)-7β, 8α-dihydroxy-9α, 10α-epoxy-7, 8, 9, 10-tetrahydro-benzo[a]pyrene or benzo[a]pyrene diol-epoxide
BPDE-G:	BPDE-adducted guanine
8-oxoG:	8-oxoguanine
HPRT:	hypoxanthine phosphoribosyltransferase
HR:	homologous recombination
Hyg^R:	hygromycin resistant
MMS:	methyl methanesulfonate
NHEJ:	non-homologous end joining
O^6-MeG:	O^6-methylguanine
Pol:	polymerase
PCNA:	proliferating cell nuclear antigen
TG:	6-thioguanine
TLS:	translesion DNA synthesis
T-T:	T-T *cis-syn* cyclobutane dimer
T-T (6-4):	T-T pyrimidine (6-4) pyrimidinone
Ubc:	ubiquitin-conjugating enzyme
XPV:	xeroderma pigmentosum variant

INTRODUCTION

Prokaryotic and eukaryotic cells are able to replicate their DNA even when it contains DNA damage. This so-called "damage tolerance" ensures that DNA replication can continue even when DNA damage has not been efficiently removed by DNA repair mechanisms. Damage tolerance is considered to encompass two pathways, i.e., translesion synthesis and damage avoidance. Translesion synthesis involves the newly discovered specialized DNA polymerases that are able to insert nucleotides across from the damaged DNA template when the major replicase, e.g., polymerase delta, is blocked by DNA lesions. This process can be either error-prone or error-free depending on the nature of the lesion and the characteristics of the particular DNA polymerase used. Damage avoidance is the alternative pathway by which cells deal with fork-blocking lesions by using an undamaged homologous copy of the replicating sequence as a temporary template to circumvent the lesion. This error-free pathway has been hypothesized (Higgins et al., 1976), but the exact mechanism and various gene products involved remain obscure.

One of my dissertation research aims was to determine whether the *hMMS2* gene is involved in damage tolerance in human cells and if so, to identify the pathway(s) in which it is involved. The yeast *MMS2* gene was cloned by Xiao and colleagues (Broomfield et al., 1998). The *mms2* deletion mutants in yeast exhibited an elevated UV-induced mutation frequency and spontaneous

mutations, although the exact mechanism was not understood (Broomfield et al., 1998). The human counterpart of *MMS2*, *hMMS2*, was cloned (Xiao et al., 1998a), which made it possible to determine the role of hMms2 in damage tolerance in human cells. The second part of my dissertation research was to determine the role of *hREV3* in error-prone translesion synthesis, i.e., carcinogen-induced mutagenesis. In yeast, the deletion mutants of *rev3* exhibit virtually no carcinogen-induced mutations and loss of the majority of spontaneous mutations (Lawrence and Hinkle, 1996). Rev3 protein, the catalytic subunit of DNA polymerase zeta, is able to perform translesion synthesis for thymidine-thymidine dimers *in vitro* (Nelson et al., 1996). The human homolog, *hREV3*, was cloned (Gibbs et al., 1998; Xiao et al., 1998b, Lin et al., 1999), which made it possible to determine the role of *hREV3* in error-prone translesion synthesis in human cells.

Chapter I reviews the background literature of translesion synthesis and damage avoidance in prokaryotic and eukaryotic cells, with the focus on eukaryotes. Chapter II consists of a manuscript by Li et al. published in the April 2002 issue of **Proceedings of the National Academy of Science U. S. A.**, 99:4459-4464. It describes the results of experiments I carried out using an antisense strategy to eliminate hMms2 protein in a human fibroblast cell strain and to identify the role of hMms2 protein in damage avoidance. In that study, I showed that hMms2 is essential for damage avoidance in human cells dealing with UV-induced replication fork-blocking lesions (providing direct evidence for use of the allelic

copy and indirect evidence for use of the newly synthesized daughter strand of sister duplex). The loss of hMms2 protein eliminated the ability of human cells to use an undamaged homologous copy as the template dealing with fork-blocking lesions and increased UV-induced mutant frequency. Chapter III consists of a manuscript by Li et al. submitted to **Mutation Research**. It describes the determination of the relative contribution of translesion synthesis versus damage avoidance for the tolerance of DNA damage induced by benzo[a]pyrene diol-epoxide (BPDE). I showed that hMms2 is essential for damage avoidance dealing with BPDE-induced fork-blocking lesions, and more importantly that translesion synthesis is used almost equally to damage avoidance for tolerating BPDE-induced fork-blocking lesions. Chapter IV consists of a manuscript by Li et al. being prepared for submission to **Mutation Research**. It describes the research I carried out using an antisense strategy to investigate the role of *hREV3* in error-prone translesion synthesis for UV- or BPDE-induced lesions.

References

Broomfield, S., Chow, B.L. and Xiao, W. (1998) MMS2, encoding a ubiquitin-conjugating-enzyme-like protein, is a member of the yeast error-free postreplication repair pathway. Proc Natl Acad Sci U S A, 95, 5678-5683.

Gibbs, P.E., McGregor, W.G., Maher, V.M., Nisson, P. and Lawrence, C.W. (1998) A human homolog of the Saccharomyces cerevisiae REV3 gene, which encodes the catalytic subunit of DNA polymerase zeta. Proc Natl Acad Sci U S A, 95, 6876-6880.

Higgins, N.P., Kato, K. and Strauss, B. (1976) A model for replication repair in mammalian cells. J Mol Biol, 101, 417-425.

Lawrence, C.W. and Hinkle, D.C. (1996) DNA polymerase zeta and the control of DNA damage induced mutagenesis in eukaryotes. Cancer Surv, 28, 21-31.

Lin, W., Wu, X. and Wang, Z. (1999) A full-length cDNA of hREV3 is predicted to encode DNA polymerase zeta for damage-induced mutagenesis in human cells. Mutat Res, 433, 89-98.

Nelson, J.R., Lawrence, C.W. and Hinkle, D.C. (1996) Thymine-thymine dimer bypass by yeast DNA polymerase zeta. Science, 272, 1646-1649.

Xiao, W., Lin, S.L., Broomfield, S., Chow, B.L. and Wei, Y.F. (1998a) The products of the yeast MMS2 and two human homologs (hMMS2 and CROC-1) define a structurally and functionally conserved Ubc-like protein family. Nucleic Acids Res, 26, 3908-3914.

Xiao, W., Lechler, T., Chow, B.L., Fontanie, T., Agustus, M., Carter, K.C. and Wei, Y.F. (1998b) Identification, chromosomal mapping and tissue-specific expression of hREV3 encoding a putative human DNA polymerase zeta. Carcinogenesis, 19, 945-949.

CHAPTER I

LITERATURE REVIEW

A. Introduction

Cells are continuously exposed to endogenous and exogenous DNA damaging agents. Certain types of DNA damage, e.g., ultraviolet (UV)-induced cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts, benzo[a]pyrene diol-epoxide (BPDE)-induced guanine adducts, and *N*-2-acetyl-2-aminofluorene (AAF)–induced lesions, will block normal DNA replication carried out by the major DNA polymerases (Friedberg et al., 1995). Cells have developed mechanisms to deal with such DNA damage. For example, cell cycle checkpoints are the mechanism by which cells temporarily inhibit normal DNA replication in order to allow time for DNA repair before replication starts. Checkpoints also prevent cells from entering mitosis until the full complement of DNA has been replicated. Excision repair mechanisms are responsible for physically removing such DNA lesions and restoring damaged DNA to its original state. In addition, cells possess damage tolerance mechanisms i.e., translesion synthesis (TLS) and damage avoidance (DA). TLS is a special replication process by which cells are able to temporarily use various specialized DNA polymerases to incorporate nucleotides opposite the damaged template. Damage avoidance is a special replication process by which cells temporarily use an undamaged alternative template to circumvent DNA lesions. I will briefly

discuss these two mechanisms in prokaryotes in Section B, and then I will discuss these two mechanisms in detail in eukaryotes in Section C.

B. Damage Tolerance Mechanisms in Prokaryotes

1. Overview of the SOS System

The SOS response is a mechanism by which *E. coli* cells deal with DNA damage, particularly fork-blocking lesions. Approximately 30 gene products are induced in the SOS response after *E. coli* cells are exposed to DNA damaging agents, and the majority of these gene products are used for damage tolerance and DNA repair (Friedberg et al., 1995; Fernandez De Henestrosa et al., 2000). Among these gene products, RecA and LexA proteins play important roles in regulating gene expression of other SOS regulon components. The LexA protein is a repressor of the SOS regulon. It binds to the SOS operon promoter region and prevents transcription of SOS response genes. Autodigesion of LexA, a process assisted by RecA protein, triggers transcription of SOS regulon genes. RecA is a single strand DNA (ssDNA) binding protein and it forms a nucleoprotein filament with ssDNA (Friedberg et al., 1995). The RecA/ssDNA filament can assist autocleavage of LexA protein, which activates the SOS-regulon. DinI, a protein that is also induced as part of the SOS response, inhibits co-protease activity of the RecA/ssDNA nucleoprotein filament by inhibiting the autodigestion of LexA protein. In summary, RecA is the activator of the SOS-regulon, whereas DinI and LexA act as repressors of the SOS regulon.

Once the SOS response is activated, the important gene products that are induced are DNA polymerase V, which is encoded by the *umuDC* gene, and polymerase IV, which is encoded by the *dinB* gene. Both polymerases IV and V are the key components in *E. coli* translesion synthesis and mutagenesis. Polymerase II, which is involved in recombination-mediated replication re-start, is also induced (see below).

2. Translesion DNA Synthesis: The UmuDC/DinB proteins

Translesion DNA synthesis (TLS) is the mechanistic basis of the SOS response. TLS allows cells to tolerate DNA damage to a certain degree by replicating DNA containing lesions. Specifically, translesion synthesis is a special type of DNA replication by which specialized DNA polymerases are able to insert nucleotides opposite the damaged template that would otherwise block normal DNA replication carried out by major replicase. In *E. coli*, TLS increases cell survival at the expense of elevated mutations. This error-prone TLS requires the *umuDC* operon, which was originally identified by screening for mutants that exhibited an extremely low frequency of UV- and other carcinogen-induced mutations, compared to the wild type (Steinborn, 1978). Translesion synthesis also requires the *E. coli dinB* gene (Wanger et al., 1999) (see below).

2.1. UmuC and UmuD' (Pol V)

E. coli DNA pol V is composed of one molecule of UmuC protein and two molecules of UmuD' protein, the post-translationally processed form of UmuD

protein. The biochemical nature of this processing is similar to that of LexA autocleavage which is assisted by the RecA/ssDNA nucleoprotein filament. The interaction between the RecA/ssDNA filament and UmuD protein activates the autodigestion of UmuD, generating a protein that is missing its N-terminal 25 amino acids (Nohmi et al., 1988; Shinagawa et al. 1988). The DinI protein inhibits this process by inhibiting the co-protease activity of RecA/ssDNA.

Goodman and colleagues (Tang et al., 1998; Tang et al., 1999; Tang et al., 2000) and Livneh and colleagues (Reuven et al., 1998; Reuven et al., 1999) have independently demonstrated *in vitro* that pol V is required for error-prone TLS past abasic sites, thymidine-thymidine *cis-syn* dimers (T-T dimers), and T-T pyrimidine (6-4) pyrimidinone [T-T (6-4)] photoproducts. Both groups found that UmuD'$_2$ alone is unable to carry out translesion synthesis *in vitro* (Tang et al., 1998; Tang et al., 1999; Reuven et al., 1998; Reuven et al., 1999). However, Reuven et al. (1999) showed that UmuC, by itself, is able to insert nucleotides across from an undamaged template with a limited efficiency *in vitro*. However, it needs UmuD'$_2$, RecA, and single-strand DNA binding protein to carry out translesion synthesis on a damaged template. These results indicate that the pol V protein complex and associated factors are the replication machinery inserting nucleotides opposite the damaged template. It is uncertain whether DNA Pol III plays an essential role in translesion synthesis, although it is considered to carry out the normal DNA replication once the lesion is bypassed.

In addition to their roles in translesion synthesis, the *umuDC* gene products may also play important roles in DNA damage checkpoints (Opperman et al., 1999). Opperman et al. (1999) observed that uncleaved UmuD in complex with UmuC acts, *in vivo*, to inhibit DNA replication after UV-irradiation. Sutton et al. (2001) showed the genetic interaction between the *umuDC* gene products and the beta processivity clamp of the replicative DNA polymerase III. *In vitro* evidence demonstrated that UmuD interacts with the pol III beta processivity clamp at its N-terminus domain, the domain that UmuD' protein is missing (Sutton et al., 2002). This interaction is essential for the role of UmuD in checkpoints. After *E. coli* cells are exposed to DNA damaging agents, the UmuD protein is probably initially involved in prohibiting cell-cycle progression before DNA replication and then it is degraded into UmuD' protein and the $UmuCD'_2$ complex triggers translesion DNA replication. Therefore, regulation of the autodigestion of UmuD is critical for cells to switch from checkpoint to translesion synthesis.

2.2. DinB (Pol IV)

The *E. coli dinB* gene is a part of the SOS regulon (Kenyon and Walker, 1980). It was later shown to encode a novel *E.coli* DNA polymerase IV (pol IV) involved in spontaneous mutagenesis (Wagner et al., 1999; Strauss et al., 2000) and induced mutagenesis (Napolitano et al., 2000). The importance of the *dinB* gene in mutagenesis was originally discovered in studies with lambda phage untargeted UV-mutagenesis (Brotcorne-Lannoye and Maenhaut-Michel, 1986), indicating that it is important for UV-induced error-prone translesion synthesis. In

addition, over-expression of the DinB protein enhanced spontaneous mutations (Kim et al., 1997). DinB was shown to be distributive, devoid of proofreading activity, and able to elongate a DNA substrate with a bulged-out nucleotide in the template *in vitro*, which resulted in -1 frameshift mutations (Wagner et al., 1999). DinB was named DNA polymerase IV (pol IV) (Wagner et al., 1999). This implies that pol IV is important for bypassing certain type of DNA lesions, although the lesion specificity for pol IV both *in vivo* and *in vitro* is not well understood. However, Kim et al. (2001) reported that DNA pol IV is involved in –1 frameshift mutagenesis induced by 4-nitroquinoline N-oxide (4-NQQ) and in *E. coli* the expression level of pol IV protein is 6 to12 times higher than those of other SOS-inducible DNA polymerases in *E. coli* such as DNA pol II and DNA pol V.

The interaction between pol IV and the processivity factor of replicative pol III strongly increases its processivity (Wanger et al., 2000) and this interaction is presumably essential for pol IV's role in translesion synthesis and mutagenesis. Lenne-Samuel et al. (2002) recently showed that five amino acids at the C-terminal of pol IV are essential for the interaction between pol IV and the beta-clamp of pol III. Disruption of this interaction obliterates the function of pol IV in both spontaneous and induced mutagenesis *in vivo* (Lenne-Samuel et al., 2002).

The crystal structure of the *Sulfolobus solfataricus* pol IV homolog in complex with DNA and an incoming nucleotide has been determined (Ling et al., 2001; Zhou et al., 2001). The results revealed that, despite having a conserved active

site and a palm-like configuration similar to all known DNA polymerases, pol IV has very limited and nonspecific contact with the replicating bases (Zhou et al., 2001). This could explain why pol IV has a low processivity and has a low fidelity in choosing the incoming nucleotides (Zhou et al., 2001).

3. SOS-regulated daughter-strand gap repair (Recombination)

In *E. coli,* recombination-mediated replication restart is a major DNA tolerance mechanism. It is an error-free pathway. Early studies demonstrated that recombination is involved in the tolerance of UV-induced DNA damage in *uvr E. coli* mutants. In 1971, Howard-Flanders and colleagues using density transfer experiments showed that recombinants form between irradiated DNA strands and strands synthesized after irradiation (Rupp et al., 1971). Later, it was shown that T-T dimer exchange occurs between strands with a DNA lesion and strands without damage (Ganesan, 1974).

In *E. coli*, when the major DNA replicase pol III is blocked by UV-induced DNA damage, the DNA replication machinery can re-establish downstream of the block and resume DNA synthesis. This results in a single-strand DNA gap between the two newly synthesized DNA fragments. The gap is then filled by a DNA recombination mechanism (Rupp and Howard-Flanders, 1968; Rupp et al., 1971) termed daughter-strand gap repair. It is the gaps in the daughter-strand, rather the lesion itself, that are repaired by this mechanism. Therefore, this is a mechanism for tolerating DNA damage rather than physically removing the

lesion. The recombination-mediated single-strand gap repair provides a mechanism that avoids the fork-blocking lesions, in contrast to translesion synthesis in which specialized DNA polymerases incorporate nucleotides across from the damaged template.

The existence of this recombination-mediated single-strand gap repair is further supported by the following observations. *E. coli* cells carrying mutations in *umuD* or *umuC*, which encodes pol V, are still capable of resuming DNA synthesis shortly after UV-irradiation (Witkin et al., 1987; Rangarajan et al., 1999). This indicates that, in addition to the translesion synthesis pathway, an additional pathway, which was later found to be dependent on damage-inducible DNA polymerase pol II, is present to deal with fork-blocking lesions in *E coli* (Rangarajan et al., 1999). Rangarajan et al. (2002) recently showed that both pol II and the origin-independent primosome-assembling function of PriA are essential for the efficient recovery of DNA synthesis after UV-irradiation, indicating that pol II and PriA are important for the recombination-mediated replication restart. They also showed that *umuDC priA* or *umuDC polB* (*polB* encodes pol II) double mutants fail to resume DNA synthesis after UV-irradiation (Rangarajan et al., 2002). These data further indicate that translesion synthesis involving pol V and the recombination-mediated error-free pathway involving RecA, pol II, and PriA are alternative pathways for tolerating fork-blocking lesions and to resume DNA replication.

Recently, increasing evidence has been obtained in prokaryotes that homologous recombination plays an important role in an error-free process dealing with replication fork-blocking lesions. DNA single-strand gaps formed during DNA replication and possibly double strand breaks caused by replication fork arrest are excellent substrates for homologous recombination (Haber, 1999; Cox et al., 2000; Kowalczykowski, 2000; Kuzminov, 2001). Thus, DNA replication, repair, and recombination are coordinated events that prevent genomic instability caused by DNA damage-induced replication arrest.

C. Damage Tolerance Mechanisms in Eukaryotes

Damage tolerance mechanisms in eukaryotes are quite different from those in prokaryotes with regard to the protein components involved. However, prokaryotic and eukaryotic organisms share the same basic strategy to deal with fork-blocking lesions induced by DNA damaging agents, i.e., translesion synthesis and damage avoidance are the major mechanisms dealing with replication fork-blocking lesions. In prokaryotes, as discussed above, damage tolerance mainly relies on translesion synthesis by pol V and pol IV, and the error-free recombination-mediated gap "repair" pathway. In eukaryotes, translesion synthesis is also a major pathway to bypass DNA damage, but it is more complex and has more components involved than the equivalent pathway in prokaryotes. Damage avoidance, similar to the recombination-mediated error-free pathway in *E. coli*, is the alternative pathway to deal with fork-blocking

lesions by circumventing DNA lesions (Higgins et al., 1976; Li et al., 2002). In eukaryotic cells, damage tolerance mechanisms are dependent on the *RAD6-RAD18* pathway (Montelone et al., 1981; di Caprio and Cox, 1981). In this pathway, multiple DNA polymerases are involved in translesion synthesis (see below). There is another sub-pathway in the *RAD6-RAD18* epistasis group called the *MMS2-UBC13* pathway. This pathway contains proteins shown to be involved in the damage avoidance pathway in human cells (Li et al., 2002) and is an error-free pathway in *S. cerevisiae* (Broomfield et al., 1998; Xiao et al., 1999).

1. Overview of *RAD6-RAD18* Pathway

In *S. cerevisiae*, both *rad6* and *rad18* mutants are sensitive to various DNA damaging agents and deficient in damage tolerance (Montelone et al., 1981; di Caprio and Cox, 1981). These mutants were initially identified by Cox and Parry (1968) in a screen of UV-sensitive *S. cerevisiae* mutants. It was later confirmed that the mutagenic effect of UV irradiation indeed depends on Rad6 and Rad18 proteins (Cassier-Chauvat and Fabre, 1991). The *RAD6-RAD18* epistasis group in yeast includes proteins involved in translesion synthesis, e.g., pol eta (Johnson et al., 1999a; Johnson et al., 1999b), pol zeta (Nelson et al., 1996a; Lawrence and Hinkle, 1996), and proteins involved in an error-free pathway, i.e., *MMS2* (Broomfield et al., 1998), *UBC13* (Brusky et al., 2000), *RAD5* (Johnson et al., 1992), and *POL30* (Torres-Ramos et al., 1996). In human cells, in addition to the human homolog of pol eta (Masutani et al., 1999a; Masutani et al., 1999b) and zeta (Gibbs et al. 1998, Gibbs et al., 2000, Murakumo et al. 2000), there are

additional kinds of DNA polymerases that yeast cells do not possess, e.g., pol iota (Tissier et al., 2000a; Tissier et al., 2000b), pol kappa (Gerlach et al., 1999; Gerlach et al., 2001), pol mu (Dominguez et al., 2000; Aoufouchi et al., 2000), and pol lambda (Garcia-Diaz et al., 2000; Aoufouchi et al., 2000). Most, if not all, of these polymerases, are involved in translesion synthesis for different DNA replication fork-blocking lesions. The damage avoidance pathway in human cells is not well understood, but a recent report indicates that the human homolog of *MMS2*, *hMMS2*, is involved (Li et al., 2002).

1.1. *RAD6*

Saccharomyces cerevisiae In budding yeast, Rad6 protein is a ubiquitin-conjugating enzyme (Jentsch et al., 1987). Rad6 forms a stable complex with Rad18 and is involved in diverse cellular functions. *rad6* mutants are extremely sensitive to various DNA damaging agents such as UV, X-ray, γ-ray, bleomycin and alkylating agents and are defective in carcinogen-induced mutagenesis (Prakash et al., 1993). The *RAD6* gene product is also important for sporulation (Morrison et al., 1988), telomere silencing (Huang et al., 1997), and N-terminal rule based protein degradation (Dohmen et al., 1991).

The ubiquitin-conjugating enzyme (Ubc) activity of Rad6 resides on the active site Cys88. All the cellular functions of Rad6 require its Ubc activity because mutation of the active site Cys88 results in a *rad6* mutant phenotype (Sung et al., 1990). Significant biochemical studies have focused on discerning regions of the

protein required for specific biological functions. The C-terminal acidic residues are important for the ubiquitination of histone H2B (Sung et al., 1988; Robzyk et al., 2000), which is required for sporulation (Morrison et al., 1988). The N-terminal region of Rad6 is involved in N-end rule protein degradation (Dohmen et al., 1991) and sporulation (Morrison et al., 1988). Deletion of N-terminal amino acids of one to nine does not disrupt Ubc-Ubiquitin thiolester formation, but prevents the interaction between Rad6 and a ligase protein (E3), Ubr1 (Watkins et al., 1993), which is required for N-end rule protein degradation (Dohmen et al., 1991; Sung et al., 1991). Compared to *rad6* deletion mutants, which are extremely sensitive to the cytotoxic effect of UV and deficient in UV-induced mutagenesis, cells expressing Rad6 with an N-terminal deletion of amino acid one to nine are moderately sensitive to the cytotoxic effect of UV but proficient in UV-induced mutagenesis (Watkins et al., 1993).

Human cells Schneider et al. (1990) discovered that the human ubiquitin carrier protein E2 is homologous to yeast Rad6, indicating the existence of a human homolog of *RAD6*. Subsequently, two human homologs of *RAD6, hHR6A* and *hHR6B*, were cloned (Koken et al., 1991). *hHR6A* is located on chromosome Xq24-25 and *hHR6A* is located on chromosome 5q23-31 (Koken et al., 1992). Co-expression of *hRAD18* and *hHR6* in yeast cells revealed the stable hRad18-hHR6A and hRad18-hHR6B protein complexes (Xin et al., 2000), indicating that the human homologs of Rad6 interact with hRad18, which is similar to what is observed in yeast.

Inactivation of *HR6B* in mice causes male sterility associated with chromatin remodeling (Roest et al., 1996; Koken et al., 1996). Interestingly, hHR6B protein is up-regulated in metastatic mammary tumor cell lines (Shekhar et al., 2002). Constitutive over-expression of exogenous *hHR6B* cDNA induces cell-cell fusion, centrosome amplification, multipolar mitotic spindles, aneuploidy, and anchorage-independent growth (Shekhar et al., 2002). These findings indicate that hRad6 plays an important role in the maintenance of genomic stability, and the loss of regulation of hRad6 protein may lead to chromosomal instability and transformation. Recently, hHR6A was shown to be phosphorylated *in vitro* by cyclin-dependent kinase 2 (CDK2) at residue Ser120. This is the first study to demonstrate regulation of ubiquitin conjugation by the phosphorylation of this conserved residue (Sarcevic et al., 2002). It suggests that CDK-mediated phosphorylation of hHR6A is an important regulatory event in the control of cell cycle progression (Sarcevic et al., 2002).

1.2. *RAD18*

Saccharomyces cerevisiae In budding yeast, Rad18 forms a complex with Rad6, a ubiquitin-conjugating enzyme, through its RING finger domain (Jentsch et al., 1987). Rad18 has an ATPase activity and is able to bind single-strand DNA (Bailly et al., 1994; Bailly et al., 1997a; Bailly et al., 1997b). The RING finger domain of Rad18 is also important for the interaction between Rad18 and the Mms2-Ubc13 complex (Ulrich and Jentsch, 2000), bringing the Rad6-Rad18 complex into contact with the Mms2-Ubc13 complex. Unlike Rad6, the role of

Rad18 seems to be limited to the damage tolerance pathway. *rad18* mutants are defective in the repair of single-stranded gaps induced by γ-rays, but proficient in repairing double-stranded breaks created during the same treatment (Geigl and Echardt-Schupp, 1991). Rad18 is also involved in maintaining the integrity of single-stranded and linear DNA created after a DNA damaging treatment (Mowat et al., 1983).

A homolog of *RAD18* in fission yeast *Schizosaccharomyces pombe*, *rhp18*, was characterized by Verkade et al. (1999). As in *Saccharomyces cerevisiae*, *rhp18* deletion mutants are hypersensitive to DNA damaging agents, and have a long DNA damage checkpoint arrest (Verkade et al., 2001). This result indicates that the function of *RAD18* is conserved in yeast.

<u>*Human cells*</u> The human homolog of *RAD18*, *hRAD18*, was cloned by Tateishi et al. (2000). *hRAD18* is located on chromosome 3p24-25. The human Rad18 protein has 484 amino acids with a calculated molecular weight of 54 kDa (Teteishi et al. 2000). hRad18 protein binds to hHR6 proteins through a conserved RING finger motif. Stable transfectants expressing hRad18 protein mutated in this RING finger motif (cystein 28 to phenylalanine) become more sensitive to the cytotoxic effect of UV, methyl methanesulfonate (MMS), and mitomycin C, and are defective in replicating UV-induced DNA lesions (Tateishi et al., 2000). These data indicate that the interaction between hRad18 and hHR6

proteins is important for damage tolerance and mutagenesis, and also suggest that hRad18 has a similar function to that of its yeast counterpart.

Mouse The mouse homolog of *RAD18*, designated *mRAD18Sc*, was identified and characterized by van der Laan et al. (2000). The *mRAD18Sc* gene is located on mouse chromosome 6F. Its open reading frame encodes a protein of 509 amino acids with a molecular weight approximately 56 kDa. High homology was found between yeast Rad18, mouse Rad18, and human Rad18, particularly with the conservation of the RING finger domain. As yet there has been no report of *RAD18* knock out mice yet.

2. Translesion Synthesis: The UmuC/DinB Superfamily

Analogous to the *E.coli* SOS system, eukaryotic cells utilize translesion synthesis as a mechanism to deal with fork-blocking lesions. The Rad6 and Rad18 proteins are essential for translesion synthesis in eukaryotic cells, although the exact mechanism is not fully understood. Recent studies have confirmed that in eukaryotic cells, translesion synthesis relies on newly discovered DNA polymerases that belong to the UmuC/DinB superfamily. Both in yeast and in human cells, polymerase zeta (pol ζ), composed of Rev3 and Rev7 proteins, and polymerase eta (pol η) are important for translesion synthesis past lesions induced by various DNA damaging agents. In higher eukaryotes, e.g., mice and humans, additional kinds of DNA polymerases exist that might be involved in translesion synthesis past DNA lesions, e.g., polymerase iota (pol ι), polymerase

kappa (pol κ), polymerase mu (pol μ), and polymerase lambda (pol λ) (see below). However, pol μ and pol λ do not belong to UmuC/DinB family.

2.1. Yeast and human polymerase zeta and Rev1

2.1.1. Polymerase zeta

Saccharomyces cerevisiae One type of translesion synthesis carried out in yeast is dependent on polymerase zeta (pol ζ), which is generally considered an error-prone polymerase dealing with a variety of DNA fork-blocking lesions. The *rev3* mutants were initially isolated in *S. cerevisiae*, along with *rev1* mutants, based on their inability to revert *arg4-17* and *lys1-1* alleles in response to UV irradiation (Lemontt 1971). Studies in yeast strongly suggest that almost all of damage-induced mutations and a large portion of spontaneous mutations are generated by Rev3 protein (Lawrence and Hinkle, 1996), the catalytic subunit of pol ζ (Morrison et al., 1989). Recently, Pavlov et al. (2001) reported that replacing each tyrosine residue with an alanine in the conserved motif of the catalytic subunit of pol ζ in yeast causes the loss of UV-induced mutations. This result confirms that the catalytic activity of pol ζ is essential for mutagenesis. The pol ζ translesion pathway is dependent the gene products of *RAD6* and *RAD18* (Lawrence and Hinkle, 1996). Genetic evidence showing that *REV3* belongs to the *RAD6-RAD18* pathway comes from the finding that *rad6-1* is epistatic to *rev3-1* for the cytotoxic effect of UV (Lawrence and Christensen, 1976). *rad6* and *rad18* mutants are extremely sensitive to the cell killing by a variety of DNA damaging agents and are defective in UV-induced mutagenesis. In contrast, *rev3*

mutants display only slightly increased sensitivity to the cytotoxic effect of UV but a greatly reduced frequency of UV-induced mutations (Lemontt 1971).

In addition to Rev3, pol ζ contains another component, the Rev7 protein. The *rev7* mutants were isolated for their deficiency in reverting the UV-induced *lys2* allele (Lawrence et al., 1985a). The effect of the *rev7* mutation was found to be target allele-specific and mutagen-specific (Lawrence et al., 1985b), indicating that, in contrast to Rev3, Rev7 is less important in some carcinogen-induced mutagenesis.

The *REV3* gene in yeast encodes a protein that has 1,504 amino acids and a molecular weight of 173 kDa with conserved DNA polymerase motifs (Morrison et al., 1989). The *REV7* gene encodes a protein with a molecular weight of 28 kDa (Torpey et al., 1994). Biochemical studies showed that the dimerization of Rev3 and Rev7 forms functional pol ζ (Nelson et al., 1996a). Translesion synthesis past T-T dimers is carried out more efficiently by DNA pol ζ than by yeast DNA pol α, indicating that pol ζ is indeed capable of inserting nucleotides across from DNA lesions. Pol ζ was also shown to perform error-prone translesion synthesis *in vitro* past a T-T (6-4) photoproduct and an acetylaminofluorene (AAF)-adducted guanine (AAF-G) (Guo et al., 2001). In addition, pol ζ extends mismatches with high efficiency including nucleotides inserted opposite a lesion (Johnson et al, 2000; Guo et al., 2001). Another example of yeast pol ζ being a mismatch extender comes from the *in vitro* efficient bypass of an abasic (AP) site

by the sequential combined action of pol δ and pol ζ (Haracska et al., 2001a). Pol δ is able to insert an A nucleotide opposite an AP site, and pol ζ subsequently extends from the inserted nucleotide, but it is not efficient in incorporating nucleotides across from the AP site (Haracska et al., 2001a). These results indicate that pol ζ has a dual-function: it can function as a special DNA polymerase that incorporates nucleotides opposite some DNA lesions, and it can sometimes function as an extension DNA polymerase. However, whether this is true *in vivo* is unclear. Nevertheless, pol ζ must be acting as either an error-prone polymerase by itself or an extender of other error-prone polymerases, or both, during translesion synthesis because deletion of *REV3* virtually eliminates carcinogen-induced mutations.

Human cells The human homolog of the yeast *REV3* gene, *hREV3*, was independently cloned by three groups (Xiao et al., 1998a; Gibbs et al., 1998; Lin et al., 1999a) and the mouse homolog was also identified (Van Sloun et al., 1999). The *hREV3* gene is localized on chromosome 6q21 (Morelli et al., 1998; Kawamura et al., 2001). Its cDNA consists of 10,716 base pairs and encodes for a predicted protein of 3,130 amino acids with a predicted molecular weight of 350 kDa, about twice the size of the yeast counterpart that has 1,504 amino acids. The deduced hRev3 protein also contains in the right order all six motifs that are the characteristic of eukaryotic DNA polymerases. The yeast and human Rev3 proteins share 29% identity at the amino terminal region of ~350 residues, 39%

identity within the carboxyl-terminal region of ~850 residues, and 29% identity within an internal region of 55 residues (Gibbs et al., 1998).

In addition to the start codon ATG for the main open reading frame (ORF), the *hREV3* gene possesses an out-of-frame ATG codon at –58 at the 5' end of untranslated region (Gibbs et al., 1998). Furthermore, a common variant transcript with an insertion of 128-bp between nucleotides +139 and +140 was found both in human (Gibbs et al., 1998; Kawamura et al., 2001) and in mouse *REV3* cDNAs (Kawamura et al., 2001). Neither the insertion variant nor the out-of-frame start codon will produce the predicted Rev3 protein, indicating that the Rev3 protein level in higher eukaryotes is strictly regulated. *hREV3* and its insertion variant transcripts are ubiquitously detected in all normal human tissues, with an additional variant species found in tissues with relatively high levels of *hREV3* expression (Kawamura et al., 2001).

Murakumo et al. (2000) reported the discovery of a putative human homolog of yeast *REV7*, *hREV7*, using a yeast two-hybrid screen. The deduced *hREV7* gene product displays 23% identity and 53% similarity with yeast Rev7 protein, as well as 23% identity and 54% similarity with the human mitotic checkpoint gene product hMad2 (Murakumo et al., 2000). *hREV7* is located on human chromosome 1p36 in a region exhibiting frequent loss of heterozygosity in human tumors (Murakumo et al., 2000). The precise domain of *hREV3* that interacts with *hREV7* is within the region of amino acid residues 1847-1892 and the precise

domain for *hREV7* that interacts with *hREV3* is within the region of amino acid residues 21-155 (Murakumo et al., 2001). Interestingly, an interaction between hRev7 and hMad2 was identified, indicating that *hREV7* may act as an adapter between damage tolerance and the spindle assembly checkpoint (Murakumo et al., 2000). The functional of this putative *hREV7 in vivo* is yet to be determined.

The translesion polymerase ζ was recently shown to be involved in immunoglobulin gene somatic hypermutation (Zan et al., 2001). In mammals, in order to produce high affinity antibodies against a specific antigen, the DNA coding for the V-region of an antibody gene has to be vigorously mutated, such that all kinds of different antibodies will be produced and ultimately one of these antibodies will be selected. This process of mutating DNA in the V-region is referred to as somatic hypermutation. Inhibition of the catalytic subunit of pol ζ, *REV3*, by specific phosphorothioate-modified oligonucleotides was found to impair immunoglobulin hypermutation (Zan et al., 2001). In addition, transgenic mice that express *REV3* antisense showed a delay of generating high affinity antibodies and a decreased somatic hypermutation in the V-region (Diaz et al., 2001). These results suggest that translesion synthesis carried out by pol ζ plays an important role in the process of generating high affinity antibodies.

Unfortunately, to date, there has been no success in knocking out the *REV3* gene in mice. Four different groups attempted to knock out the *REV3* gene in mice and discovered that *REV3*$^{-/-}$ mice exhibited early embryonic lethality

(Esposito et al., 2000; Wittschieben et al., 2000; Bemark et al., 2000; Van Sloun et al., 2002). Mouse fibroblasts derived from the *REV3*$^{-/-}$ embryos could not be propagated. A significant increase in double-strand DNA breaks as well as chromatid and chromosome aberrations was observed in cells from *REV3*$^{-/-}$ embryos (Van Sloun et al., 2002), indicating that *REV3* might be involved in dealing with double-strand breaks and maintaining genomic stability. These studies with *REV3* provide the evidence that an enzyme involved in translesion synthesis and mutagenesis is critical for mammalian embryonic development.

Because no one has succeeded in knocking out *REV3* gene in mice, it is very difficult to study an *in vivo* model system to fully explore the biological function of *REV3* gene. There has been no report of Rev3 protein expression, purification, and biochemical characterization in mammalian cells, either. It is very important to break through these two barriers to extend the *REV3* research.

2.1.2. *REV1*

Saccharomyces cerevisiae rev1 mutants were initially isolated in *S. cerevisiae* along with *rev3* mutants one the basis of their inability to revert *arg4-17* and *lys1-1* alleles in response to UV irradiation (Lemontt 1971). The 3.1 Kb *REV1* transcript encodes a 985 amino acid-protein with a molecular weight of 112 kDa. *rev1* deletion mutants exhibit a reduced frequency of UV-induced mutations compared to wild type cells (Larimer et al., 1989). *REV1* is a nonessential gene and its gene product is an auxiliary factor of pol ζ (Lawrence and Hinkle, 1996).

A 152-residue internal segment of Rev1 protein shows 25% identity with *E. coli* UmuC protein (Larimer et al., 1989). Rev1 has a deoxycytidyl transferase activity on a template containing an AP site (Nelson et al., 1996b). Insertion of cytosine by Rev1 opposite an AP site produces a terminus that is efficiently extended by pol ζ (Nelson et al., 1996b). In addition to its role as a deoxycytidyl transferase, Rev1 appears to have a second function. Nelson et al. (2000) reported that Rev1 function is needed for the bypass of a T-T (6-4) UV photoproduct, a process in which dCMP incorporation occurs very rarely. Replication past this lesion is much reduced in the G193R *rev1-1* mutant, which retains substantial levels of deoxycytidyl transferase activity. This mutant is, therefore, deficient principally in the second function which might be the DNA polymerase activity. Consistent with this, Haracska et al., (2002a) showed recently that Rev1 is a G template-specific DNA polymerase, specifically inserts a C residue opposite template G, and that Rev1 is approximately 25-, 40-, and 400-fold less efficient at inserting a C residue opposite an AP site, an O^6-methylguanine (O^6-MeG), and an 8-oxoguanine (8-oxoG) lesion, respectively. Rev1 misincorporates G, A, and T residues opposite template G with a frequency of approximately 10^{-3} to 10^{-4} (Haracska et al., 2002a).

Human cells The human homolog of yeast *REV1* gene, *hREV1*, was identified by two different groups (Lin et al., 1999b; Gibbs et al., 2000). *hREV1* is localized between 2q11.1 and 2q11.2 (Lin et al., 1999b). Its cDNA consists of 4,225bp and codes for 1,251 amino residues with a calculated molecular weight of 140 kDa.

Significant homology exists between hRev1 and Rev1 proteins. In two regions of their N-terminus (about 100 amino acids in length), these proteins exhibit 41% and 20% identity. A centrally located motif that is highly conserved in Rev1 proteins is also found in *E. coli* pol IV (Gibbs et al., 2000). The level of *hREV1* mRNA is very low (Gibbs et al., 2000). Like the *hREV3* transcript, *hREV1* mRNA has a 5' out-of-frame ATG at position –35 upstream of the main translation start codon, which could explain why the hRev1 protein level is low in human cells.

hREV1 interacts with *hREV7* in a yeast two-hybrid system using *hREV7* as the bait (Murakumo et al., 2001). *hREV1* interacts with the region of amino acid residues 21-155 of *hREV7* and the minimum interaction domain of *hREV1* is within amino acid residues 1130-1251. It appears that hRev7 interacts with hRev3 and hRev1 proteins in the same region (Murakumo et al., 2001). The interaction between hRev1 and hRev7 was confirmed by *in vitro* and *in vivo* binding assays (Murakumo et al., 2000). Although *hREV7* interacts with both *hREV1* and *hREV3*, a stable complex formation of these three proteins has not been detected *in vitro*, and no direct interaction between *hREV1* and *hREV3* has been found (Murakumo et al., 2000). These findings suggest that hRev7 plays an important role in bringing hRev1 and hRev3 proteins together for translesion synthesis.

Like yeast Rev1, hRev1 protein was shown to have a deoxycytidyl transferase activity and to efficiently insert a dCMP opposite a DNA template with an AP site

(Lin et al., 1999b). Deletion and point mutation analysis of the hRev1 protein revealed that the domain required for deoxycytidyl transferase and DNA binding activities is located in a conserved domain of translesion DNA polymerase (Masuda et al., 2001). This result indicates that structure of the catalytic site of deoxycytidyl transferase closely resembles a translesion DNA polymerase. However, this result is contradictory to what is described in yeast in which it seems that dCTP transferase domain and polymerase domain are separate (Nelson et al., 2000). Recently, Zhang et al. (2002) showed *in vitro* that purified hRev1 lacks a 3' to 5' proofreading exonuclease activity and exhibits a DNA polymerase activity on a repeating template G sequence, similar to that of its yeast counterpart (Haracska et al., 2002a). hRev1 is able to insert a dCMP efficiently opposite a template containing 8-oxoG, BPDE-adducted guanine (BPDE-G), or 1,N6-ethenoadenine adducts, and very inefficiently opposite an AAF-G, but is unresponsive to a template containing a T-T dimer or T-T (6-4) photoproduct (Zhang et al., 2002). This is not consistent with what is described in yeast (Nelson et al., 2000). Nevertheless, both the yeast Rev1 and the human Rev1 possess a polymerase activity on a repeating G-template.

Pioneering biological function studies of *hREV1 in vivo* were carried out using the expression of *hREV1* antisense and the results showed that human cells expressing a high level of antisense *hREV1* have a greatly reduced UV-induced mutation frequency (Gibbs et al., 2000). The biological data strongly support the

idea that hRev1 is an auxiliary factor of human pol ζ that is involved in an error-prone translesion synthesis.

2.2. Yeast and human polymerase eta

Saccharomyces cerevisiae The alternative translesion synthesis pathway in yeast is the *RAD30* or pol η pathway. Earlier studies showed that mutations in *RAD30* increased the cytotoxic effect of UV and enhanced UV-induced mutability (McDonald et al., 1997; Roush et al., 1998) but showed minor sensitivity to other DNA damaging agents (Roush et al., 1998). Rad30 is a DinB-like protein. Like *dinB* in *E. coli*, *RAD30* is UV-inducible in yeast. Genetic analysis showed that *RAD30* belongs to the *RAD6* epistasis group because *rad6* or *rad18* deletion mutants are epistatic to *rad30* deletion mutants, and *rad30* mutants are additive to *rev1*, *rev3*, and *rev7* mutants for UV-sensitivity (McDonald et al., 1997). These results indicate that for UV-irradiated DNA, Rad30 protein is involved in an error-free pathway parallel to the error-prone translesion synthesis pathway of pol ζ dealing with fork-blocking lesions. Later, Johnson et al. (1999b) demonstrated that Rad30 is able to carry out error-free translesion synthesis past T-T dimmers, and therefore, Rad30 protein was named polymerase eta (pol η).

The polymerase activity of pol η is required for the resistance to cell killing and prevention of mutagenesis in response to UV-irradiation in yeast (Johnson et al., 1999a), indicating the error-free nature of pol η in damage tolerance. Interestingly, it was suggested that pol δ is more error-prone than pol η during

the translesion synthesis past N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced lesions (O^6-MeG) *in vivo* (Haracska et al., 2000a) because a *rad30 pol32* double mutant shows an abolished MNNG-induced mutagenesis whereas a *rad30* single mutant shows no defect [Pol32 is a non-essential subunit of the pol δ complex (Gerik et al., 1998)]. Pol η is ten times more efficient inserting bases across from O^6-MeG *in vitro* than is pol δ. Pol δ has strong stall sites both immediately preceding the lesion, and directly across from it, whereas these stall sites are much weaker for pol η (Haracska et al., 2000a). Pol η inserts a C across from O^6-MeG twice as frequently as pol δ, although both polymerases can be error-prone for this kind of lesion (Haracska et al., 2000a). These results indicate that, when pol δ fails to replicate DNA lesions, pol η might be recruited to bypass these lesions with a reasonable accuracy. Washington et al. (2001a) showed that pol η utilizes an induced-fit mechanism to selectively incorporate the correct nucleotide, which explains the error-free nature of pol η during translesion synthesis for a lot of DNA lesions. During the initial nucleotide-binding step, pol η is not selective between the correct and incorrect nucleotide. The subsequent "induced-fit" conformational change step allows pol η to select the correct base (Washington et al., 2001a). Pol η interacts with the proliferating cell nuclear antigen (PCNA), and this interaction is essential for the function of pol η *in vivo*. Pol η is highly inefficient at inserting a nucleotide opposite an AP site, but interaction with PCNA greatly stimulates its ability for nucleotide incorporation opposite this lesion (Haracska et al., 2001b).

The accuracy of pol η replicating a T-T dimer is almost the same as replicating an undamaged T-T template (Washington et al., 2000). Genetic studies also indicate that pol η is error-free replicating cyclobutane dimers and (6-4) photoproducts formed at CC and TC sites *in vivo* (Yu et al., 2001). Opposite the 3' T of a (6-4) T-T photoproduct, both yeast and human η preferentially insert a G residue, but they are unable to extend from the inserted nucleotide (Johnson et al., 2001). Pol η can also efficiently insert C and A opposite 8-oxoG, a C opposite an AAF-G, and a G or less frequently an A opposite an AP site, but it is unable to extend in both cases (Yuan et al., 2000). These results indicate that pol η can be error-prone when replicating certain types of lesions. However, Haracska et al. (2000b) showed that yeast and human pol η replicate 8-oxoG efficiently by inserting a cytosine across from the lesion (error-free). This result is not consistent with the finding of Yuan et al. (2000).

Domains essential for pol η function were identified. Deleting the last 54 amino acids of pol η has no effect on its DNA polymerase or T-T dimer bypass activity *in vitro*, but abolishes its biological function *in vivo* (Kondratick et al., 2001). There is a bipartite nuclear targeting sequence within C-terminal, indicating that portion of Rad30 is for nuclear localization. Acidic residues Asp30, Glu39, and Asp155 are critical for the catalytic activity and biological function of pol η (Kondratick et al., 2001). These residues are able to bind two metal ions required for the reaction of the incoming deoxynucleoside 5'-triphosphate with the 3'-hydroxyl in the primer terminus. Trincao et al. (2001) reported the crystal

structure of pol η, which revealed a novel polydactyl right hand-shaped molecule with a unique polymerase-associated domain. The fingers and thumb domains are unusually small and stubby. The openness of the active site is the critical feature that enables pol η to replicate past DNA lesions, such as T-T dimers.

Human cells The human homolog of *RAD30*, *hRAD30*, was discovered by two research groups (Masutani et al., 1999a; Johnson et al., 1999c). *hRAD30* gene is located at chromosome 6p21.1-6p12, has 11 exons covering the entire coding sequence, and lacks a TATA sequence in the upstream region of the transcription initiation site (Yuasa et al., 2000). Like Rad30 in yeast, hRad30 is a DNA polymerase η (hpol η) that is able to perform accurate TLS past T-T dimers *in vitro* (Masutani et al., 1999b; Washington et al., 2001b). Interestingly, the *hRAD30* gene is either truncated or has a reduced expression in xeroderma pigmentosum variant (XPV) (Masutani et al., 1999a; Johnson et al., 1999c). This explains why XPV cells are hypermutable in UV-induced mutagenesis (Maher et al., 1976) and exhibit a greatly reduced ability to perform TLS *in vitro* (Ensch-Simon et al., 1998; Svoboda et al., 1998; Cordonnier et al., 1999). It is generally considered that, for reactions *in vitro*, hpol η is error-free for UV-induced lesions (Masutani et al., 1999b) and error-prone for some other lesions (Zhang et al., 2000a; Chiapperino et al., 2002).

Although hpol η is able to perform accurate TLS past certain types of lesions *in vitro* (Masutani et al., 1999b; Washington et al., 2001b; Haracska et al., 2000b), it

replicates undamaged DNA with much lower fidelity than any other template-dependent DNA polymerases. It lacks an intrinsic proofreading exonuclease activity and makes one base substitution error for every 18 to 380 nucleotides synthesized (Matsuda et al., 2000). In studies designed to elucidate the mechanism of the error-free nature of hpol η, Masutani et al. (2000) discovered that hpol η preferentially puts A or G opposite AP sites and C opposite AAF-G and cisplatin-GG lesions. However, if hpol η incorporates an incorrect nucleotide opposite a lesion, it is not able to continue chain elongation. If hpol η incorporates a correct nucleotide opposite a lesion, elongation continues (Masutani et al., 2000). This result indicates that hpol η has a special mechanism to ensure the correct nucleotides can be incorporated during translesion synthesis. However, increasing evidence suggests that hpol η may be acting in an error-prone fashion past some DNA lesions *in vitro*. Zhang et al. (2000a) demonstrated that hpol η preferentially inserts A or C opposite a template 8-oxoG, inserts A opposite AP sites, and inserts A opposite BPDE-G lesions. Chiapperino et al. (2002) also showed that hpol η preferentially incorporates G or A opposite BPDE-G adducts.

hpol η is mainly localized in the nucleus and is associated with replication foci during S phase. After cells are exposed to UV light, hpol η accumulates at replication foci stalled at DNA lesions (Kannouche et al., 2001). The C-terminal 70 amino acids are required for nuclear localization, and 120 amino acids are needed for relocation into foci. hpol η missing this domain fails to correct XP-V

defects (Kannouche et al., 2001). Glick et al. (2001) showed that Tyr52 and Ala54 are the key residues for hpol η function in translesion synthesis. Interestingly, they described an hpol η mutant S62G that has an increased activity to bypass lesions. The physical interaction of hpol η with PCNA is important for the function of hpol η, and mutations in the PCNA binding motif of hpol η abolish this interaction and decrease the activity of hpol η (Haracska et al., 2001c).

2.3. Polymerase iota (human/mouse)

Human and mouse cells contain a second *RAD30* homolog, *RAD30B* (McDonald et al., 1999). *hRAD30B* is localized on chromosome 18q21.1 in a region that is often implicated in the etiology of many human cancers. The mouse homolog *Rad30b* is located on chromosome 18E2. The second human *RAD30* homolog, *hRAD30B*, encodes a novel DNA polymerase, pol iota (pol ι) (Tissier et al. 2000a). Pol ι is a highly error-prone and distributive DNA polymerase when replicating undamaged DNA. At template G or C, the average error frequency was approximately 1 x 10^{-2}. Most errors occurred at template T, where the misincorporation of G was significantly preferred (Tissier et al. 2000a). Zhang et al. (2000b) confirmed that pol ι preferentially inserts G opposite an undamaged template T and inserts T, A, or C opposite an undamaged template C. Pol ι is also a very error-prone DNA polymerase replicating damaged DNA. Tissier et al. (2000b) showed that pol ι replicates T-T dimers and T-T (6-4) photoproducts in an error-prone fashion. Pol ι predominantly inserts T or G opposite the 3' T of T-T

dimers and readily misinserts any two bases opposite T-T (6-4) photoproducts (Tissier et al., 2000b). Zhang et al. (2001) demonstrated that pol ι at a low concentration is not able to incorporate nucleotides across from 8-oxoG lesions, but incorporates C or A opposite these lesions at a higher concentration. Pol ι efficiently incorporates a G opposite an AP site, a C opposite an AAF-G, and an A opposite a T-T (6-4) photoproduct (Zhang et al., 2001). However, contrary to the finding of Tissier et al. (2000b), Zhang et al. (2001) reported that pol ι was not responsive to T-T dimmers. Taken together, the data indicate that pol ι might play a significant role in the error-prone and error-free translesion synthesis past UV-induced and other carcinogen-induced lesions. Haracska et al. (2001d) reported the physical interaction of pol ι with PCNA and showed that PCNA, together with replication factor C (RFC) and replication protein A (RPA), stimulates the DNA synthetic activity of pol ι. Whether pol ι is involved in carcinogen-induced mutagenesis *in vivo* is yet to be determined.

Although Johnson et al. (2000) reported that pol ι is not able to extend mispairs after it incorporates incorrect bases opposite DNA lesions, Vaisman et al. (2001) showed that pol ι extends all 12 possible mispairs and four correct pairs in different sequence contexts. They showed that extension from both matched and mismatched primer termini is generally efficient and accurate when A is the next template base. In contrast, extension occurs less efficiently and accurately when T is the target template base.

Pol ι was also found to contain an intrinsic 5'-deoxyribose phosphate lyase activity *in vitro*, an important enzymatic activity for polymerase beta which is involved in base excision repair (Bebenek et al., 2001). This result implies that pol ι may have multiple functions *in vivo*.

2.4. Polymerase kappa (human/mouse)

The human homolog of *DINB* has been identified. It encodes DNA polymerase kappa (pol κ). The human *DINB1* gene is localized to chromosome 5q13 and is ubiquitously expressed (Gerlach et al., 1999). Interestingly, pol κ is over-expressed in lung cancer patients (O-Wang et al., 2001). The mouse and human pol κ share significant identity with *E. coli* DinB, including distinct motifs implicated in catalysis (Gerlach et al., 1999). Ogi et al. (1999) transiently expressed the mouse *DINB* cDNA in cultured mouse cells and discovered that it resulted in a nearly 10-fold increase in the incidence of spontaneous point mutations, ~30% of which were frameshift mutations, indicating that pol κ plays a role in translesion synthesis and mutagenesis.

Gerlach et al. (2001) reported that human pol κ lacks detectable 3' to 5' proofreading exonuclease activity and is not stimulated by recombinant PCNA *in vitro*. However, Haracska et al. (2002b) provided evidence for the physical interaction between pol κ and PCNA and showed that PCNA along with RFC and RPA promotes the DNA synthesis activity of pol κ. These results contradict to

each other. The difference could be due to the different conditions used in those assays.

Human pol κ is able to incorporate mismatched bases on an undamaged template with a high frequency (Ohashi et al., 2000a; Zhang et al., 2000c). With damaged templates, pol κ is unable to bypass a cisplatin adduct, a *cis-syn* T-T dimer, or a T-T (6-4) photoproduct (Gerlach et al., 2001; Ohashi et al., 2000b; Zhang et al., 2000d; Suzuki et al., 2002). Pol κ can bypass of an AAF-G with low efficiency and incorporate C or T across from this lesion, suggesting that this process is potentially mutagenic (Zhang et al., 2000d; Gerlach et al., 2001; Suzuki et al., 2002). Moreover, pol κ incorporates A opposite an AP site (Ohashi et al., 2000b; Zhang et al., 2000d; Suzuki et al., 2002), and efficiently inserts A opposite an 8-oxoG and C opposite a BPDE-G (Zhang et al., 2000d; Suzuki et al., 2002). These *in vitro* studies indicate that pol κ can perform both error-prone and error-free translesion synthesis for different DNA lesions.

2.5. Other DNA polymerases potentially involved in translesion synthesis

Recently, two additional kinds of DNA polymerases were identified in human cells: pol μ and pol λ. These polymerases do not belong to the UmuC/DinB/Rad30/Rev1 superfamily, but belong to the X-family. Pol μ is similar to terminal deoxynucleotidyl transferase and has a high rate of error *in vitro*. Because of its elevated level in tissues of the immune system, it was considered to be involved in the generation of somatic hypermutations in the immunoglobulin

genes (Dominguez et al., 2000; Aoufouchi et al., 2000). However, Recent work indicates that pol μ and pol λ are not involved in somatic hypermutation because mice inactivated for either of these two polymerases are viable and fertile and display a normal hypermutation pattern (Bertocci et al., 2002). Pol λ is a pol β-like DNA polymerase and an accurate polymerase. It might be functioning in error-free repair in meiosis (Garcia-Diaz et al., 2000).

3. Damage Avoidance

3.1. Overview of damage avoidance

Rupp and Howard-Flanders, 1968 first showed that in *E.coli*, recombinational strand transfer of DNA from the damaged duplex to the homologous strand of the undamaged duplex is one mechanism for tolerating UV damage. Recently, increasing evidence has been obtained that the homologous recombination pathway plays an important role in this error-free process for dealing with replication fork-blocking lesions in prokaryotic cells. DNA gaps and possibly double strand breaks caused by replication fork arrest are excellent substrates for homologous recombination (Haber, 1999; Cox et al., 2000; Kowalczykowski, 2000; Kuzminov, 2001). In eukaryotes, in addition to the translesion synthesis pathway that is carried out by the newly discovered specialized DNA polymerases, cells have an additional error-free pathway that is also dependent on Rad6 and Rad18 proteins (see below). This pathway is usually referred to as damage avoidance by which cells temporary utilize an undamaged homologous copy as the alternative template to circumvent DNA lesions that block normal

replication carried out by the DNA polymerase δ complex. The undamaged copy can be either the newly synthesized daughter strand of the sister duplex or the homologous allele. Sometimes referred to as "copy choice" or "strand switch", this pathway was originally proposed as a mechanism by which DNA polymerases (e.g., pol δ) temporarily use the newly synthesized daughter strand of the undamaged complementary sequence as a template to avoid the lesion (Higgins et al., 1976). Once the replication fork has moved beyond the lesion, the replicating pol δ switches back to continue replicating using the original template strand. However, the exact mechanism of this process and the various gene products involved remain obscure. Recently, Nikolaishvili-Feinberg and Cordeiro-Stone (2000), using bisulfite treatment followed by methylation-specific polymerase chain reaction *in vitro*, were able to distinguish the nascent DNA strand synthesized from the damage avoidance pathway and the strand replicated from the plasmid itself. The results of their studies indicated the use of a template switch mechanism.

The phenotype of several newly identified mutants in the *RAD6-RAD18* epistasis group clearly suggests that these genes are involved in the damage avoidance pathway, e.g., *MMS2* (Broomfield et al., 1998), *UBC13* (Brusky et al., 2000), *RAD5* (Johnson et al., 1992), and *POL30* (Torres-Ramos et al., 1996) (see below). In this section, I will discuss the homologous recombination repair in yeast and in mammalian cells as a mechanism to deal with double-strand breaks. I will also discuss the error-free damage tolerance pathway that depends on the

RAD6-RAD18-RAD5-POL30 epistasis group dealing with replication fork-blocking lesions, although the majority data are from *S. cerevisiae.*

3.2. Double-strand break repair

Saccharomyces cerevisiae Double-strand breaks (DSBs) are potent inducers of mutations and of cell death and are predominantly repaired by homologous recombination (HR) and non-homologous end joining (NHEJ) in yeast (Paques and Haber, 1999). During HR, the damaged chromosome is repaired using the genetic information from an undamaged DNA molecule with which it shares extensive sequence homology. Thus, it is a relatively error-free repair process. In contrast, NHEJ ligates two DNA DSBs without requiring the extensive sequence homology between the DNA ends and thus it often alters the DNA sequence of the involved molecules.

Homologous recombination in yeast can involve gene conversion and/or crossing over. The preferred substrate for homologous recombination repair is the sister chromatid (Kadyk and Hartwell, 1992). Since sister chromatids are identical to each other, DNA damage is repaired faithfully, with no genetic consequences. Although the sister chromatid is preferred, homologs can serve as the repair template at a frequency 2-3-fold less lower (Kadyk and Hartwell, 1992). Gene conversion is defined as a non-reciprocal transfer of genetic information from one molecule to its homolog. Usually this occurs between two alleles of a gene, but it can embrace many contiguous genes, including the entire distal part of a

chromosome arm. Szostak et al. (1983) proposed a model to account for the frequent association of gene conversion with crossing-over. Central to this model is the formation of a recombination intermediate containing two Holliday junctions and the resolution of these Holliday junctions results in either non-crossover or crossover gene conversion products. It is now taken for granted that most, if not all, crossovers arise from the same transfers of DNA strands that cause gene conversion.

Genes important for the homologous recombination repair of DSBs were identified primarily by identifying mutants sensitive to ionizing radiation. These genes were classified as the *RAD52* epistasis group. A mutation in *rad52* was as radiation sensitive as a double mutant consisting of *rad52* and one of the other *rad* mutations (Game and Mortimer, 1974), indicating that *RAD52* is epistatic to the other *RAD* genes in the same pathway. Currently 10 genes fall into this group: *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD56*, *RAD57*, *MRE11*, and *XRS2* (Paques and Haber, 1999). *RAD52* is required for all homologous recombination events. *RAD51*, *RAD54*, *RAD55*, *RAD57*, *RAD50*, *MRE11*, and *XRS2* are required for some homologous recombination events but dispensable or less necessary for others (Paques and Haber, 1999).

Non-homologous end joining (NEHJ) is the alternative pathway to repair double-strand breaks. It requires the second DNA ligase of yeast (Schar et al., 1997; Wilson et al., 1997), termed LIG4 (Teo and Jackson, 1997). Re-ligation also

requires *RAD50*, *XRS2*, and *MRE11* (Milne and Weaver, 1996; Moore and Haber, 1996; Tsukamoto et al., 1996) and the two subunits of the yeast homologs of the mammalian Ku80 and Ku70 proteins, HDF1 and HDF2, respectively (Boulton and Jackson, 1996a; Boulton and Jackson, 1996b; Milne and Weaver, 1996). Ku protein, a heterodimer of Ku70 and Ku80, binds DNA in a non-sequence-dependent fashion and in a manner that relies on DNA DSBs (Dynan and Yoo, 1998). Ku forms an open ring-type structure that can be threaded onto a DNA end. One side of ring forms a cradle to protect the surface of the DNA double helix, whereas the other side is to allow other NHEJ components to access DSB.

Mammals In mammalian cells, the repair of DNA double-strand breaks (DSBs) also occurs by both homologous recombination and non-homologous end joining. It was considered that non-homologous mechanisms were used significantly more frequently than homologous one. However, more recently, homologous recombination has also been shown to be a major DSB repair pathway in mammalian cells (Liang et al., 1998).

The homologous recombination pathway is conserved in mammalian cells although the details of HR are considerably more complex. Homologs of the *S. cerevisiae* gene products in *RAD52* epistasis group are identified. The mammalian homologs of *RAD51*, *RAD52*, *RAD54*, *RAD54B*, *RAD50*, *MRE11*, *RAD51B*, *RAD51C*, *RAD51D*, *XRCC2*, and *XRCC3* have been shown to be

implicated in HR (Johnson and Jasin, 2001). In addition, the breast cancer susceptibility genes, *BRCA1* and *BRCA2* (Scully and Livingston, 2000), which do not appear to have yeast homologs, are also involved (Moynahan et al., 1999; Moynahan et al., 2001). A deficiency in homologous recombination is correlated with high rates of spontaneous chromosomal abnormalities (Johnson et al., 1999d; Pierce et al., 1999).

To introduce a double strand break at a specific locus, the I-*Sce*I endonuclease experimental assay in mammalian cells commonly used to investigate the molecular events of homologous recombination and the protein components involved makes use of I-*Sce*I, a rare cutting endonuclease. I-*Sce*I recognizes an 18-bp DNA substrate, which allows one to generate a site-specific double-strand break on an artificial DNA substrate without cutting other chromosomal DNA. Using this system, Jasin and colleagues showed that homologous recombination accounts for 30-40% of the double-strand break repair products, whereas NHEJ accounts for the remainder (Liang et al., 1998; Johnson and Jasin, 2000), indicating that homologous recombination is a prominent pathway for double-strand break repair. As in yeast, homologs, as well as sequence repeats located at ectopic positions on heterologs, are able to serve as repair templates for HR in double-strand break repair. However, in contrast to yeast, the frequency of using the repair templates from homologs and heterologs is 2-3 orders of magnitude lower than that of using the sister chromatid (Richardson et al., 1998; Johnson and Jasin, 2000). In addition, unlike in yeast, gene conversion events in

mammalian cells are not associated with crossing-over. This lack of crossing-over is observed whether repair involves sister chromatids or two different chromosomes (Richardson et al., 1998; Johnson and Jasin, 2000).

Non-homologous end joining (NHEJ), the rejoining of DNA ends with the use of little or no sequence homology, involves the processing of ends such that nucleotides are often deleted or inserted at the break site prior to ligation (Jeggo, 1998). Several processes exist in which repair of a double-strand break is restricted to either NHEJ or HR. For example, double-strand breaks introduced by the RAG proteins to generate antigen receptor diversity during V(D)J rearrangement are repaired by the NHEJ pathway (Jeggo, 1998), whereas those introduced during meiosis by the mammalian homolog of Spo11 are repaired by the HR pathway (Keeney, 2001). The proteins involved in NHEJ include Ku70, Ku80, DNA-PKcs and the XRCC4/DNA ligase IV complex. Ku70, Ku80, and DNA-PKcs form the DNA-dependent protein kinase complex (Jackson, 2002). The XRCC4/DNA ligase IV complex has been implicated more broadly in the general repair of chromosomal DSBs.

3.3. Ubc13-Mms2 pathway

3.3.1. *MMS2* and *UBC13* in yeast

In yeast, tolerating UV-induced DNA damage needs an additional pathway that is separate from error-free or error-prone translesion synthesis. This error-free pathway relies on the *UBC13* and *MMS2* gene products. Xiao and colleagues

(Broomfield et al., 1998; Brusky et al., 2000) cloned *MMS2* gene and *UBC13* gene in *S. cerevisiae*. *MMS2*, codes for a ubiquitin-conjugating-enzyme-like protein. Although Mms2 shares strong homology with most ubiquitin conjugating (Ubc) enzymes (E2), it does not possess the defining E2 active-site residues and thus a Ubc activity (Broomfield et al., 1998). Mms2 forms a specific heteromeric complex with Ubc13, a real E2. This interaction is required for the Ubc13-dependent assembly of polyubiquitin chain formation via Lys-63 (Hofmann and Pickart, 1999) instead of the conventional Lys-48 chain assembly mediated by most other Ubcs (Dubiel and Gordon, 1999). Evidence that *MMS2* functions in the *RAD6* pathway comes from the fact that *rad6* and *rad18* mutations are epistatic to *mms2* for UV- and MMS-sensitivity. Loss of Mms2 significantly increases the rate of spontaneous mutations in *S. cerevisiae* but only moderately increases the cytotoxic and mutagenic effects of UV_{254nm} radiation (Broomfield et al., 1998). These data indicate that Mms2 plays a role in an error-free damage avoidance pathway.

Brusky et al. (2000) showed that *UBC13* belongs to the *RAD6-RAD18* pathway, and is not involved in the same pathway as *REV3*, which is required for mutagenesis. The *ubc13* mutant displays up to a 30-fold increase in the spontaneous mutation rate and is proficient in UV-induced mutagenesis. These data indicate that *UBC13* is a member of the error-free damage tolerance pathway. Further genetic evidence showed that *UBC13* and *MMS2* belong to the same error-free damage tolerance pathway (Brusky et al., 2000). However, there

are no data yet in yeast demonstrating that the *MMS2* and *UBC13* gene products are involved in a damage avoidance pathway dealing with DNA lesions.

The crystal structure of the Mms2/Ubc13 heterodimer indicates that the active site of Ubc13 is located at the intersection of two channels, potential binding sites for two ubiquitins. Mutations that destabilize the heterodimer interface confer an increased UV sensitivity, providing direct evidence that the intact heterodimer is necessary for damage tolerance (VanDemark et al., 2001), although the mutagenesis effect of this destabilization was not investigated. In this *MMS2-UBC13* pathway, there are two sub-pathways, i.e., *RAD5* and *POL30* (Xiao et al., 2000).

RAD5 *RAD5* encodes a protein of 1,169 amino acids with the molecular weight of 134 kDa that has a single-strand DNA ATPase activity (Johnson et al., 1992; Johnson et al., 1994). Rad5 does not possess helicase activity, although it contains all seven conserved domains associated with DNA helicases (Johnson et al., 1994). Deletion of *rad5* mutants in yeast results in a ten-fold increase in spontaneous mutagenesis as indicated by the instability of poly(GT) repeat sequences (Johnson et al., 1992). *rad5* mutants are moderately sensitive to UV-irradiation and proficient in UV-induced mutagenesis, but are synergistic with *rev3* mutants for killing by UV. *RAD5* was assigned to the error-free branch of the *RAD6* pathway (Johnson et al., 1992). *rad5* mutants show an increased frequency of non-homologous end joining of DNA double-strand breaks (Ahne et

al., 1997). *rad5* and *rad18* mutants are hyper-recombinant, and the level of recombination of the *rad5 rad18* double mutant does not exceed levels seen in either single mutant (Liefshitz et al., 1998).

Ulrich and Jentsch (2000) showed that in yeast, Rad5 recruits the Mms2/Ubc13 complex to DNA through its RING finger domain, and that Rad5 interaction with Rad18 brings the Mms2/Ubc13 complex into contact with the Rad6/Rad18 complex. This result indicates that Rad5 acts as the adaptor protein to bring Mms2/Ubc3 into the contact of Rad6/Rad18.

POL30 *POL30* encodes, proliferating cell nuclear antigen (PCNA), an auxiliary subunit of DNA polymerase delta. The yeast *POL30* gene, cloned by Bauer and Burgers (1990), codes for an essential protein of 29 kDa and has 35% homology with human PCNA. Ayyagari et al. (1995) reported an interesting *pol30* mutant, *pol30-46*, which exhibits an increased sensitivity to the cytotoxic effect of UV and MMS but does not exhibit a defect in replication. In addition, the *pol30-46* mutation has no effect on growth or cell cycle progression, and the mutant PCNA protein interacts normally with DNA polymerase δ and ε. Genetic studies indicate that the *pol30-46* mutation is specifically defective in the *RAD6*-dependent pathway, and that this mutation impairs the error-free mode of post-replication repair. Furthermore, *pol30-46* does not affect UV-induced mutagenesis and is not involved in the same pathway as *REV3* (Torres-Ramos et al., 1996). These observations strongly indicate that *POL30* plays a role in an error-free pathway

dealing with DNA damage, similar to the role of *RAD5.* Furthermore, epistasis analyses reveal a synergistic effect of UV sensitivity between *rad5* and *pol30-46*, and this effect is similar to that of *mms2* mutants (Xiao et al., 2000). Taken together, these data indicate that *MMS2* and *UBC13* promote both error-free sub-pathways of *RAD5* and *POL30.*

3.3.2. *MMS2* and *UBC13* in human cells

The human homolog of *MMS2*, *hMMS2*, was cloned by Xiao et al. (1998). It encodes a 145 amino acids, 16.4 kDa protein with 50.4% identity to Mms2 throughout the entire length. In particular, the N-terminal half of the two proteins have 65% identity, including two highly conserved stretches with 13/13 and 13/16 amino acid sequence identity (Xiao et al., 1998). As in yeast, the human Mms2 and Ubc13 proteins form a stable complex *in vitro* (McKenna et al., 2001). hMms2 can associate with a Ubc13-ubiquitin thiol ester to form an activated heterodimer, which is capable of covalently binding of an untethered ubiquitin molecule, resulting in di-ubiquitin formation through Lys-63 (McKenna et al., 2001). The crystal structure of the hMms2-hUbc13 complex and a free hMms2 molecule reveals that the hMms2 monomer undergoes a conformational change upon interaction with hUbc13 (Moraes et al., 2001). The crystal structure also indicates that hMms2 prefers hUbc13 as a partner over a variety of other structurally similar ubiquitin-conjugating enzymes (Moraes et al., 2001). These relationships are very similar to what is known about the yeast homolog. However, no human homolog of *RAD5* has been identified yet.

Evidence of use of a homologous copy of a gene for damage avoidance in human cells comes from the earlier studies by Maher and McCormick and colleagues (Tsujimura et al., 1990; Bhattacharyya et al., 1990a and b; Zhang et al., 2000e). Bhattacharyya et al., (1990a) utilized the thymidine kinase (tk)-deficient human cell lines containing a single integrated copy of a plasmid carrying two copies of the herpes simplex virus thymidine kinase (Htk) gene, each containing an 8-base-pair XhoI linker inserted at a unique site. Expression of a functional Htk enzyme requires a productive recombinational event between the two nonfunctional genes. Following UV or BPDE treatment, wild type tk (tk+) cells were formed in a dose dependent manner, and analysis of tk+ cells indicated that majority of these cells were generated through gene conversion events unaccompanied by crossing-over. These results indicate that human cells are able to use a homologous allelic copy as an alternative template to circumvent DNA lesions. Cells containing a functional copy of the tk gene were resistant to HAT medium. A similar system in human cells that select for the formation of a wild type *hyg* gene generated from two non-functional *hyg* copies by homologous recombination provided similar results (Tsujimura et al., 1990). Using that assay system, they showed that un-excised DNA lesions, rather than excision repair processes, stimulate intrachromosomal recombination.

Using a human fibroblasts cell strain containing a *hyg* intrachromosomal recombination substrate stably integrated into the genome and using antisense RNA technology to eliminate hMms2 protein in human cells, Li et al. (2002)

recently showed that cells lacking hMms2 are devoid of UV-induced intrachromosomal gene conversion. The loss significantly increased the frequency of UV-induced *HPRT* mutants. These results indicate that when the ability to use an undamaged homologous copy as an alternative template is eliminated, human cells make increased use of translesion synthesis to tolerate DNA lesions.

References

Ahne, F., Jha, B. and Eckardt-Schupp, F. (1997) The RAD5 gene product is involved in the avoidance of non-homologous end-joining of DNA double strand breaks in the yeast Saccharomyces cerevisiae. Nucleic Acids Res, 25, 743-749.

Aoufouchi, S., Flatter, E., Dahan, A., Faili, A., Bertocci, B., Storck, S., Delbos, F., Cocea, L., Gupta, N., Weill, J.C. and Reynaud, C.A. (2000) Two novel human and mouse DNA polymerases of the polX family. Nucleic Acids Res, 28, 3684-3693.

Ayyagari, R., Impellizzeri, K.J., Yoder, B.L., Gary, S.L. and Burgers, P.M. (1995) A mutational analysis of the yeast proliferating cell nuclear antigen indicates distinct roles in DNA replication and DNA repair. Mol Cell Biol, 15, 4420-4429.

Bailly, V., Lamb, J., Sung, P., Prakash, S. and Prakash, L. (1994) Specific complex formation between yeast RAD6 and RAD18 proteins: a potential mechanism for targeting RAD6 ubiquitin-conjugating activity to DNA damage sites. Genes Dev, 8, 811-820.

Bailly, V., Lauder, S., Prakash, S. and Prakash, L. (1997a) Yeast DNA repair proteins Rad6 and Rad18 form a heterodimer that has ubiquitin conjugating, DNA binding, and ATP hydrolytic activities. J Biol Chem, 272, 23360-23365.

Bailly, V., Prakash, S. and Prakash, L. (1997b) Domains required for dimerization of yeast Rad6 ubiquitin-conjugating enzyme and Rad18 DNA binding protein. Mol Cell Biol, 17, 4536-4543.

Bauer, G.A. and Burgers, P.M. (1990) Molecular cloning, structure and expression of the yeast proliferating cell nuclear antigen gene. Nucleic Acids Res, 18, 261-265.

Bebenek, K., Tissier, A., Frank, E.G., McDonald, J.P., Prasad, R., Wilson, S.H., Woodgate, R. and Kunkel, T.A. (2001) 5'-Deoxyribose phosphate lyase activity of human DNA polymerase iota in vitro. Science, 291, 2156-2159.

Bemark, M., Khamlichi, A.A., Davies, S.L. and Neuberger, M.S. (2000) Disruption of mouse polymerase zeta (Rev3) leads to embryonic lethality and impairs blastocyst development in vitro. Curr Biol, 10, 1213-1216.

Bertocci, B., De Smet, A., Flatter, E., Dahan, A., Bories, J.C., Landreau, C., Weill, J.C. and Reynaud, C.A. (2002) Cutting edge: DNA polymerases mu and lambda are dispensable for Ig gene hypermutation. J Immunol, 168, 3702-3706.

Bhattacharyya, N.P., Maher, V.M. and McCormick, J.J. (1990a) Intrachromosomal homologous recombination in human cells which differ in nucleotide excision-repair capacity. Mutat Res, 234, 31-41.

Bhattacharyya, N.P., Maher, V.M. and McCormick, J.J. (1990b) Effect of nucleotide excision repair in human cells on intrachromosomal homologous recombination induced by UV and 1-nitrosopyrene. Mol Cell Biol, 10, 3945-3951.

Boulton, S.J. and Jackson, S.P. (1996a) Identification of a Saccharomyces cerevisiae Ku80 homolog: roles in DNA double strand break rejoining and in telomeric maintenance. Nucleic Acids Res, 24, 4639-4648.

Boulton, S.J. and Jackson, S.P. (1996b) Saccharomyces cerevisiae Ku70 potentiates illegitimate DNA double-strand break repair and serves as a barrier to error-prone DNA repair pathways. EMBO J, 15, 5093-5103.

Broomfield, S., Chow, B.L. and Xiao, W. (1998) MMS2, encoding a ubiquitin-conjugating-enzyme-like protein, is a member of the yeast error-free postreplication repair pathway. Proc Natl Acad Sci U S A, 95, 5678-5683.

Brotcorne-Lannoye, A. and Maenhaut-Michel, G. (1986) Role of RecA protein in untargeted UV mutagenesis of bacteriophage lambda: evidence for the requirement for the dinB gene. Proc Natl Acad Sci U S A, 83, 3904-3908.

Brusky, J., Zhu, Y. and Xiao, W. (2000) UBC13, a DNA-damage-inducible gene, is a member of the error-free postreplication repair pathway in Saccharomyces cerevisiae. Curr Genet, 37, 168-174.

Cassier-Chauvat, C. and Fabre, F. (1991) A similar defect in UV-induced mutagenesis conferred by the rad6 and rad18 mutations of Saccharomyces cerevisiae. Mutat Res, 254, 247-253.

Chiapperino, D., Kroth, H., Kramarczuk, I.H., Sayer, J.M., Masutani, C., Hanaoka, F., Jerina, D.M. and Cheh, A.M. (2002) Preferential misincorporation of purine nucleotides by human DNA polymerase eta opposite benzo[a]pyrene 7,8-diol 9,10-epoxide deoxyguanosine adducts. J Biol Chem, 277, 11765-11771.

Cordonnier, A.M., Lehmann, A.R. and Fuchs, R.P. (1999) Impaired translesion synthesis in xeroderma pigmentosum variant extracts. Mol Cell Biol, 19, 2206-2211.

Cox, B.S. and Parry, J.M. (1968) The isolation, genetics and survival characteristics of ultraviolet light-sensitive mutants in yeast. Mutat Res, 6, 37-55.

Cox, M.M., Goodman, M.F., Kreuzer, K.N., Sherratt, D.J., Sandler, S.J. and Marians, K.J. (2000) The importance of repairing stalled replication forks. Nature, 404, 37-41.

di Caprio, L. and Cox, B.S. (1981) DNA synthesis in UV-irradiated yeast. Mutat Res, 82, 69-85.

Diaz, M., Verkoczy, L.K., Flajnik, M.F. and Klinman, N.R. (2001) Decreased frequency of somatic hypermutation and impaired affinity maturation but intact germinal center formation in mice expressing antisense RNA to DNA polymerase zeta. J Immunol, 167, 327-335.

Dohmen, R.J., Madura, K., Bartel, B. and Varshavsky, A. (1991) The N-end rule is mediated by the UBC2(RAD6) ubiquitin-conjugating enzyme. Proc Natl Acad Sci U S A, 88, 7351-7355.

Dominguez, O., Ruiz, J.F., Lain de Lera, T., Garcia-Diaz, M., Gonzalez, M.A., Kirchhoff, T., Martinez, A.C., Bernad, A. and Blanco, L. (2000) DNA polymerase mu (Pol mu), homologous to TdT, could act as a DNA mutator in eukaryotic cells. EMBO J, 19, 1731-1742.

Dubiel, W. and Gordon, C. (1999) Ubiquitin pathway: another link in the polyubiquitin chain? Curr Biol, 9, R554-557.

Dynan, W.S. and Yoo, S. (1998) Interaction of Ku protein and DNA-dependent protein kinase catalytic subunit with nucleic acids. Nucleic Acids Res, 26,1551-1559.

Ensch-Simon, I., Burgers, P.M. and Taylor, J.S. (1998) Bypass of a site-specific cis-Syn thymine dimer in an SV40 vector during in vitro replication by HeLa and XPV cell-free extracts. Biochemistry, 37, 8218-8226.

Esposito, G., Godindagger, I., Klein, U., Yaspo, M.L., Cumano, A. and Rajewsky, K. (2000) Disruption of the Rev3l-encoded catalytic subunit of polymerase zeta in mice results in early embryonic lethality. Curr Biol, 10, 1221-1224.

Fernandez De Henestrosa, A.R., Ogi, T., Aoyagi, S., Chafin, D., Hayes, J.J., Ohmori, H. and Woodgate, R. (2000) Identification of additional genes belonging to the LexA regulon in Escherichia coli. Mol Microbiol, 35, 1560-1572.

Friedberg, E.C., Walker, G.C., and Siede, W. (1995) DNA Repair and Mutagenesis, Am. Soc. Microbiol. Press, Washington, D.C.

Game, J.C. and Mortimer, R.K. (1974) A genetic study of x-ray sensitive mutants in yeast. Mutat Res, 24, 281-292.

Ganesan, A.K. (1974) Persistence of pyrimidine dimers during post-replication repair in ultraviolet light-irradiated Escherichia coli K12. J Mol Biol, 87, 103-119.

Garcia-Diaz, M., Dominguez, O., Lopez-Fernandez, L.A., de Lera, L.T., Saniger, M.L., Ruiz, J.F., Parraga, M., Garcia-Ortiz, M.J., Kirchhoff, T., del Mazo, J., Bernad, A. and Blanco, L. (2000) DNA polymerase lambda (Pol lambda), a novel eukaryotic DNA polymerase with a potential role in meiosis. J Mol Biol, 301, 851-867.

Geigl, E.M. and Eckardt-Schupp, F. (1991) Repair of gamma ray-induced S1 nuclease hypersensitive sites in yeast depends on homologous mitotic recombination and a RAD18-dependent function. Curr Genet, 20, 33-37.

Gerik, K.J., Li, X., Pautz, A. and Burgers, P.M. (1998) Characterization of the two small subunits of Saccharomyces cerevisiae DNA polymerase delta. J Biol Chem, 273, 19747-19755.

Gerlach, V.L., Aravind, L., Gotway, G., Schultz, R.A., Koonin, E.V. and Friedberg, E.C. (1999) Human and mouse homologs of Escherichia coli DinB (DNA polymerase IV), members of the UmuC/DinB superfamily. Proc Natl Acad Sci U S A, 96, 11922-11927.

Gerlach, V.L., Feaver, W.J., Fischhaber, P.L. and Friedberg, E.C. (2001) Purification and characterization of pol kappa, a DNA polymerase encoded by the human DINB1 gene. J Biol Chem, 276, 92-98.

Gibbs, P.E., McGregor, W.G., Maher, V.M., Nisson, P. and Lawrence, C.W. (1998) A human homolog of the Saccharomyces cerevisiae REV3 gene, which encodes the catalytic subunit of DNA polymerase zeta. Proc Natl Acad Sci U S A, 95, 6876-6880.

Gibbs, P.E., Wang, X.D., Li, Z., McManus, T.P., McGregor, W.G., Lawrence, C.W. and Maher, V.M. (2000) The function of the human homolog of Saccharomyces cerevisiae REV1 is required for mutagenesis induced by UV light. Proc Natl Acad Sci U S A, 97, 4186-4191.

Glick, E., Vigna, K.L. and Loeb, L.A. (2001) Mutations in human DNA polymerase eta motif II alter bypass of DNA lesions. EMBO J, 20, 7303-12.

Guo, D., Wu, X., Rajpal, D.K., Taylor, J.S. and Wang, Z. (2001) Translesion synthesis by yeast DNA polymerase zeta from templates containing lesions of ultraviolet radiation and acetylaminofluorene. Nucleic Acids Res, 29, 2875-2883.

Haber, J.E. (1999) DNA recombination: the replication connection. Trends Biochem Sci, 24, 271-275.

Haracska, L., Prakash, S. and Prakash, L. (2000a) Replication past O(6)-methylguanine by yeast and human DNA polymerase eta. Mol Cell Biol, 20, 8001-8007.

Haracska, L., Yu, S.L., Johnson, R.E., Prakash, L. and Prakash, S. (2000b) Efficient and accurate replication in the presence of 7,8-dihydro-8-oxoguanine by DNA polymerase eta. Nat Genet, 25, 458-461.

Haracska, L., Unk, I., Johnson, R.E., Johansson, E., Burgers, P.M., Prakash, S. and Prakash, L. (2001a) Roles of yeast DNA polymerases delta and zeta and of Rev1 in the bypass of abasic sites. Genes Dev, 15, 945-954.

Haracska, L., Kondratick, C.M., Unk, I., Prakash, S. and Prakash, L. (2001b) Interaction with PCNA is essential for yeast DNA polymerase eta function. Mol Cell, 8, 407-415.

Haracska, L., Johnson, R.E., Unk, I., Phillips, B., Hurwitz, J., Prakash, L. and Prakash, S. (2001c) Physical and functional interactions of human DNA polymerase eta with PCNA. Mol Cell Biol, 21, 7199-7206.

Haracska, L., Johnson, R.E., Unk, I., Phillips, B.B., Hurwitz, J., Prakash, L. and Prakash, S. (2001d) Targeting of human DNA polymerase iota to the replication machinery via interaction with PCNA. Proc Natl Acad Sci U S A, 98, 14256-14261.

Haracska, L., Prakash, S. and Prakash, L. (2002a) Yeast Rev1 Protein Is a G Template-specific DNA Polymerase. J Biol Chem, 277, 15546-15551.

Haracska, L., Unk, I., Johnson, R.E., Phillips, B.B., Hurwitz, J., Prakash, L. and Prakash, S. (2002b) Stimulation of DNA synthesis activity of human DNA polymerase kappa by PCNA. Mol Cell Biol, 22, 784-791.

Higgins, N.P., Kato, K. and Strauss, B. (1976) A model for replication repair in mammalian cells. J Mol Biol, 101, 417-425.

Hofmann, R.M. and Pickart, C.M. (1999) Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. Cell, 96, 645-653.

Huang, H., Kahana, A., Gottschling, D.E., Prakash, L. and Liebman, S.W. (1997) The ubiquitin-conjugating enzyme Rad6 (Ubc2) is required for silencing in Saccharomyces cerevisiae. Mol Cell Biol, 17, 6693-6699.

Jackson, S.P. (2002) Sensing and repairing DNA double-strand breaks. Carcinogenesis, 23, 687-696.

Jeggo, P.A. (1998) Identification of genes involved in repair of DNA double-strand breaks in mammalian cells. Radiat Res, 150, S80-91.

Jentsch, S., McGrath, J.P. and Varshavsky, A. (1987) The yeast DNA repair gene RAD6 encodes a ubiquitin-conjugating enzyme. Nature, 329, 131-134.

Johnson, R.D. and Jasin, M. (2000) Sister chromatid gene conversion is a prominent double-strand break repair pathway in mammalian cells. EMBO J, 19, 3398-407.

Johnson, R.D. and Jasin, M. (2001) Double-strand-break-induced homologous recombination in mammalian cells. Biochem Soc Trans, 29, 196-201.

Johnson, R.E., Henderson, S.T., Petes, T.D., Prakash, S., Bankmann, M. and Prakash, L. (1992) Saccharomyces cerevisiae RAD5-encoded DNA repair protein contains DNA helicase and zinc-binding sequence motifs and affects the stability of simple repetitive sequences in the genome. Mol Cell Biol, 12, 3807-3818.

Johnson, R.E., Prakash, S. and Prakash, L. (1994) Yeast DNA repair protein RAD5 that promotes instability of simple repetitive sequences is a DNA-dependent ATPase. J Biol Chem, 269, 28259-28262.

Johnson, R.E., Prakash, S. and Prakash, L. (1999a) Requirement of DNA polymerase activity of yeast Rad30 protein for its biological function. J Biol Chem, 274, 15975-15977.

Johnson, R.E., Prakash, S. and Prakash, L. (1999b) Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Poleta. Science, 283, 1001-1004.

Johnson, R.E., Kondratick, C.M., Prakash, S. and Prakash, L. (1999c) hRAD30 mutations in the variant form of xeroderma pigmentosum. Science, 285, 263-265.

Johnson, R.D., Liu, N. and Jasin, M. (1999d) Mammalian XRCC2 promotes the repair of DNA double-strand breaks by homologous recombination. Nature, 401, 397-399.

Johnson, R.E., Washington, M.T., Haracska, L., Prakash, S. and Prakash, L. (2000) Eukaryotic polymerases iota and zeta act sequentially to bypass DNA lesions. Nature, 406, 1015-1019.

Johnson, R.E., Haracska, L., Prakash, S. and Prakash, L. (2001) Role of DNA polymerase zeta in the bypass of a (6-4) TT photoproduct. Mol Cell Biol, 21, 3558-3563.

Kadyk, L.C. and Hartwell, L.H. (1992) Sister chromatids are preferred over homologs as substrates for recombinational repair in Saccharomyces cerevisiae. Genetics, 132, 387-402.

Kannouche, P., Broughton, B.C., Volker, M., Hanaoka, F., Mullenders, L.H. and Lehmann, A.R. (2001) Domain structure, localization, and function of DNA polymerase eta, defective in xeroderma pigmentosum variant cells. Genes Dev, 15, 158-172.

Kawamura, K., J, O.W., Bahar, R., Koshikawa, N., Shishikura, T., Nakagawara, A., Sakiyama, S., Kajiwara, K., Kimura, M. and Tagawa, M. (2001) The error-prone DNA polymerase zeta catalytic subunit (Rev3) gene is ubiquitously expressed in normal and malignant human tissues. Int J Oncol, 18, 97-103.

Keeney, S. (2001) Mechanism and control of meiotic recombination initiation. Curr Top Dev Biol, 52, 1-53.

Kenyon, C.J. and Walker, G.C. (1980) DNA-damaging agents stimulate gene expression at specific loci in Escherichia coli. Proc Natl Acad Sci U S A, 77, 2819-2823.

Kim, S.R., Maenhaut-Michel, G., Yamada, M., Yamamoto, Y., Matsui, K., Sofuni, T., Nohmi, T. and Ohmori, H. (1997) Multiple pathways for SOS-induced mutagenesis in Escherichia coli: an overexpression of dinB/dinP results in strongly enhancing mutagenesis in the absence of any exogenous treatment to damage DNA. Proc Natl Acad Sci U S A, 94, 13792-13797.

Kim, S.R., Matsui, K., Yamada, M., Gruz, P. and Nohmi, T. (2001) Roles of chromosomal and episomal dinB genes encoding DNA pol IV in targeted and untargeted mutagenesis in Escherichia coli. Mol Genet Genomics, 266, 207-215.

Koken, M.H., Hoogerbrugge, J.W., Jasper-Dekker, I., de Wit, J., Willemsen, R., Roest, H.P., Grootegoed, J.A. and Hoeijmakers, J.H. (1996) Expression of the ubiquitin-conjugating DNA repair enzymes HHR6A and B suggests a role in spermatogenesis and chromatin modification. Dev Biol, 173, 119-132.

Koken, M.H., Reynolds, P., Jaspers-Dekker, I., Prakash, L., Prakash, S., Bootsma, D. and Hoeijmakers, J.H. (1991) Structural and functional conservation of two human homologs of the yeast DNA repair gene RAD6. Proc Natl Acad Sci U S A, 88, 8865-8869.

Koken, M.H., Smit, E.M., Jaspers-Dekker, I., Oostra, B.A., Hagemeijer, A., Bootsma, D. and Hoeijmakers, J.H. (1992) Localization of two human homologs, HHR6A and HHR6B, of the yeast DNA repair gene RAD6 to chromosomes Xq24-q25 and 5q23-q31. Genomics, 12, 447-4453.

Kondratick, C.M., Washington, M.T., Prakash, S. and Prakash, L. (2001) Acidic residues critical for the activity and biological function of yeast DNA polymerase eta. Mol Cell Biol, 21, 2018-2025.

Kowalczykowski, S.C. (2000) Initiation of genetic recombination and recombination-dependent replication. Trends Biochem Sci, 25, 156-165.

Kusumoto, R., Masutani, C., Iwai, S. and Hanaoka, F. (2002) Translesion synthesis by human DNA polymerase eta across thymine glycol lesions. Biochemistry, 41, 6090-6099.

Kuzminov, A. (2001) DNA replication meets genetic exchange: chromosomal damage and its repair by homologous recombination. Proc Natl Acad Sci U S A, 98, 8461-8468.

Larimer, F.W., Perry, J.R. and Hardigree, A.A. (1989) The REV1 gene of Saccharomyces cerevisiae: isolation, sequence, and functional analysis. J Bacteriol, 171, 230-237.

Lawrence, C.W. and Christensen, R. (1976) UV mutagenesis in radiation-sensitive strains of yeast. Genetics, 82, 207-232.

Lawrence, C.W. and Hinkle, D.C. (1996) DNA polymerase zeta and the control of DNA damage induced mutagenesis in eukaryotes. Cancer Surv, 28, 21-31.

Lawrence, C.W., Das, G. and Christensen, R.B. (1985a) REV7, a new gene concerned with UV mutagenesis in yeast. Mol Gen Genet, 200, 80-85.

Lawrence, C.W., Nisson, P.E. and Christensen, R.B. (1985b) UV and chemical mutagenesis in rev7 mutants of yeast. Mol Gen Genet, 200, 86-91.

Lemontt, J.F. (1971) Pathways of ultraviolet mutability in Saccharomyces cerevisiae. I. Some properties of double mutants involving uvs9 and rev. Mutat Res, 13, 311-7.

Lenne-Samuel, N., Wagner, J., Etienne, H. and Fuchs, R.P. (2002) The processivity factor beta controls DNA polymerase IV traffic during spontaneous mutagenesis and translesion synthesis in vivo. EMBO Rep, 3, 45-49.

Levine, R.L., Miller, H., Grollman, A., Ohashi, E., Ohmori, H., Masutani, C., Hanaoka, F. and Moriya, M. (2001) Translesion DNA synthesis catalyzed by human pol eta and pol kappa across 1,N6-ethenodeoxyadenosine. J Biol Chem, 276, 18717-18721.

Li, Z., Xiao, W., McCormick, J.J. and Maher, V.M. (2002) Identification of a protein essential for a major pathway used by human cells to avoid UV- induced DNA damage. Proc Natl Acad Sci U S A, 99, 4459-4464.

Liang, F., Han, M., Romanienko, P.J. and Jasin, M. (1998) Homology-directed repair is a major double-strand break repair pathway in mammalian cells. Proc Natl Acad Sci U S A, 95, 5172-5177.

Liefshitz, B., Steinlauf, R., Friedl, A., Eckardt-Schupp, F. and Kupiec, M. (1998) Genetic interactions between mutants of the 'error-prone' repair group of Saccharomyces cerevisiae and their effect on recombination and mutagenesis. Mutat Res, 407, 135-145.

Lin, W., Wu, X. and Wang, Z. (1999a) A full-length cDNA of hREV3 is predicted to encode DNA polymerase zeta for damage-induced mutagenesis in human cells. Mutat Res, 433, 89-98.

Lin, W., Xin, H., Zhang, Y., Wu, X., Yuan, F. and Wang, Z. (1999b) The human REV1 gene codes for a DNA template-dependent dCMP transferase. Nucleic Acids Res, 27, 4468-4475.

Ling, H., Boudsocq, F., Woodgate, R. and Yang, W. (2001) Crystal structure of a Y-family DNA polymerase in action: a mechanism for error-prone and lesion-bypass replication. Cell, 107, 91-102.

Maher, V.M., Ouellette, L.M., Curren, R.D. and McCormick, J.J. (1976) Frequency of ultraviolet light-induced mutations is higher in xeroderma pigmentosum variant cells than in normal human cells. Nature, 261, 593-595.

Masuda, Y., Takahashi, M., Tsunekuni, N., Minami, T., Sumii, M., Miyagawa, K. and Kamiya, K. (2001) Deoxycytidyl transferase activity of the human REV1 protein is closely associated with the conserved polymerase domain. J Biol Chem, 276, 15051-15058.

Masutani, C., Kusumoto, R., Yamada, A., Dohmae, N., Yokoi, M., Yuasa, M., Araki, M., Iwai, S., Takio, K. and Hanaoka, F. (1999a) The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta. Nature, 399, 700-704.

Masutani, C., Araki, M., Yamada, A., Kusumoto, R., Nogimori, T., Maekawa, T., Iwai, S. and Hanaoka, F. (1999b) Xeroderma pigmentosum variant (XP-V) correcting protein from HeLa cells has a thymine dimer bypass DNA polymerase activity. EMBO J, 18, 3491-3501.

Masutani, C., Kusumoto, R., Iwai, S. and Hanaoka, F. (2000) Mechanisms of accurate translesion synthesis by human DNA polymerase eta. EMBO J, 19, 3100-3109.

Matsuda, T., Bebenek, K., Masutani, C., Hanaoka, F. and Kunkel, T.A. (2000) Low fidelity DNA synthesis by human DNA polymerase-eta. Nature, 404, 1011-1013.

McDonald, J.P., Levine, A.S. and Woodgate, R. (1997) The Saccharomyces cerevisiae RAD30 gene, a homolog of Escherichia coli dinB and umuC, is DNA damage inducible and functions in a novel error-free postreplication repair mechanism. Genetics, 147, 1557-1568.

McDonald, J.P., Rapic-Otrin, V., Epstein, J.A., Broughton, B.C., Wang, X., Lehmann, A.R., Wolgemuth, D.J. and Woodgate, R. (1999) Novel human and mouse homologs of Saccharomyces cerevisiae DNA polymerase eta. Genomics, 60, 20-30.

McKenna, S., Spyracopoulos, L., Moraes, T., Pastushok, L., Ptak, C., Xiao, W. and Ellison, M.J. (2001) Noncovalent interaction between ubiquitin and the human DNA repair protein Mms2 is required for Ubc13-mediated polyubiquitination. J Biol Chem, 276, 40120-40126.

Milne, G.T., Jin, S., Shannon, K.B. and Weaver, D.T. (1996) Mutations in two Ku homologs define a DNA end-joining repair pathway in Saccharomyces cerevisiae. Mol Cell Biol, 16, 4189-4198.

Montelone, B.A., Prakash, S. and Prakash, L. (1981) Recombination and mutagenesis in rad6 mutants of Saccharomyces cerevisiae: evidence for multiple functions of the RAD6 gene. Mol Gen Genet, 184, 410-415.

Moore, J.K. and Haber, J.E. (1996) Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in Saccharomyces cerevisiae. Mol Cell Biol, 16, 2164-2173.

Moraes, T.F., Edwards, R.A., McKenna, S., Pastushok, L., Xiao, W., Glover, J.N. and Ellison, M.J. (2001) Crystal structure of the human ubiquitin conjugating enzyme complex, hMms2-hUbc13. Nat Struct Biol, 8, 669-673.

Morelli, C., Mungall, A.J., Negrini, M., Barbanti-Brodano, G. and Croce, C.M. (1998) Alternative splicing, genomic structure, and fine chromosome localization of REV3L. Cytogenet Cell Genet, 83, 18-20.

Morrison, A., Christensen, R.B., Alley, J., Beck, A.K., Bernstine, E.G., Lemontt, J.F. and Lawrence, C.W. (1989) REV3, a Saccharomyces cerevisiae gene

whose function is required for induced mutagenesis, is predicted to encode a nonessential DNA polymerase. J Bacteriol, 171, 5659-5667.

Morrison, A., Miller, E.J. and Prakash, L. (1988) Domain structure and functional analysis of the carboxyl-terminal polyacidic sequence of the RAD6 protein of Saccharomyces cerevisiae. Mol Cell Biol, 8, 1179-1185.

Mowat, M.R., Jachymczyk, W.J., Hastings, P.J. and von Borstel, R.C. (1983) Repair of gamma-ray induced DNA strand breaks in the radiation-sensitive mutant rad18-2 of Saccharomyces cerevisiae. Mol Gen Genet, 189, 256-262.

Moynahan, M.E., Chiu, J.W., Koller, B.H. and Jasin, M. (1999) Brca1 controls homology-directed DNA repair. Mol Cell, 4, 511-518.

Moynahan, M.E., Pierce, A.J. and Jasin, M. (2001) BRCA2 is required for homology-directed repair of chromosomal breaks. Mol Cell, 7, 263-272.

Murakumo, Y., Ogura, Y., Ishii, H., Numata, S., Ichihara, M., Croce, C.M., Fishel, R. and Takahashi, M. (2001) Interactions in the error-prone postreplication repair proteins hREV1, hREV3, and hREV7. J Biol Chem, 276, 35644-35651.

Murakumo, Y., Roth, T., Ishii, H., Rasio, D., Numata, S., Croce, C.M. and Fishel, R. (2000) A human REV7 homolog that interacts with the polymerase zeta catalytic subunit hREV3 and the spindle assembly checkpoint protein hMAD2. J Biol Chem, 275, 4391-4397.

Napolitano, R., Janel-Bintz, R., Wagner, J. and Fuchs, R.P. (2000) All three SOS-inducible DNA polymerases (Pol II, Pol IV and Pol V) are involved in induced mutagenesis. EMBO J, 19, 6259-6265.

Nelson, J.R., Lawrence, C.W. and Hinkle, D.C. (1996a) Thymine-thymine dimer bypass by yeast DNA polymerase zeta. Science, 272, 1646-1649.

Nelson, J.R., Lawrence, C.W. and Hinkle, D.C. (1996b) Deoxycytidyl transferase activity of yeast REV1 protein. Nature, 382, 729-731.

Nelson, J.R., Gibbs, P.E., Nowicka, A.M., Hinkle, D.C. and Lawrence, C.W. (2000) Evidence for a second function for Saccharomyces cerevisiae Rev1p. Mol Microbiol, 37, 549-554.

Nikolaishvili-Feinberg, N. and Cordeiro-Stone, M. (2000) Discrimination between translesion synthesis and template switching during bypass replication of thymine dimers in duplex DNA. J Biol Chem, 275, 30943-30950.

Nohmi, T., Battista, J.R., Dodson, L.A. and Walker, G.C. (1988) RecA-mediated cleavage activates UmuD for mutagenesis: mechanistic relationship between

transcriptional derepression and posttranslational activation. Proc Natl Acad Sci U S A, 85, 1816-1820.

Ogi, T., Kato, T., Jr., Kato, T. and Ohmori, H. (1999) Mutation enhancement by DINB1, a mammalian homolog of the Escherichia coli mutagenesis protein dinB. Genes Cells, 4, 607-618.

Ohashi, E., Bebenek, K., Matsuda, T., Feaver, W.J., Gerlach, V.L., Friedberg, E.C., Ohmori, H. and Kunkel, T.A. (2000a) Fidelity and processivity of DNA synthesis by DNA polymerase kappa, the product of the human DINB1 gene. J Biol Chem, 275, 39678-39684.

Ohashi, E., Ogi, T., Kusumoto, R., Iwai, S., Masutani, C., Hanaoka, F. and Ohmori, H. (2000b) Error-prone bypass of certain DNA lesions by the human DNA polymerase kappa. Genes Dev, 14, 1589-1594.

Opperman, T., Murli, S., Smith, B.T. and Walker, G.C. (1999) A model for a umuDC-dependent prokaryotic DNA damage checkpoint. Proc Natl Acad Sci U S A, 96, 9218-9223.

O-Wang, J., Kawamura, K., Tada, Y., Ohmori, H., Kimura, H., Sakiyama, S. and Tagawa, M. (2001) DNA polymerase kappa, implicated in spontaneous and DNA damage-induced mutagenesis, is overexpressed in lung cancer. Cancer Res, 61, 5366-5369.

Paques, F. and Haber, J.E. (1999) Multiple pathways of recombination induced by double-strand breaks in Saccharomyces cerevisiae. Microbiol Mol Biol Rev, 63, 349-404.

Pavlov, Y.I., Shcherbakova, P.V. and Kunkel, T.A. (2001) In vivo consequences of putative active site mutations in yeast DNA polymerases alpha, epsilon, delta, and zeta. Genetics, 159, 47-64.

Pierce, A.J., Johnson, R.D., Thompson, L.H. and Jasin, M. (1999) XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. Genes Dev, 13, 2633-2638.

Prakash, S., Sung, P. and Prakash, L. (1993) DNA repair genes and proteins of Saccharomyces cerevisiae. Annu Rev Genet, 27, 33-70.

Rangarajan, S., Woodgate, R. and Goodman, M.F. (1999) A phenotype for enigmatic DNA polymerase II: a pivotal role for pol II in replication restart in UV-irradiated Escherichia coli. Proc Natl Acad Sci U S A, 96, 9224-9229.

Rangarajan, S., Woodgate, R. and Goodman, M.F. (2002) Replication restart in UV-irradiated Escherichia coli involving pols II, III, V, PriA, RecA and RecFOR proteins. Mol Microbiol, 43, 617-628.

Reuven, N.B., Tomer, G. and Livneh, Z. (1998) The mutagenesis proteins UmuD' and UmuC prevent lethal frameshifts while increasing base substitution mutations. Mol Cell, 2, 191-199.

Reuven, N.B., Arad, G., Maor-Shoshani, A. and Livneh, Z. (1999) The mutagenesis protein UmuC is a DNA polymerase activated by UmuD', RecA, and SSB and is specialized for translesion replication. J Biol Chem, 274, 31763-31766.

Richardson, C., Moynahan, M.E. and Jasin, M. (1998) Double-strand break repair by interchromosomal recombination: suppression of chromosomal translocations. Genes Dev, 12, 3831-3842.

Robzyk, K., Recht, J. and Osley, M.A. (2000) Rad6-dependent ubiquitination of histone H2B in yeast. Science, 287, 501-504.

Roest, H.P., van Klaveren, J., de Wit, J., van Gurp, C.G., Koken, M.H., Vermey, M., van Roijen, J.H., Hoogerbrugge, J.W., Vreeburg, J.T., Baarends, W.M., Bootsma, D., Grootegoed, J.A. and Hoeijmakers, J.H. (1996) Inactivation of the HR6B ubiquitin-conjugating DNA repair enzyme in mice causes male sterility associated with chromatin modification. Cell, 86, 799-810.

Roush, A.A., Suarez, M., Friedberg, E.C., Radman, M. and Siede, W. (1998) Deletion of the Saccharomyces cerevisiae gene RAD30 encoding an Escherichia coli DinB homolog confers UV radiation sensitivity and altered mutability. Mol Gen Genet, 257, 686-692.

Rupp, W.D. and Howard-Flanders, P. (1968) Discontinuities in the DNA synthesized in an excision-defective strain of Escherichia coli following ultraviolet irradiation. J Mol Biol, 31, 291-304.

Rupp, W.D., Wilde, C.E., 3rd, Reno, D.L. and Howard-Flanders, P. (1971) Exchanges between DNA strands in ultraviolet-irradiated Escherichia coli. J Mol Biol, 61, 25-44.

Sarcevic, B., Mawson, A., Baker, R.T. and Sutherland, R.L. (2002) Regulation of the ubiquitin-conjugating enzyme hHR6A by CDK-mediated phosphorylation. EMBO J, 21, 2009-2018.

Schar, P., Herrmann, G., Daly, G. and Lindahl, T. (1997) A newly identified DNA ligase of Saccharomyces cerevisiae involved in RAD52-independent repair of DNA double-strand breaks. Genes Dev, 11, 1912-1924.

Schneider, R., Eckerskorn, C., Lottspeich, F. and Schweiger, M. (1990) The human ubiquitin carrier protein E2(Mr = 17,000) is homologous to the yeast DNA repair gene RAD6. EMBO J. 9, 1431-1435.

Scully, R. and Livingston, D.M. (2000) In search of the tumour-suppressor functions of BRCA1 and BRCA2. Nature, 408, 429-432.

Shekhar, M.P., Lyakhovich, A., Visscher, D.W., Heng, H. and Kondrat, N. (2002) Rad6 overexpression induces multinucleation, centrosome amplification, abnormal mitosis, aneuploidy, and transformation. Cancer Res, 62, 2115-2124.

Shinagawa, H., Iwasaki, H., Kato, T. and Nakata, A. (1988) RecA protein-dependent cleavage of UmuD protein and SOS mutagenesis. Proc Natl Acad Sci U S A, 85, 1806-1810.

Singh, J., Goel, V. and Klar, A.J. (1998) A novel function of the DNA repair gene rhp6 in mating-type silencing by chromatin remodeling in fission yeast. Mol Cell Biol, 18, 5511-5522.

Steinborn, G. (1978) Uvm mutants of Escherichia coli K12 deficient in UV mutagenesis. I. Isolation of uvm mutants and their phenotypical characterization in DNA repair and mutagenesis. Mol Gen Genet, 165, 87-93.

Strauss, B.S., Roberts, R., Francis, L. and Pouryazdanparast, P. (2000) Role of the dinB gene product in spontaneous mutation in Escherichia coli with an impaired replicative polymerase. J Bacteriol, 182, 6742-6750.

Sung, P., Prakash, S. and Prakash, L. (1988) The RAD6 protein of Saccharomyces cerevisiae polyubiquitinates histones, and its acidic domain mediates this activity. Genes Dev, 2, 1476-1485.

Sung, P., Prakash, S. and Prakash, L. (1990) Mutation of cysteine-88 in the Saccharomyces cerevisiae RAD6 protein abolishes its ubiquitin-conjugating activity and its various biological functions. Proc Natl Acad Sci U S A, 87, 2695-2699.

Sung, P., Berleth, E., Pickart, C., Prakash, S. and Prakash, L. (1991) Yeast RAD6 encoded ubiquitin conjugating enzyme mediates protein degradation dependent on the N-end-recognizing E3 enzyme. EMBO J, 10, 2187-93.

Sutton, M.D., Farrow, M.F., Burton, B.M. and Walker, G.C. (2001) Genetic interactions between the Escherichia coli umuDC gene products and the beta processivity clamp of the replicative DNA polymerase. J Bacteriol, 183, 2897-2909.

Sutton, M.D., Narumi, I. and Walker, G.C. (2002) Posttranslational modification of the umuD-encoded subunit of Escherichia coli DNA polymerase V regulates its interactions with the beta processivity clamp. Proc Natl Acad Sci U S A, 99, 5307-5312.

Suzuki, N., Ohashi, E., Kolbanovskiy, A., Geacintov, N.E., Grollman, A.P., Ohmori, H. and Shibutani, S. (2002) Translesion synthesis by human DNA polymerase kappa on a DNA template containing a single stereoisomer of dG-(+)- or dG-(-)-anti-N(2)-BPDE (7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene). Biochemistry, 41, 6100-6106.

Svoboda, D.L., Briley, L.P. and Vos, J.M. (1998) Defective bypass replication of a leading strand cyclobutane thymine dimer in xeroderma pigmentosum variant cell extracts. Cancer Res, 58, 2445-2448.

Szostak, J.W., Orr-Weaver, T.L., Rothstein, R.J. and Stahl, F.W. (1983) The double-strand-break repair model for recombination. Cell, 33, 25-35.

Tang, M., Bruck, I., Eritja, R., Turner, J., Frank, E.G., Woodgate, R., O'Donnell, M. and Goodman, M.F. (1998) Biochemical basis of SOS-induced mutagenesis in Escherichia coli: reconstitution of in vitro lesion bypass dependent on the UmuD'2C mutagenic complex and RecA protein. Proc Natl Acad Sci U S A, 95, 9755-9760.

Tang, M., Shen, X., Frank, E.G., O'Donnell, M., Woodgate, R. and Goodman, M.F. (1999) UmuD'(2)C is an error-prone DNA polymerase, Escherichia coli pol V. Proc Natl Acad Sci U S A, 96, 8919-8924.

Tang, M., Pham, P., Shen, X., Taylor, J.S., O'Donnell, M., Woodgate, R. and Goodman, M.F. (2000) Roles of E. coli DNA polymerases IV and V in lesion-targeted and untargeted SOS mutagenesis. Nature, 404, 1014-1018.

Tateishi, S., Sakuraba, Y., Masuyama, S., Inoue, H. and Yamaizumi, M. (2000) Dysfunction of human Rad18 results in defective postreplication repair and hypersensitivity to multiple mutagens. Proc Natl Acad Sci U S A, 97, 7927-7932.

Teo, S.H. and Jackson, S.P. (1997) Identification of Saccharomyces cerevisiae DNA ligase IV: involvement in DNA double-strand break repair. EMBO J, 16, 4788-4795.

Tissier, A., McDonald, J.P., Frank, E.G. and Woodgate, R. (2000a) poliota, a remarkably error-prone human DNA polymerase. Genes Dev, 14, 1642-1650.

Tissier, A., Frank, E.G., McDonald, J.P., Iwai, S., Hanaoka, F. and Woodgate, R. (2000b) Misinsertion and bypass of thymine-thymine dimers by human DNA polymerase iota. EMBO J, 19, 5259-5266.

Torpey, L.E., Gibbs, P.E., Nelson, J. and Lawrence, C.W. (1994) Cloning and sequence of REV7, a gene whose function is required for DNA damage-induced mutagenesis in Saccharomyces cerevisiae. Yeast, 10, 1503-1509.

Torres-Ramos, C.A., Yoder, B.L., Burgers, P.M., Prakash, S. and Prakash, L. (1996) Requirement of proliferating cell nuclear antigen in RAD6-dependent postreplicational DNA repair. Proc Natl Acad Sci U S A, 93, 9676-9681.

Torres-Ramos, C.A., Prakash, S. and Prakash, L. (1997) Requirement of yeast DNA polymerase delta in post-replicational repair of UV-damaged DNA. J Biol Chem, 272, 25445-25448.

Trincao, J., Johnson, R.E., Escalante, C.R., Prakash, S., Prakash, L. and Aggarwal, A.K. (2001) Structure of the catalytic core of S. cerevisiae DNA polymerase eta: implications for translesion DNA synthesis. Mol Cell, 8, 417-426.

Tsujimura, T., Maher, V.M., Godwin, A.R., Liskay, R.M. and McCormick, J.J. (1990) Frequency of intrachromosomal homologous recombination induced by UV radiation in normally repairing and excision repair-deficient human cells. Proc Natl Acad Sci U S A, 87, 1566-1570.

Tsukamoto, Y., Kato, J. and Ikeda, H. (1996) Hdf1, a yeast Ku-protein homolog, is involved in illegitimate recombination, but not in homologous recombination. Nucleic Acids Res. 24, 2067-2072.

Ulrich, H.D. and Jentsch, S. (2000) Two RING finger proteins mediate cooperation between ubiquitin-conjugating enzymes in DNA repair. EMBO J, 19, 3388-3397.

Vaisman, A., Tissier, A., Frank, E.G., Goodman, M.F. and Woodgate, R. (2001) Human DNA polymerase iota promiscuous mismatch extension. J Biol Chem, 276, 30615-30622.

van der Laan, R., Roest, H.P., Hoogerbrugge, J.W., Smit, E.M., Slater, R., Baarends, W.M., Hoeijmakers, J.H. and Grootegoed, J.A. (2000) Characterization of mRAD18Sc, a mouse homolog of the yeast postreplication repair gene RAD18. Genomics, 69, 86-94.

Van Sloun, P.P., Romeijn, R.J. and Eeken, J.C. (1999) Molecular cloning, expression and chromosomal localisation of the mouse Rev3l gene, encoding the catalytic subunit of polymerase zeta. Mutat Res, 433, 109-116.

Van Sloun, P.P., Varlet, I., Sonneveld, E., Boei, J.J., Romeijn, R.J., Eeken, J.C. and De Wind, N. (2002) Involvement of mouse Rev3 in tolerance of endogenous and exogenous DNA damage. Mol Cell Biol, 22, 2159-2169.

VanDemark, A.P., Hofmann, R.M., Tsui, C., Pickart, C.M. and Wolberger, C. (2001) Molecular insights into polyubiquitin chain assembly: crystal structure of the Mms2/Ubc13 heterodimer. Cell, 105, 711-720.

Verkade, H.M., Bugg, S.J., Lindsay, H.D., Carr, A.M. and O'Connell, M.J. (1999) Rad18 is required for DNA repair and checkpoint responses in fission yeast. Mol Biol Cell, 10, 2905-2918.

Verkade, H.M., Teli, T., Laursen, L.V., Murray, J.M. and O'Connell, M.J. (2001) A homolog of the Rad18 postreplication repair gene is required for DNA damage responses throughout the fission yeast cell cycle. Mol Genet Genomics, 265, 993-1003.

Wagner, J., Gruz, P., Kim, S.R., Yamada, M., Matsui, K., Fuchs, R.P. and Nohmi, T. (1999) The dinB gene encodes a novel E. coli DNA polymerase, DNA pol IV, involved in mutagenesis. Mol Cell, 4, 281-286.

Wagner, J., Fujii, S., Gruz, P., Nohmi, T. and Fuchs, R.P. (2000) The beta clamp targets DNA polymerase IV to DNA and strongly increases its processivity. EMBO Rep, 1, 484-488.

Washington, M.T., Johnson, R.E., Prakash, S. and Prakash, L. (2000) Accuracy of thymine-thymine dimer bypass by Saccharomyces cerevisiae DNA polymerase eta. Proc Natl Acad Sci U S A, 97, 3094-3099.

Washington, M.T., Prakash, L. and Prakash, S. (2001a) Yeast DNA polymerase eta utilizes an induced-fit mechanism of nucleotide incorporation. Cell, 107, 917-927.

Washington, M.T., Johnson, R.E., Prakash, L. and Prakash, S. (2001b) Accuracy of lesion bypass by yeast and human DNA polymerase eta. Proc Natl Acad Sci U S A, 98, 8355-8360.

Watkins, J.F., Sung, P., Prakash, S. and Prakash, L. (1993) The extremely conserved amino terminus of RAD6 ubiquitin-conjugating enzyme is essential for amino-end rule-dependent protein degradation. Genes Dev, 7, 250-261.

Wilson, T.E., Grawunder, U. and Lieber, M.R. (1997) Yeast DNA ligase IV mediates non-homologous DNA end joining. Nature, 388, 495-498.

Witkin, E.M., Roegner-Maniscalco, V., Sweasy, J.B. and McCall, J.O. (1987) Recovery from ultraviolet light-induced inhibition of DNA synthesis requires umuDC gene products in recA718 mutant strains but not in recA+ strains of Escherichia coli. Proc Natl Acad Sci U S A, 84, 6805-6809.

Wittschieben, J., Shivji, M.K., Lalani, E., Jacobs, M.A., Marini, F., Gearhart, P.J., Rosewell, I., Stamp, G. and Wood, R.D. (2000) Disruption of the developmentally regulated Rev3l gene causes embryonic lethality. Curr Biol, 10, 1217-1220.

Xiao, W., Lechler, T., Chow, B.L., Fontanie, T., Agustus, M., Carter, K.C. and Wei, Y.F. (1998a) Identification, chromosomal mapping and tissue-specific expression of hREV3 encoding a putative human DNA polymerase zeta. Carcinogenesis, 19, 945-949.

Xiao, W., Lin, S.L., Broomfield, S., Chow, B.L. and Wei, Y.F. (1998b) The products of the yeast MMS2 and two human homologs (hMMS2 and CROC-1) define a structurally and functionally conserved Ubc-like protein family. Nucleic Acids Res, 26, 3908-3914.

Xiao, W., Chow, B.L., Fontanie, T., Ma, L., Bacchetti, S., Hryciw, T. and Broomfield, S. (1999) Genetic interactions between error-prone and error-free postreplication repair pathways in Saccharomyces cerevisiae. Mutat Res, 435, 1-11.

Xiao, W., Chow, B.L., Broomfield, S. and Hanna, M. (2000) The Saccharomyces cerevisiae RAD6 group is composed of an error-prone and two error-free postreplication repair pathways. Genetics, 155, 1633-1641.

Xin, H., Lin, W., Sumanasekera, W., Zhang, Y., Wu, X. and Wang, Z. (2000) The human RAD18 gene product interacts with HHR6A and HHR6B. Nucleic Acids Res, 28, 2847-2854.

Yu, S.L., Johnson, R.E., Prakash, S. and Prakash, L. (2001) Requirement of DNA polymerase eta for error-free bypass of UV-induced CC and TC photoproducts. Mol Cell Biol, 21, 185-188.

Yuan, F., Zhang, Y., Rajpal, D.K., Wu, X., Guo, D., Wang, M., Taylor, J.S. and Wang, Z. (2000) Specificity of DNA lesion bypass by the yeast DNA polymerase eta. J Biol Chem, 275, 8233-8239.

Yuasa, M., Masutani, C., Eki, T. and Hanaoka, F. (2000) Genomic structure, chromosomal localization and identification of mutations in the xeroderma pigmentosum variant (XPV) gene. Oncogene, 19, 4721-4728.

Zan, H., Komori, A., Li, Z., Cerutti, A., Schaffer, A., Flajnik, M.F., Diaz, M. and Casali, P. (2001) The translesion DNA polymerase zeta plays a major role in Ig and bcl-6 somatic hypermutation. Immunity, 14, 643-653.

Zhang, Y., Yuan, F., Wu, X., Rechkoblit, O., Taylor, J.S., Geacintov, N.E. and Wang, Z. (2000a) Error-prone lesion bypass by human DNA polymerase eta. Nucleic Acids Res, 28, 4717-4724.

Zhang, Y., Yuan, F., Wu, X. and Wang, Z. (2000b) Preferential incorporation of G opposite template T by the low-fidelity human DNA polymerase iota. Mol Cell Biol, 20, 7099-7108.

Zhang, Y., Yuan, F., Xin, H., Wu, X., Rajpal, D.K., Yang, D. and Wang, Z. (2000c) Human DNA polymerase kappa synthesizes DNA with extraordinarily low fidelity. Nucleic Acids Res, 28, 4147-4156.

Zhang, Y., Yuan, F., Wu, X., Wang, M., Rechkoblit, O., Taylor, J.S., Geacintov, N.E. and Wang, Z. (2000d) Error-free and error-prone lesion bypass by human DNA polymerase kappa in vitro. Nucleic Acids Res, 28, 4138-4146.

Zhang, H., Marra, G., Jiricny, J., Maher, and V.M., McCormick, J.J. (2000e), Mismatch repair is required for O(6)-methylguanine-induced homologous recombination in human fibroblasts. Carcinogenesis, 21, 1639-46.

Zhang, Y., Yuan, F., Wu, X., Taylor, J.S. and Wang, Z. (2001) Response of human DNA polymerase iota to DNA lesions. Nucleic Acids Res, 29, 928-935.

Zhang, Y., Wu, X., Rechkoblit, O., Geacintov, N.E., Taylor, J.S. and Wang, Z. (2002) Response of human REV1 to different DNA damage: preferential dCMP insertion opposite the lesion. Nucleic Acids Res, 30, 1630-1638.

Zhou, B.L., Pata, J.D. and Steitz, T.A. (2001) Crystal structure of a DinB lesion bypass DNA polymerase catalytic fragment reveals a classic polymerase catalytic domain. Mol Cell, 8, 427-437.

CHAPTER II

Identification of a protein essential for a major pathway used by human cells to avoid UV-induced DNA damage

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Abbreviations: *HPRT*, hypoxanthine phosphoribosyltransferase gene; *hyg,* hygromycin phosphotransferase gene; hyg^R, hygromycin resistant; TG, 6-thioguanine; TG^R, 6-thioguanine resistant.

Abstract

When DNA replication stalls at a fork-blocking lesion, cells use damage tolerance pathways to continue replication. One pathway, "translesion synthesis", involves specialized DNA polymerases that can use damaged DNA as a template. Translesion synthesis can result in mutations (i.e., can be error-prone), but it can also be error-free. An alternative pathway, sometimes called "damage avoidance", has been hypothesized, by which cells make temporary use of an undamaged copy of the blocked sequence as a template, i.e., the newly-synthesized daughter strand of the sister duplex or the allelic copy. This pathway is error-free. Direct evidence of the use of this alternative pathway in intact mammalian cells has not been available. To determine whether hMms2, a ubiquitin-conjugating-enzyme-like protein [Xiao, W., Lin, S. L., Broomfield, S., Chow, B. L. & Wei, Y. F. (1998) *Nucleic Acids Res.* 26, 3908-3914], plays a critical role in such damage avoidance, a human fibroblast cell strain in which both error-prone translesion synthesis and error-free damage avoidance can be detected and quantified simultaneously, and several derivative strains in which expression of hMms2 protein had been eliminated or greatly decreased, were compared for their ability to avoid translesion synthesis past UV_{254nm}-induced DNA photoproducts. Loss of hMms2 protein eliminated the ability of the latter strains to use an allelic copy of a target gene for damage avoidance, i.e., to produce a wild type gene from two non-functional allelic copies of that gene. Molecular analysis of the wild type gene showed that

this process involves gene conversion unassociated with crossing-over. That loss of hMms2 also eliminated use of the daughter strand of the sister duplex as a template for damage avoidance could be inferred from the fact that the frequency of mutations induced by UV in the single copy *HPRT* gene of the derivative strains was significantly higher than that observed in the parental strain. These data indicate that *hMMS2* is essential for human cells to carry out damage avoidance using either type of homolog and that damage avoidance and translesion synthesis are alternative pathways for tolerating fork-blocking photoproducts.

Introduction

DNA is constantly exposed to damaging agents. If the damage is not removed e.g., by excision repair, prior to onset of S-phase, certain kinds of lesions can block replication by the major DNA polymerase complex (1). The damage tolerance mechanisms developed by prokaryotic and eukaryotic cells to overcome such replication blocks fall into two categories: translesion synthesis and damage avoidance. Evidence suggests that translesion synthesis is a process in which specialized, distributive DNA polymerases take over for the major DNA polymerase complex to carry out DNA replication using the damaged DNA as a template (2-5). After distributive incorporation of nucleotides past the damage, the major DNA replication complex resumes its replication activity. Translesion synthesis can be either "error-prone" or "error-free" depending upon such factors as the type of damage, its sequence context in the DNA, and the availability of specialized distributive translesion synthesis polymerases, e.g., hPol zeta, hPol eta, Pol iota (6-9) or their auxiliary proteins (10). The alternative

pathway to tolerate DNA damage, sometimes referred to as damage avoidance, temporarily uses a homologous undamaged copy of DNA instead of the damaged DNA as a template to continue replication (11-13). By its very nature, damage avoidance is error-free. The mechanisms involved in mammalian cells' avoidance of fork-blocking lesions are not well characterized, but unlike what occurs in bacteria (12), mammalian cells do not transfer parental DNA strands containing UV photoproducts into daughter strands (14). It has been proposed that in mammalian cells, the 3' end of a blocked leading strand separates from its original template and makes use of an intact homologous copy of the DNA as a template for continuing replication past the block. If the newly-synthesized daughter strand of the sister duplex is to provide the temporary template for the blocked leading strand, synthesis of the lagging strand must have continued for a short distance beyond where the leading strand was blocked, and this lagging strand must become dissociated from its partner. After replication has proceeded past the block, the paired structure must uncouple so that normal DNA replication using the original template can resume. Although the newly-synthesized daughter strand of the sister duplex may provide the most convenient homologous copy for the blocked fork, the homologous allelic gene could also serve as an alternative template for circumventing such a replication block. Evidence that intact mammalian cells make use of such damage avoidance mechanisms to circumvent fork-blocking lesions and identification of the various gene products has not been available.

Xiao and his colleagues (15) cloned a gene from *S. cerevisiae* that complements the sensitivity of yeast mutant *mms2-1*, originally identified (16) for its sensitivity to methyl methanesulfonate. This gene, *MMS2*, codes for a ubiquitin-conjugating-enzyme-like protein (15) that forms a stable complex with

Ubc13 protein (17-19), enabling *in vitro* di-ubiquitin formation via Lys-63 instead of the conventional Lys-48 chain assembly (20). Loss of Mms2 significantly increases the rate of spontaneous mutations in *S. cerevisiae* and moderately increases the cytotoxic and mutagenic effects of UV_{254nm} radiation (15), indicating that it plays a role in an error-free pathway.

Xiao *et al.* (21) isolated the human homolog of the *S. cerevisiae MMS2* gene, designated *hMMS2*. The present study was designed to determine whether the *hMMS2* gene plays a critical role in the ability of human cells to tolerate DNA damage, and if so, to identify the pathway in which it functions and determine the nature of the molecular events involved. To do so, an infinite life span, chromosomally-stable, human fibroblast cell strain (22) was engineered to contain a chromosomally-integrated substrate that allows detection of the production of a selectable wild type gene from two non-functional homologous genes (23). Three strains in which expression of hMms2 protein was eliminated or greatly reduced using antisense were identified and compared with the parental strain for their ability to carry out damage avoidance following the exposure to UV_{254nm} irradiation. The results indicate that human cells can use both kinds of homologous templates to avoid fork-blocking lesions and that hMms2 protein is essential for the damage avoidance process.

Materials and Methods

Media Used. Cells to be used for experiments involving assaying cell survival, or selection for resistance to 6-thioguanine (TG, Sigma), or hygromycin (Calbiochem), were cultured in Eagle's minimal medium supplemented with L-aspartic acid (0.2 mM), L-serine (0.2 mM), sodium pyruvate (1 mM), hydrocortisone (1 μg/ml), and supplemented calf serum (10% vol/vol) (culture

medium). This medium containing the appropriate selection drug, i.e., TG (40 μM) or hygromycin (100 U/ml) was used for selection of TG resistant or hygromycin resistant cells, respectively. The parental cell strain, which contains a transfected *tTAk* gene that was transfected on a plasmid carrying the hisD gene as its selectable marker (see text), was cultured in McM medium (24) lacking histidine, but containing histidinol (1mM), supplemented calf serum (10% vol/vol) (Hyclone), penicillin (100 U/ml), streptomycin (100 μg/ml), and hydrocortisone (1 μg/ml). Cell strains that contain a transfected antisense *hMMS2* gene that was tranfected on a plasmid carrying the gene coding for puromycin resistance (see text) as its selectable marker, were cultured in this same McM medium, but also containing puromycin (1 μg/ml).

DNA Transfection. Cells were transfected using LipofectAMINE (Life Technologies, GibcoBRL). After 48 h, transfectants were selected in medium containing the appropriate drug. The selection medium was renewed every five days. When drug-resistant clones had formed, they were isolated, expanded in the appropriate selection medium, and stored frozen until needed.

Preparation of Purified hMms2 and Mouse Polyclonal hMms2 Antibodies. The human *hMMS2* open reading frame was PCR amplified and cloned into pGEX6 (Pharmacia). The GST-hMms2 fusion protein was over-produced in Epicurian coli BL21-CodonPlus-RIL cells (Stratagene), and standard protocols were followed to purify the fusion protein using a 5ml GSTrap column (Pharmacia). After PreScission protease (Pharmacia) cleavage, hMms2 was eluted from a GSTrap column (Pharmacia) and further purified using a Superdex-75 size column (Pharmacia). Polyclonal mouse serum was raised against the purified hMms2 protein in five BALB/c mice. Three weeks after the second boost,

the mice were bled, and the anti-serum was pooled and characterized by ELISA and Western analysis using the purified protein.

Northern and Western Analysis. For Northern analysis, RNA from each cell strain was extracted using RNAzol^{TM}B (TEL-TEST, INC.) according to manufacturer's recommendations. For each sample, 15 μg of total RNA was fractionated in 1.2% formaldehyde agrose gel and transferred to Hybond-N^{+} (Amersham) nylon membranes. The RNA was crosslinked to the membrane using a UV stratalinker 2400 (Stratagene). To determine the level of antisense RNA expression, hybridization was performed according to the standard procedures using a probe specific to *hMMS2*. The membrane was stripped and re-probed with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) to compare the relative amount of total RNA loaded for each sample. For Western analysis, nuclear protein was extracted from the cell strains as described (25). For each cell strain, 50 μg of nuclear protein was subjected to Western analysis using hMms2 mouse polyclonal antibody. Purified hMms2 protein was included as the positive control, and Ku-80 protein was detected using rabbit polyclonal Ku-80 antibody to compare the relative amount of total nuclear extracts loaded for each sample.

Assay for Cytotoxicity. Cells in exponential growth were detached from the dishes using trypsin, plated at cloning density, and allowed 12 h to attach before being irradiated by UV_{254nm} as described (26). The culture medium was renewed one day after irradiation and again after seven days. After 14 days, when the surviving cells had formed clones, they were stained. The survival was determined by comparing the cloning efficiency of the irradiated cells with that of the sham-irradiated control cells, and the value was expressed as percent of that of those control cells.

Assay for the Frequency of UV-Induced Wild Type Hygromycin Resistant Cells. This assay was carried out as described previously (27). Briefly, sufficient sets of cells plated at 0.5×10^6 cells per dish were used to have at least 2×10^6 surviving target cells per dose and allowed 12 h to attach. The number of target cells attached to the dishes at the time of irradiation was determined electronically. Forty-eight hours after irradiation, the culture medium was changed to medium containing hygromycin (100 U/ml). This selection medium was replaced every five days, and the clones were stained 3 wk after irradiation. The frequency was calculated as follows: total hygromycin resistant clones divided by total number of surviving cells, determined as described in the above paragraph, taking into account the number of attached target cells. The induced frequencies were calculated by subtracting the background frequencies

Characterization of *hyg* Genes. Hygromycin resistant clones were analyzed using PCR and *Hin*dIII digestion as described previously (23). Briefly, cells from each clone were used for PCR analysis using a set of primers that amplify a 1,167-bp fragment from each copy of the *hyg* gene. The PCR products were digested with *Hin*dIII and subjected to gel electrophoresis. PCR products from wild type copies of the *hyg* gene are resistant to *Hin*dIII digestion. When gene conversion events occur, one or other of the mutant *hyg* genes is restored to wild type while the second mutant *hyg* gene remains unchanged. *Hin*dIII digestion of PCR products generates 1,167-bp, 278-bp and 889-bp fragments or 1,167-bp, 695-bp and 472-bp fragments depending on which *hyg* gene has been restored to the wild type. However, if a single reciprocal exchange were to occur between two mutant *hyg* genes, this would result in a single wild type copy of the *hyg* gene.

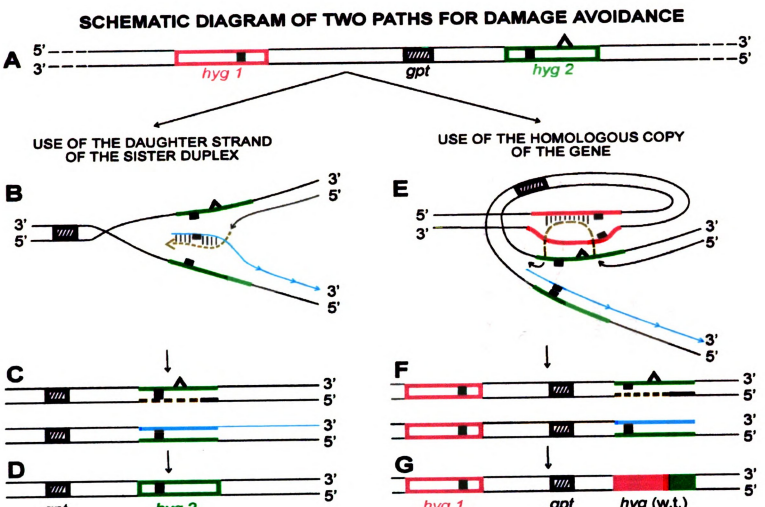
Assay for the Frequency of UV-induced Mutations in the *HPRT* Gene. The methods used to determine the frequency of UV-induced TG resistant (TG^R) cells has been described (28). Briefly, sufficient sets of cells, plated at 0.5×10^6 to 2×10^6 per 150-mm diameter dish were used to have at least 1×10^6 surviving target cells per dose. The culture medium was changed the day after irradiation. Cells used to detect induced mutations were allowed an 8-day expression period before at least 1×10^6 cells were plated at 25,000 cells per 100-mm diameter dish to assay for TG resistance. Selection medium containing 40 μM TG was replaced after seven days. At the time of selection, for each dose, a separate set of cells, plated at cloning density and fed with medium lacking TG, was used to determine the cloning efficiency of the cells. The frequency of mutants was calculated as follows: total TG resistant clones divided by the total number of clonable cells selected. The induced frequencies were calculated by subtracting the background frequencies in the sham-irradiated control population.

Results and Discussion

Assay System for Detecting Damage Tolerance in the Same Target Cell Strains. Damage tolerance pathways allow human cells with fork-blocking lesions to continue DNA replication and avoid cell death. To detect use of translesion synthesis, we measure the frequency of error-prone translesion synthesis, using the *HPRT* gene as the target for mutations and quantifying the frequency of induced 6-thioguanine-resistant (TG^R) cells (28). [Error-free translesion synthesis cannot be detected directly, but can be inferred by comparing the frequency of mutations induced in cells that lack such a process with that of cells that have it]. To detect use of damage avoidance, we employ a substrate (stably integrated into the genome of the target cells) that allows quantification of the frequency with which cells produce a selectable wild type

copy of a gene by combining information from two defective homologous genes. The substrate (diagrammed in Fig. 1*A*) consists of two copies of the hygromycin phosphotransferase (*hyg*) gene conferring resistance to hygromycin, each inactivated by a 10-bp *Hin*dIII linker inserted at a different site (27). The genes, separated by ~6000 bp (23), are arranged in *cis* with one another, mimicking the situation for allelic genes in human cells. If a fork-blocking DNA lesion (represented by the triangle) interrupts replication of one of the genes, e.g., the *hyg2* gene shown in Figure 1*A*, use of the corresponding section of the newly-synthesized daughter strand (shown in blue in Figure 1*B*) of the sister duplex as a template will allow the cell to avoid the damage and continue DNA replication. However, the newly-synthesized DNA strand (shown in brown) of the *hyg2* gene resulting from such damage avoidance will still contain the 10-bp *Hin*dIII linker (Fig. 1*C*, top panel), even after the lesion has been removed or after another round of replication has occurred (Fig. 1*D*). Therefore, this process will not yield a cell that is resistant to hygromycin. However, as diagrammed in Fig. 1*E*, if cells use the homologous copy of the gene, *hyg1*, as a template to avoid the blocking lesion, the newly-synthesized strand (shown in brown) can obtain the information from the corresponding section of the *hyg1* gene. The strand will no longer contain the 10-bp *Hin*dIII linker (Fig. 1*F*, top panel). The next round of replication of that strand will yield a selectable wild type copy of the *hyg* gene (Fig. 1*G*). An added value of this substrate is that it allows one to infer the nature of the mechanisms involved by analyzing the structure of the *hyg* gene(s) in the hygromycin resistant (hyg^R) cells.

Figure 1: The two possible paths for avoiding DNA damage by making use of an undamaged homolog to continue replication, i.e, a daughter strand of the sister duplex or a homologous copy. (This image in this dissertation is presented in color). (*A*) The intrachromosomal substrate contains two copies of the *hyg* gene (*hyg1*, red; and *hyg2*, green), orientated in the same direction. Each has a *Hin*dIII linker insertion (■) at the different position. The open triangle in the *hyg2* gene represents a UV-induced lesion. (*B*) The blocked strand of the *hyg2* gene is shown copying information from the newly-synthesized daughter strand (blue) of the sister duplex, rather than the damaged template. (*C*) The outcome of this process. The newly-replicated leading strand (brown) of the *hyg2* gene still contains the *Hin*dIII linker sequence (top panel). The replicated product not containing the UV lesion also retains the original *Hin*dIII linker DNA sequence (bottom panel). The section containing the *hyg1* gene (red) is not shown for these products. (*D*) The outcome after one round of replication. The *hyg2* gene has been replicated, but still contains the linker. (*E*) The *hyg2* gene is shown copying information from the homologous copy, *hyg1*, rather than the damaged template. (*F*) The outcome of this process. The DNA sequence of the *hyg1* gene remains intact. The newly replicated leading strand of the *hyg2* gene (brown) has obtained information from the corresponding section of the *hyg1* gene, so it no longer contains the *Hin*dIII linker sequence (top panel). The replicated product not containing the lesion retains its original DNA sequence (bottom panel). (*G*) The outcome after one round of replication. A wild type (w.t.) copy of the *hyg* gene has been generated.


SCHEMATIC DIAGRAM OF TWO PATHS FOR DAMAGE AVOIDANCE
A
5'
3'
hyg 1
gpt
hyg 2
3'
5'
USE OF THE DAUGHTER STRAND OF THE SISTER DUPLEX
USE OF THE HOMOLOGOUS COPY OF THE GENE
B
C
D
E
F
G
gpt
hyg 2
hyg 1
gpt
hyg (w.t.)

Preparation and Identification of Human Cells Expressing Antisense *hMMS2* and Devoid of hMms2 Protein. We successfully used an antisense *hMMS2* RNA strategy to deplete human cells of hMms2 protein. To do so, we transfected an MSU-1.2 human fibroblast-derived cell strain, designated E7.2 (23), containing the substrate as diagrammed in Figure 1*A*, with a plasmid designated pTet-tTak containing the tetracycline promotor (tetP) (29, 30), the tetracycline transactivator (tTA) gene under the control of tetP, and the hisD gene coding for histidinol resistance. A clone of histidinol-resistant cells that expressed tTA at a high level and exhibited the same UV-induced frequency of TG^R cells and hyg^R cells as cell strain E7.2 was chosen as the parental strain for the present study. This parental cell strain expressing tTA was transfected with a plasmid carrying antisense *hMMS2* gene under control of the tetP promoter. The *hMMS2* antisense expression construct contains a 430-bp *Spe*I-*Hin*dIII fragment carrying the first 400-bp *hMMS2* open reading frame and 20-bp 5' untranslated region of the *hMMS2* cDNA. This fragment was cloned into the *Spe*I-*Hin*dIII sites of pTet-Puro plasmid (6, 10), in antisense orientatation. The construct also contains a gene coding for puromycin resistance. Sixty puromycin-resistant transfectants were isolated and screened for expression of antisense *hMMS2* RNA by RT-PCR using a set of antisense-specific primers. Sixteen clones were found to express *hMMS2* antisense RNA. These clones were expanded into large populations, and the cell strains were further analyzed by Northern blotting to quantify the level of expression of antisense RNA (data not shown). Nuclear extracts from the parental cell strain and the 16 antisense RNA-expressing cell

strains were analyzed by Western blotting for the level of hMms2 protein. Purified hMms2 protein was included as the positive control. Three cell strains, designated 2A-M2, 3C-M2, and 13D-M2, which express high levels of antisense *hMMS2* RNA (Fig. 2*A*), showed an undetectable level or a very low level of expression of hMms2 protein compared to the parental cell strain (Fig. 2*B*). We verified that these three cell strains lacking hMms2 protein still retained an intact copy of the substrate shown in Figure 1*A* and then used them to investigate the role of hMms2 in damage tolerance.

Effect of Depletion of hMms2 Protein on Cell Survival. These three cell strains were compared with their parental strain for the effect of loss of hMms2 on UV-induced cytotoxicity. As shown in Figure 3*A*, there was no significant difference between cells expressing or not expressing hMms2 protein in their sensitivity to the cytotoxic effect of UV. This indicated that cells expressing or not expressing hMms2 protein were equally capable of tolerating the UV photoproducts. The implications of this finding are discussed below. The majority of the data points shown in Figure 3*A* were derived from experiments in which cell strains were also assayed for the frequency of UV-induced hyg^R cells and TG^R cells (see below).

Effect of Depletion of hMms2 Protein on the Frequency of UV-Induced Hyg^R Cells. As expected (27), there was a dose-dependent increase in the frequency of UV-induced hyg^R cells in the parental cell strain (Fig. 3*B*). However, in the three cell strains lacking hMms2 protein, that response to UV was virtually eliminated. The frequency of UV-induced hyg^R cells was not significantly greater

Figure 2: Northern and Western blots showing the correlation between expression of antisense *hMMS2* RNA and loss of hMms2 protein. (*A*) Northern analysis showing that clones 2A-M2, 3C-M2, and 13D-M2 express high levels of *hMMS2* antisense RNA. *GAPDH* transcript was used as the loading control for all the samples. *hMMS2 AS* indicates the transcript of *hMMS2* antisense. (*B*) Western analysis showing the level of expression of hMms2 protein in nuclear extracts from these *hMMS2* antisense-expresssing transfectants and their parental strain. Ku-80 was used as the loading control. Purified hMms2 was included as the positive control.

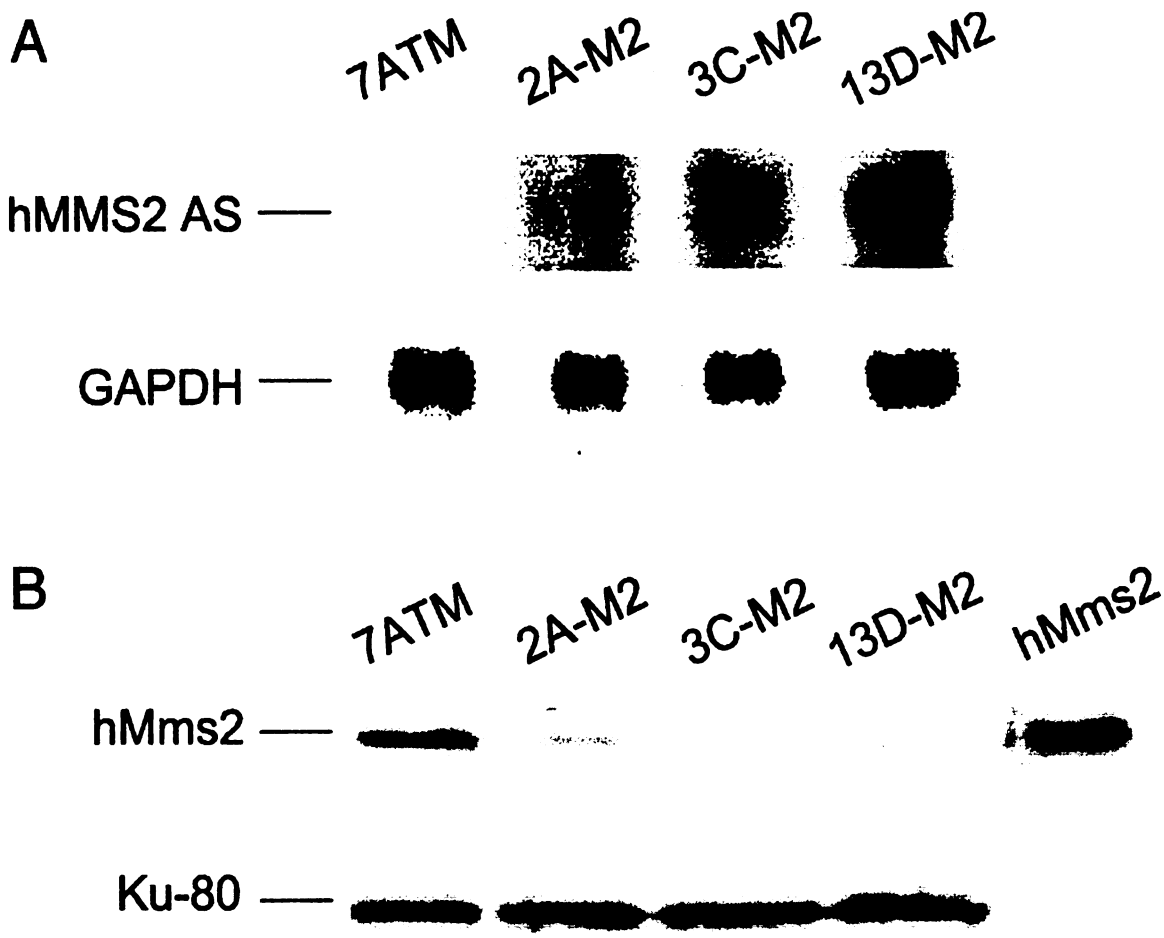

A
7ATM
2A-M2
3C-M2
13D-M2
hMMS2 AS
GAPDH
B
7ATM
2A-M2
3C-M2
13D-M2
hMms2
hMms2
Ku-80

Figure 2

Figure 3: The biological effects of inhibition of *hMMS2* expression. Open symbol, the parental cell strain; closed symbols, transfectant cell strains showing an undetectable or very low level of hMms2 protein: circle, strain 3C-M2; triangle, strain 2A-M2; and square, strain 13D-M2. (*A*) The UV-induced cytotoxicity in these cell strains. Some points have been offset slightly in order to be visible. (*B*) The frequency of hyg^R cells induced by UV in the parental cell strain and the three cell strains lacking hMms2 protein. Some points have been offset slightly in order to be visible. (*C*) The frequency of TG^R cells induced by UV in the parental cell strain and the three cell strains lacking hMms2 protein. For the majority of the data points generated, at least 2×10^6 cells were assayed. The observed frequency of TG^R clones has been corrected for the cloning efficiency of the cells at the time of selection as described (28). For the majority of the data points, the cloning efficiency of the cells at the time of selection ranged from 45-65%, so the correction factor was less than 3-fold. The induced frequencies were calculated by subtracting the background frequencies, observed with the sham-irradiated populations. The latter were less than or equal to 10 per 10^6 clonable cells. The solid line represents the least-squares line for the data obtained from the parental cell strain, and the dashed line represents the least-squares line for all the data points generated from the three cell strains deficient in hMms2 protein, taken together.

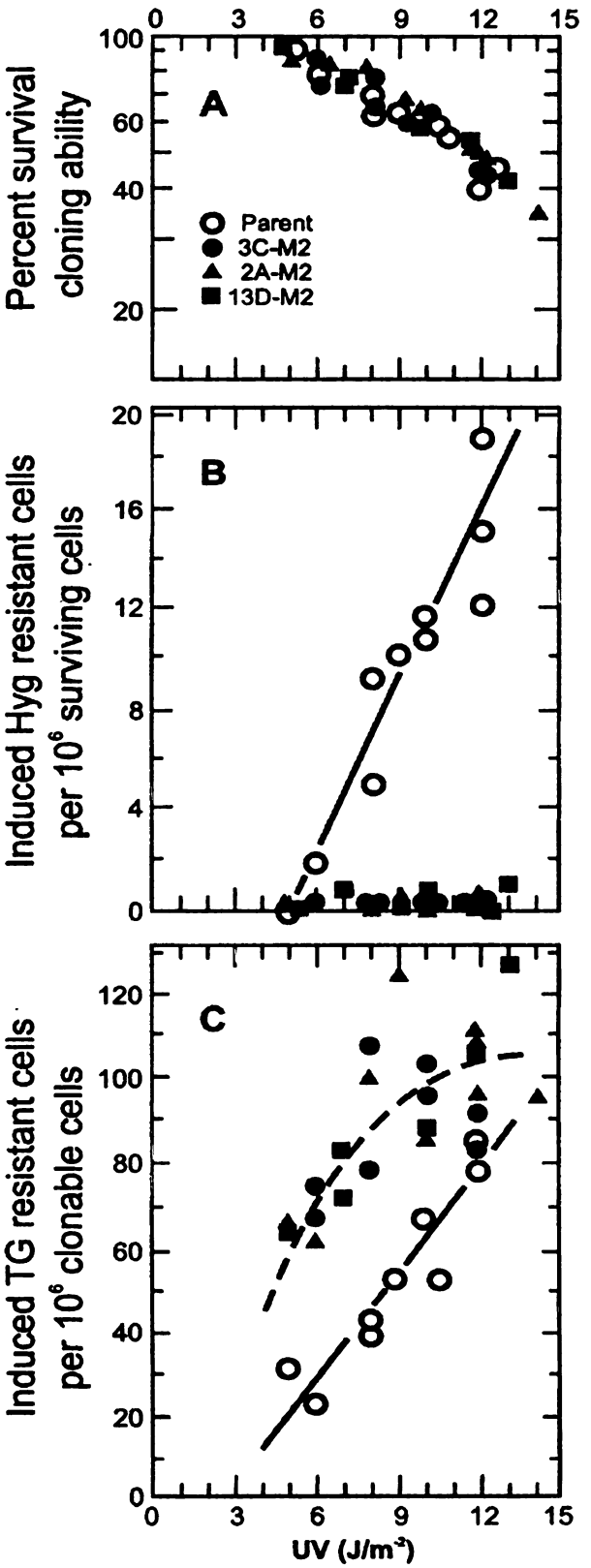

A
Percent survival
cloning ability
Parent
3C-M2
2A-M2
13D-M2
B
Induced Hyg resistant cells
per 10^6 surviving cells
C
Induced TG resistant cells
per 10^6 clonable cells
UV (J/m^-2)

Figure 3

than the background frequency, i.e., $< 1\times10^{-6}$. This decrease is not the result of mere expression of antisense, rather than the loss of hMms2 protein, because a cell strain exhibiting a very high level of antisense RNA, but a normal level of hMms2 protein, gave an UV-induced frequency of hyg^R cells equal to that found in the parental strain (data not shown). The data in Figure 3*B* indicate that hMms2 protein plays an essential role in the damage avoidance pathway dealing with photoproducts, i.e., during DNA replication, human cells can make temporary use of the allelic copy of a gene as a template to avoid fork-blocking lesions, but cells lacking hMms2 protein are virtually incapable of using this damage avoidance pathway. PCR and restriction enzyme digestion was used to analyze 30 of the hyg^R clones induced by UV, or arising spontaneously, for the structure of their *hyg* genes. The results showed that each wild type *hyg* gene had been generated by a gene conversion event unaccompanied by crossing-over.

Effect of Depletion of hMms2 Protein on the Frequency of UV-Induced TG^R Cells. As expected, UV radiation caused a dose-dependent increase in the frequency of TG^R cells in the parental cell strain (Fig. 3*C*), indicating that these cells had carried out translesion synthesis. The three cell strains lacking hMms2 protein also gave a dose-dependent induction of TG^R cells for doses up to 8 J/m^2, but the frequency of TG^R cells was twice as high as that induced in the parental cells, indicating that the strains lacking hMms2 carried out translesion synthesis more frequently. This increased use of translesion synthesis as a means of tolerating photoproducts explains why the cells lacking hMms2 were not more sensitive to than the parental cells to the cytotoxic effect of UV.

Increased sensitivity to cell killing in the cells lacking hMms2 should only be seen if they are unable, or less able than the parent cells to continue to replicate DNA containing fork-blocking lesions. If the human cells lacking hMms2 were to increase their use of various specialized translesion synthesis polymerases, they could continue to replicate their DNA even though all use of an undamaged homologous copy of the DNA as a template had been eliminated. S. *cerevisiae* cells lacking Mms2 are slightly more sensitive to the cytotoxic effect of UV than are wild type cells. However, human cells have additional translesion synthesis polymerases, e.g., they have DNA pol iota (9) and should be more capable than yeast of replicating past fork-blocking lesions.

At higher doses of UV, the frequency of mutants in the three cell strains lacking hMms2 leveled off. The dashed line is the least-squares line for all the data points obtained from these three cell strains. One possible explanation for why cells lacking hMms2 protein did not exhibit a dose-dependent increase in mutant frequency at higher UV doses is that at those doses, the number of mutations continued to increase, but some of these occurred within the same cell. We tested that possibility by sequencing the coding region of the *HPRT* gene of 8 independent mutants from these cell strains irradiated with the highest doses of UV. There was only one mutation in each gene. Another possible explanation is that, at those higher doses, the level of a DNA polymerase(s) involved in error-free translesion synthesis past photoproducts is increased in cells lacking hMms2 protein. Western analysis of the level of expression of polymerase eta after UV-irradiation did not demonstrate such induction (data not shown).

Indirect Evidence That Human Cells Use the Newly-Synthesized Daughter Strand of the Sister Duplex for Damage Avoidance. The data in Figure 3*A* and 3*C* showed that the parental cells expressing hMms2 and the three strains lacking hMms2 are equally able to tolerate UV-induced damage, but the latter are twice as sensitive to the induction of mutations in the *HPRT* gene. *HPRT* is located on the X-chromosome and the target cells are from a male donor, there is only one copy of this gene in the cell. Therefore, cells have only two options for tolerating fork-blocking lesions in the *HPRT* gene: 1) use the newly-synthesized daughter strand of the sister duplex of the *HPRT* gene as the template to avoid the damage (error-free) or 2) carry out potentially mutagenic translesion synthesis. Our data indicate that the parental cells must have used option 1 and that this option was not available in cells lacking hMms2.

We recognize that UV induces several kinds of fork-blocking photoproducts and that some types of lesions are more likely than others to result in mutations. However, for the sake of simplicity, let us assume there is only one type of fork-blocking lesion and that cells make equal use of the two major pathways for tolerating such lesions, i.e., translesion synthesis and damage avoidance. If the latter pathway is completely eliminated, e.g., by knocking out of expression of hMms2 protein, the number of fork-blocking lesions that have to be dealt with by translesion synthesis polymerases should double. Even though some translesion synthesis might not result in mutations, the frequency of mutations introduced during translesion synthesis should double because the total number of lesions to be passed has doubled. This is the extent of increase we observed when we eliminated hMms2 protein.

Possible Role of hMms2 plays in Damage Avoidance The role that the hMms2 play3 in the observed loss of ability of the human cells to carry out gene conversion events to avoid DNA fork-blocking lesions remains elusive. Hofmann and Pickart (17) showed that in yeast, Mms2 forms a complex with Ubc13, and the complex is required for the assembly of polyubiquitin chains linked through lysine 63 in Ubc13-depepndent manner. They proposed that this complex acts as a signal transducer to recruit target proteins to the site of DNA damage for repair. Ulrich and Jentsch (18) later on showed that in yeast, Rad5 recruits the Mms2/Ubc13 complex to DNA through its RING finger domain, and that Rad5 interaction with Rad18 brings the Mms2/Ubc13 complex into contact with the Rad6/Rad18 complex. At the same time, Xiao *et al.* (31) proposed that in yeast the Mms2/Ubc13 complex is required to promote both *RAD5* and *POL30* error-free repair pathways. Recently, McKenna *et al.* (32) showed that in human cells, hMms2 forms a complex with hUbc13, and this interaction is required for hUbc13-mediated polyubiquitination through lysine 63. The crystal structure of this complex was also determined recently (33). Our data support the hypothesis that the hMms2/hUbc13 complex is required to recruit or activate proteins needed by human cells to be able to make use of an undamaged homologous copy of DNA as an alternative template to avoid fork-blocking lesions and continue DNA replication.

In summary, our data provide direct evidence that human cells can carry out damage avoidance using an allelic copy of a gene as a temporary template for DNA replication and that this process involves gene conversion,

unaccompanied by crossing-over. Our mutagenesis data provide indirect evidence to support the model, proposed by Strauss and colleagues (11), that the newly-synthesized daughter strand of the sister duplex can also be used as a temporary template for DNA replication to avoid DNA damage. Our data indicate that *hMMS2* gene is essential in both cases. Our assay system provides a basis for identifying other proteins involved in the damage avoidance pathway dealing with fork-blocking lesions.

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References

1. Friedberg, E. C., Walker, G. C. & Siede, W. (1995) *DNA Repair and Mutagenesis.* (Am. Soc. Microbiol., Washington DC).

2. Nelson, J. R., Lawrence, C. W. & Hinkle, D. C. (1996) *Science* **272,** 1646-1649.

3. Woodgate, R. (1999) *Genes Dev.* **13**, 2191-2195.

4. Friedberg, E. C., Feaver, W. J. & Gerlach, V. L. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 5681-5683.

5. Pham, P., Bertram, J. G., O'Donnell, M., Woodgate, R. & Goodman, M. F. (2001) *Nature (London)* **409**, 366-370.

6. Gibbs, P. E., McGregor, W. G., Maher, V. M., Nisson, P. & Lawrence, C. W. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 6876–6880.

7. Masutani, C., Araki, M., Yamada, A., Kusumoto, R., Nogimori, T., Maekawa, T., Iwai, S. & Hanaoka, F. (1999) *EMBO J.* **18,** 3491-3501.

8. Masutani, C., Kusumoto, R., Iwai, S. & Hanaoka, F. (2000) *EMBO J.* **19,** 3100-3109.

9. Tissier, A., McDonald, J. P., Frank, E. G. & Woodgate, R. (2000) *Genes Dev.* **14,**1642-1650.

10. Gibbs, P. E., Wang, X. -D., Li, Z., McManus, T. P., McGregor, W. G., Lawrence C. W. & Maher, V. M. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 4186-4191.

11. Higgins, N. P., Kato, K. & Strauss, B. (1976) *J. Mol. Biol.* **101,** 417-425.

12. West, S. C., Cassuto, E. & Howard-Flanders, P. (1981) *Nature (London)* **294**, 659-662

13. Echols, H. & Goodman, M. F. (1991) *Annu. Rev. Biochem.* **60,** 477-511.

14. Spivak, G. & Hanawalt, P. C. (1992) *Biochemistry* **31**, 6794-6800.

15. Broomfield, S., Chow, B. L. & Xiao, W. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 5678-5683.

16. Prakash, L. & Prakash, S. (1977) *Genetics* **86,** 33-55.

17. Hofmann, R. M. & Pickart, C. M. (1999) *Cell* **96,** 645-653.

18. Ulrich, H. D. & Jentsch, S. (2000) *EMBO J.* **19,** 3388-3397.

19. VanDemark, A. P., Hofmann, R. M., Tsui, C., Pickart, C. M. & Wolberger, C. (2001) *Cell* **105,** 711-720.

20. Dubiel, W. & Gordon, C. (1999) *Curr. Biol.* **9**, R554-R557.

21. Xiao, W., Lin, S. L., Broomfield, S., Chow, B. L. & Wei, Y. F. (1998) *Nucleic Acids Res.* **26,** 3908-3914.

22. Lin, C. C., Wang, Q., Maher, V. M. & McCormick, J. J. (1994) *Cell Growth Differ.* **5,** 1381-1387.

23. Zhang, H., Tsujimura, T., Bhattacharyya, N. P., Maher, V. M. & McCormick, J. J. (1996) *Carcinogenesis* **17**, 2229-2235.

24. Ryan, P. A., Maher, V. M., & McCormick, J. J. (1987) *Exp. Cell Res.* **172**, 318-328.

25. Schreiber, E., Matthias, P., Muller, M. M. & Schaffner, W. (1989) *Nucleic Acids Res.* **17**, 6419.

26. Patton, J. D., Maher, V. M., & McCormick, J. J. (1986) *Carcinogenesis* **7,** 89-93.

27. Tsujimura, T., Maher, V. M., Godwin, A. R., Liskay, R. M. & McCormick, J. J. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1566-1570.

28. Maher, V. M. & McCormick, J. J. (1996) in *Technologies for Detection of DNA Damage and Mutagenesis*, ed. Pfeifer G. (Plenum, New York), pp. 381-390.

29. Gossen, M. & Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5547-5551.

30. Shockett, P., Difilippantonio, M., Hellman, N. & Schatz, D. G. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6522-6526.

31. Xiao, W., Chow, B. L., Broomfield, S., Hanna, M. (2000) *Genetics* **155**, 1633-1641.

32. McKenna, S., Spyracopoulos, L., Moraes, T., Pastushok, L., Ptak, C., Xiao, W., Ellison, M. J. (2001) *J Biol Chem.* **276**, 40120-40126.

33. Moraes, T. F., Edwards, R. A., McKenna, S., Pastushok, L., Xiao, W., Glover, J. N. & Ellison, M. J. (2001) *Nat. Struct. Biol.* **8**, 669-673.

CHAPTER III

Translesion synthesis and damage avoidance contribute almost equally to the tolerance of BPDE-adducts in human cells

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Abstract

We showed recently (Li et al., Proc. Natl. Acad. Sci. U.S.A. 99, 2002:4459) that hMms2 plays a critical role in a pathway by which human cells avoid translesion synthesis past fork-blocking lesions induced by UV by making temporary use of an undamaged copy of the blocked sequence, i.e., either the newly synthesized daughter strand of the sister duplex or an allelic copy, as a template to continue DNA replication. However, because UV produces several different kinds of photoproducts, and cells may differ significantly in their relative use of a particular pathway for each type of photoproduct, it is not possible to use UV as the DNA-damaging agent to determine the relative frequency with which human cells make use of damage avoidance (DA), rather than translesion synthesis (TLS). The present study was designed to estimate the ratio with which human fibroblasts use TLS versus DA to continue DNA replication past a major (~94%) type of fork-blocking adduct formed by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE). Three cell strains lacking hMms2 were compared with their hMms2-proficient parental strain for the ability to make temporary use of an undamaged homologous copy of DNA as a template to avoid such damage, rather than using translesion synthesis. The results show that loss of hMms2 did not increase the cytotoxic effect of BPDE, but completely eliminated the cells' ability to use an undamaged homologous copy of a reporter gene as a template for DNA replication past BPDE-adducts and increased the frequency of BPDE-

induced mutants in the single copy *HPRT* gene ~2.5-fold. These results indicate that human cells make approximately equal use of DA and TLS in order to continue replicating DNA containing BPDE adducts.

***Keywords: hMMS2*; damage avoidance; translesion synthesis; BPDE; homologous recombination; mutagenesis**

1. Introduction

Cells are continuously exposed to endogenous and exogenous DNA damaging agents. Certain kinds of lesions block replication carried out by the major DNA polymerase complex, but cells have developed mechanisms for tolerating such damage. For example, they can make temporary use of specialized distributive polymerases that are capable of inserting nucleotides opposite a damaged template (translesion synthesis) [1-6]. Alternatively, cells can make temporary use of an undamaged homolog of the blocked sequence, e.g., the newly synthesized daughter strand of the sister duplex [7], which was suggested by Higgins et al. [8], or the allelic gene [7], as a temporary template to circumvent the lesion (damage avoidance). Damage avoidance is error-free. In contrast, translesion synthesis can be either "error-prone" or "error-free" depending upon the type of damage, its sequence context in the DNA, and the availability of these various specialized distributive DNA polymerases in the cell. Evidence indicates that after a nucleotide(s) has been incorporated opposite a fork-blocking lesion and extended for a limited number of nucleotides, the major DNA replication

complex can resume replication. The relative use of these two processes in human cells is the subject of the present study.

Using a parental human fibroblast cell strain containing a chromosomally-integrated substrate that allows quantification of the frequency by which cells produce a wild type copy of a target *hyg* gene by combining genetic information from two defective homologous *hyg* genes [9-11] and its three derivative cell strains, we recently showed that hMms2, a ubiquitin-conjugating-enzyme-like protein [12], is essential if cells are to make temporary use of an undamaged homologous copy of a gene to circumvent UV photoproducts and continue DNA replication. Elimination of hMms2 protein from the derivative cell strains did not increase their sensitivity to the cytotoxic effect of UV, but completely eliminated the induction of Hyg-resistant cells, i.e., eliminated use of a homologous copy of a blocked gene to carry out damage avoidance. What is more, the frequency of UV-induced mutations in the target *HPRT* gene of the cells lacking hMms2 increased significantly above that induced in the parental cells, indicating increased use of error-prone translesion synthesis [7]. Because the target gene for detecting mutations is located on the X-chromosome, there is no allelic copy of the *HPRT* gene in these male cells. Therefore, the increase in *HPRT* mutations when hMms2 was eliminated strongly suggests that when it is present, hMms2 facilitates temporary use of the newly synthesized daughter strand of the sister duplex as an undamaged template for completing replication (damage avoidance).

Because UV produces several different kinds of photoproducts, and cells the potential for translesion synthesis, rather than temporary use of an undamaged template, may well depend on the type of photoproduct encountered by the replication fork [13], it is not possible to use UV as the DNA damaging agent to determine cells' relative use of these two pathways. Therefore, in the present study, designed to estimate this ratio, BPDE was used as the DNA-damaging agent. Approximately 94% of the adducts formed in DNA by BPDE involve guanine [14, 15], and consist of a covalent bond between the C10 of BPDE and the exocyclic 2-amino position of guanine [16]. This adduct strongly blocks the replication fork [17, 18] and is highly mutagenic in human cells [18-21]. (Of the mutations induced by BPDE in the *HPRT* gene of finite life span human fibroblasts, 110 of the 121 analyzed (≥ 90%) involved G:C base pairs; 95% of these were base substitutions, with the majority (70%) being G:C to T:A transversions [19-21]).

We compared the parental human cell strain and its three derivative strains lacking hMms2 for their response to BPDE. Loss of hMms2 did not increase the sensitivity of the cell strains to the cytotoxic effect of BPDE. In the parental cell strain, BPDE induced a dose-dependent increase in the frequency of hygromycin–resistant (Hyg^R) cells, indicating that the parental cells were able to combine information from two non-functional homologous copies of the *hyg* gene (damage avoidance). In the cell strains lacking hMms2, this process was completely eliminated. BPDE-induced a dose-dependent increase in the

frequency of mutations in the single copy *HPRT* gene of the parent cell strain, but in the cell strains lacking hMms2, the frequency of such mutations was 2.5-fold higher. These results indicate that loss of hMms2 not only eliminates use of a homologous allelic copy of a gene, but also use of the newly synthesized daughter strand of the sister duplex. They also indicate that human cells make approximately equal use of translesion synthesis and damage avoidance in order to tolerate BPDE-induced fork-blocking lesions.

2. Materials and methods

2.1. Cell strains and media used

The parental cell strain and its three derivative strains 2A-M2, 3C-M2, and 13D-M2, deficient in hMms2 protein, were described previously [7]. Unless otherwise indicated, cells were cultured in Eagle's minimal medium supplemented with L-aspartic acid (0.2 mM), L-serine (0.2 mM), sodium pyruvate (1 mM), hydrocortisone (1 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and supplemented bovine calf serum (Hyclone, Logan, UT) (10% vol/vol) (culture medium). This medium containing the appropriate selection drug, i.e., TG (40 μM) or hygromycin (100 U/ml) was used for selection of TG resistant or hygromycin resistant cells, respectively. When these cell strains were removed from storage in liquid nitrogen to be used in biological experiments, they were cultured for several days in selective culture medium containing histidinol or histidinol plus puromycin [7] to be sure that they retained the transfected genes.

2.2. BPDE treatment

Exposure of the cells to BPDE was carried out as described [21]. Briefly, cells in exponential growth were plated at 10^4 cells/cm^2 12 h before treatment to allow cells to attach to the culture dishes. The medium was removed from the dishes, cells were rinsed twice with phosphate-buffered saline (PBS), and Eagle's minimal essential medium lacking serum was added. BPDE was dissolved in anhydrous dimethylsulfoxide (DMSO) immediately before treatment and delivered to each dish by micropipette to yield the desired concentrations. An appropriate amount of DMSO was added to the dishes to ensure that all the cells were exposed to the same concentration of DMSO (<0.5%). After 1 h incubation at 37°C, in a humidified 5% CO_2 incubator, the medium containing BPDE was removed, and the cells were assayed for the biological effects of BPDE.

2.3. Assay for cytotoxicity

Cells were washed with PBS, and detached from the dishes using trypsin, suspended in culture medium, and plated into 100-mm diameter dishes at various cloning densities. The culture medium was renewed 7 days later. When the surviving cells had formed colonies, ~14 days after the plating, the cells were stained and the number of clones on each dish was scored. Cell survival was calculated as follows: for each dose, the cloning efficiency of the BPDE-treated cells was divided by that of the untreated control cells, and the value was expressed as percent of that of control cells.

2.4. Assay for the frequency of BPDE-induced hygromycin resistant cells

Cells were plated at 10^4 cells/cm^2 and sufficient sets of dishes were used to have at least 2×10^6 surviving target cells per dose. Just prior to exposure to BPDE, the cells in at least two dishes were detached and counted electronically to determine the percentage of cell attachment. After 1 h of BPDE treatment, the medium was removed, the cells were rinsed twice with PBS, and the medium was replaced with appropriate selective culture medium. Approximately 48 h after BPDE treatment, the medium was changed to culture medium containing hygromycin (100 U/ml), which was replaced every five days. Cells were stained 3 weeks after treatment. The frequency of recombination for each dose was calculated as follows: total hygromycin resistant clones divided by total number of surviving cells corrected by the percentage of attachment. The induced frequencies were calculated by subtracting the background frequencies.

2.5. Assay for the frequency of BPDE-induced mutations in the HPRT gene

Cells were plated at 10^4 cells/cm^2 into sufficient dishes to have at least 1×10^6 surviving target cells per dose, according to the expected survival. After 1 h exposure to BPDE, the cells were rinsed twice with PBS. The medium was replaced with appropriate selective culture medium, and cells were allowed an 8-day expression period to be depleted of wild type Hprt protein before at least 1×10^6 cells per dose were assayed for the frequency of TG resistance as described [22]. Culture medium containing 40 μM TG was renewed after 7 days. For each dose, a separate set of dishes with cells plated at cloning density in

medium lacking TG, was used to determine the cloning efficiency of the cells at the time of TG selection. The culture medium in these dishes was replaced 7 days after the plating. The cells for determining cloning efficiency and TG resistance were stained 7 days later. The number of clones on each dish was scored. For each cell strain, the frequency of mutants was calculated as follows: the number of colonies of TG resistant cells divided by the number of clonable cells selected (determined as above). The induced frequencies for each cell strain were calculated by subtracting the background frequencies determined using the mock-treated cells.

3. Results and discussion

3.1. Determining the relative use of translesion synthesis vs. damage avoidance

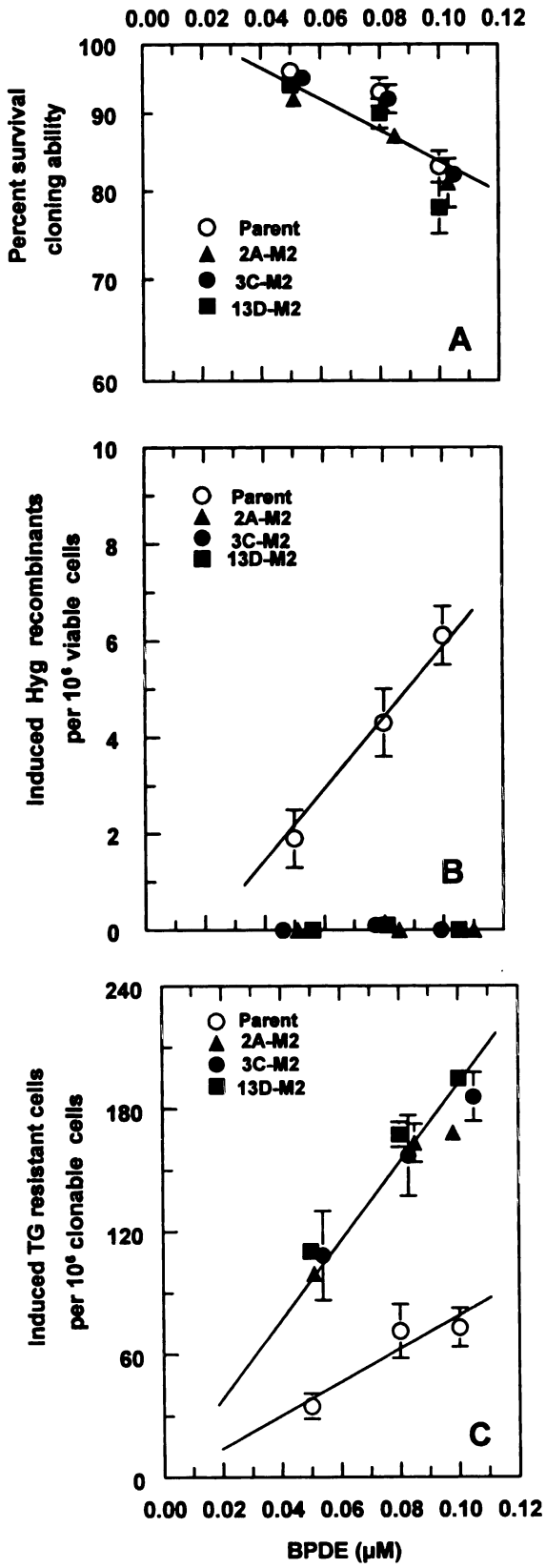
The underlying assumption an approach is that if an agent forms only one major type of fork-blocking adduct in DNA, and this adduct is mutagenic, and if one can eliminate use of the damage avoidance, error-free pathway for dealing with such damage, the relative use of translesion synthesis versus damage avoidance can be determined by comparing the frequency of mutations induced by that agent in cells in which use of damage avoidance has been blocked to that found in the wild type parental cells that retain the use of this latter pathway. One cannot estimate cells' relative use of damaged DNA directly as a template versus use of an undamaged homologous copy as a template to continue replication of DNA containing fork-blocking lesions if the DNA damage consists of several different kinds of fork-blocking lesions, e.g., UV-photoproducts, each with a

different potential for using one or the other of these two processes. Use of BPDE as the DNA-damaging agent eliminates this difficulty. In contrast to UV, BPDE induces only one very predominant adduct. Approximately 94% of BPDE adducts involve guanine [14, 15], and consist of a covalent bond between the C10 of the hydrocarbon molecule and the exocyclic N^2 of guanine [16]. Therefore, BPDE was used for the present study designed to estimate how frequently human cells make use of damage avoidance rather than translesion synthesis. To detect use of an undamaged copy of the blocked sequence as a template (damage avoidance), we employed as our target a substrate stably integrated into the genome of the target cells that allows quantification of the frequency with which cells produce a selectable wild type *hyg* gene, coding for resistance to hygromycin (Hyg^R), by combining information from two defective *hyg* genes [7, 9, 11]. To detect error-prone translesion synthesis, we employed as our target the *HPRT* gene, present as a single copy in our male target cells.

3.2. Effect of depletion of hMms2 protein to the cytotoxic effect of BPDE

As shown in Figure 1A, there was no significant difference between the parental cell strain and the three derivative cell strains devoid of hMms2 in sensitivity to the cytotoxic effect of BPDE. This indicates that the parental cells containing hMms2 and the derivative cell strains lacking hMms2 are equally capable of replicating the damaged DNA. This result is consistent with the previous finding that these cell strains deficient of hMms2 are not more sensitive to the cytotoxic effect of UV than their parental strain [8].

Figure 1. The biological effects of loss of hMms2 protein on BPDE-induced recombination and mutations. Open symbol, the parental cell strain; closed circle, strain 3C-M2; closed triangle, strain 2A-M2; and closed square, strain 13D-M2. Each data point is derived from the average of three independent experiments. For some data points, the standard deviation is too small to be visible. Some points are offset slightly in order to be visible. (A) The BPDE-induced cytotoxicity in these cell strains. The solid line represents the least-squares line for all the data points. (B) The BPDE-induced frequency of hyg^R cells in the parental cell strain and the cell strains lacking hMms2 protein. For the data points generated, at least 3×10^6 surviving cells were assayed in each experiment. The solid line represents the least-squares line for the data points obtained from the parental cell strain. (C) The BPDE-induced frequency of TG^R cells the wild type cell strain and the three cell strains lacking hMms2 protein. For the data points generated, at least 1×10^6 cells were assayed in each experiment. The observed frequency of TG^R clones was corrected by the cloning efficiency of the cells at the time of selection and the induced mutation frequency was obtained as described [22]. For the majority of the data points, the cloning efficiency of the cells at the time of selection ranged from 40-52%, so the correction factor was less than 3-fold. The solid lines represent the least-squares lines for the data obtained from the parental cell strain and cell strains lacking hMms2, respectively.


0.00 0.02 0.04 0.06 0.08 0.10 0.12
Percent survival cloning ability
100
90
80
70
60
Parent
2A-M2
3C-M2
13D-M2
A
Induced Hyg recombinants per 10⁶ viable cells
10
8
6
4
2
0
Parent
2A-M2
3C-M2
13D-M2
B
Induced TG resistant cells per 10⁶ clonable cells
240
180
120
60
0
Parent
2A-M2
3C-M2
13D-M2
C
0.00 0.02 0.04 0.06 0.08 0.10 0.12
BPDE (μM)

3.3. Effect of depletion of hMms2 protein on the frequency of BPDE-induced hyg^R recombinant cells

As shown in Figure 1B, the parental cell strain exhibited a dose-dependent increase in the frequency of BPDE-induced hyg^R cells (recombinants), indicating that this strain used the undamaged homologous copy of the *hyg* gene as a template to circumvent BPDE-induced adducts in one or the other of the two nonfunctional *hyg* genes. In contrast, this response to BPDE was eliminated from cell strains 2A-M2, 3C-M2, and 13D-M2 which are virtually devoid of hMms2 protein. These data indicate that hMms2 protein plays an essential role in allowing human cells to make use of a homologous copy of a blocked sequence as a temporary template to tolerate BPDE-induced fork-blocking lesions (damage avoidance). This is consistent with our previous finding in human cells containing UV-induced DNA damage [7].

3.4. Effect of depletion of hMms2 protein on the frequency of BPDE-induced TG^R cells

BPDE treatment also caused a dose-dependent increase in the frequency of *HPRT* mutants (TG^R cells) in the parental cell strain (Fig. 1C), indicating that these cells carry out translesion synthesis. The three cell strains lacking hMms2 protein also gave a dose-dependent induction of TG^R cells, but the frequency of TG^R cells at each dose was ~2.5 times higher than that induced in the parental cells, which are capable of carrying out damage avoidance. These data can be interpreted as indicating that loss of hMms2 protein resulted in lack of the ability

to use an undamaged copy of the blocked *HPRT* sequence as a template and, therefore, forced the cells to use the translesion synthesis pathway. Because there is only one copy of the *HPRT* gene in these target cell strains, the increased frequency of mutations in the *HPRT* gene provides indirect evidence that when hMms2 is present, it not only allows human cells to make use of a homologous copy, e.g., an allele, but also allows them to make use of the newly synthesized daughter strand of the sister duplex as an undamaged template to avoid DNA damage. The data in Figure 1A, 1B, and 1C strongly suggest that when normal human cells replicate DNA containing BPDE adducts, they make approximately equal use of damage avoidance and translesion synthesis, and that loss of hMms2, with the resulting loss of the ability to copy an undamaged homolog, channels DNA lesions into the translesion synthesis pathway, resulting in a 2.5-fold increase in frequency of mutants.

3.5. Significance for future studies

The present data obtained using BPDE, together with our earlier data using UV as the DNA damaging agent, indicate that translesion synthesis and recombination are the major means for tolerating fork-blocking lesions in human cells. Our data with the *hyg* gene indicate that damage avoidance is achieved via a recombination-mediated specialized DNA replication process that predominantly involves gene conversion, unaccompanied by crossing-over [9-11]. Both sets of data obtained using cells lacking hMms2, i.e., those exposed to UV [7] or to BPDE, indicate that hMms2 protein is absolutely essential for this

process, although the exact mechanisms and various components remain to be explored. Use of homologous recombination by mammalian cells to repair double-strand breaks has been extensively studied, and the mammalian homologs of the yeast *RAD52* epistasis group, *RAD51*, *RAD52*, *RAD54*, *RAD54B*, *RAD50*, *MRE11*, *NBS1*, *RAD51B*, *RAD51C*, *RAD51D*, *XRCC2* and XRCC3, have been implicated in this latter process [23]. The breast cancer susceptibility genes *BRCA1* and *BRCA2*, which do not appear to have yeast homologs, are also involved [24, 25]. It will be important to determine whether these gene products are involved in damage avoidance as a means of tolerating replication fork-blocking lesions.

How the hMms2/hUbc13 complex is connected to the recombination process is not yet clear. It has been shown in yeast that Ubc13 is required for the assembly of polyubiquitin chains linked through lysine 63 in a Mms2-dependent manner [26], and similar findings were also obtained in human cells [27]. A most intriguing finding was that by Ulrich and Jentsch [28] who demonstrated that in yeast, Rad5 interacts with both Ubc13 and Rad18 through its RING finger domain. These interactions bring the Mms2/Ubc13 complex into contact with the Rad6/Rad18 complex. They proposed that the Mms2/Ubc13 complex acts as a signal transducer to recruit target proteins to the site of DNA damage for repair. However, a human homolog of *RAD5* has not been identified. It will be important to find out whether a functional counterpart of yeast Rad5 exists in human cells. In addition, the connection between the novel polyubiquitin pathway through

lysine 63 catalyzed by the Mms2/Ubc13 complex and the damage tolerance pathways demonstrated in the present studies needs to be addressed. Our data suggest that the hMms2/hUbc13 complex is required to recruit and/or activate proteins needed for human cells to avoid fork-blocking lesions by using an undamaged homologous copy of DNA as an alternative template to continue DNA replication. The data implicate the novel polyubiquitin pathway through lysine 63, mediated by the hMms2/hUbc13 complex, as being essential for this recombination/gene conversion process.

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References

[1] J.R. Nelson, C.W. Lawrence, D.C. Hinkle, Thymine-thymine dimer bypass by yeast DNA polymerase zeta, Science 272 (1996) 1646-1649.

[2] W. Xiao, T. Lechler, B.L. Chow, T. Fontanie, M. Agustus, K.C. Carter, Y.F. Wei, Identification, chromosomal mapping and tissue-specific expression of hREV3 encoding a putative human DNA polymerase zeta, Carcinogenesis 19 (1998) 945-949.

[3] P.E. Gibbs, W.G. McGregor, V.M. Maher, P. Nisson, C.W. Lawrence, A human homolog of the Saccharomyces cerevisiae REV3 gene, which encodes the catalytic subunit of DNA polymerase zeta, Proc Natl Acad Sci U S A 95 (1998) 6876-6880.

[4] C. Masutani, R. Kusumoto, A. Yamada, N. Dohmae, M. Yokoi, M. Yuasa, M. Araki, S. Iwai, K. Takio, F. Hanaoka, The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta, Nature 399 (1999) 700-704.

[5] A. Tissier, E.G. Frank, J.P. McDonald, S. Iwai, F. Hanaoka, R. Woodgate, Misinsertion and bypass of thymine-thymine dimers by human DNA polymerase iota, EMBO J 19 (2000) 5259-5266.

[6] V.L. Gerlach, W.J. Feaver, P.L. Fischhaber, E.C. Friedberg, Purification and characterization of pol kappa, a DNA polymerase encoded by the human DINB1 gene, J Biol Chem 276 (2001) 92-98.

[7] Z. Li, W. Xiao, J.J. McCormick, V.M. Maher, Identification of a protein essential for a major pathway used by human cells to avoid UV- induced DNA damage, Proc Natl Acad Sci U S A 99 (2002) 4459-4464.

[8] N.P. Higgins, K. Kato, B. Strauss, A model for replication repair in mammalian cells, J Mol Biol 101 (1976) 417-425.

[9] N.P. Bhattacharyya, V.M. Maher, J.J. McCormick, Effect of nucleotide excision repair in human cells on intrachromosomal homologous recombination induced by UV and 1-nitrosopyrene, Mol Cell Biol 10 (1990) 3945-3951

[10] T. Tsujimura, V.M. Maher, A.R. Godwin, R.M. Liskay, J.J. McCormick, Frequency of intrachromosomal homologous recombination induced by UV radiation in normally repairing and excision repair-deficient human cells, Proc Natl Acad Sci U S A 87 (1990) 1566-1570.

[11] H. Zhang, G. Marra, J. Jiricny, V.M. Maher, J.J. McCormick, Mismatch repair is required for O(6)-methylguanine-induced homologous recombination in human fibroblasts, Carcinogenesis 21 (2000) 1639-1646.

[12] W. Xiao, S.L. Lin, S. Broomfield, B.L. Chow, Y.F. Wei, The products of the yeast MMS2 and two human homologs (hMMS2 and CROC-1) define a structurally and functionally conserved Ubc-like protein family, Nucleic Acids Res 26 (1998) 3908-3914.

[13] N. Nikolaishvili-Feinberg, M. Cordeiro-Stone, Bypass replication in vitro of UV-induced photoproducts blocking leading or lagging strand synthesis, Biochemistry 40 (2001) 15215-15223.

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

[15] T. Meehan, K. Straub, M. Calvin, Benzo[alpha]pyrene diol epoxide covalently binds to deoxyguanosine and deoxyadenosine in DNA, Nature 269 (1977) 725-727.

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

[17] M. Cordeiro-Stone, J.C. Boyer, B.A. Smith, W.K. Kaufmann, Effect of benzo[a]pyrene-diol-epoxide-I on growth of nascent DNA in synchronized human fibroblasts, Carcinogenesis 7 (1986) 1775-1781.

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

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

[20] R.H. Chen, V.M. Maher, J.J. McCormick, Effect of excision repair by diploid human fibroblasts on the kinds and locations of mutations induced by (+/-)-7 beta,8 alpha-dihydroxy-9 alpha,10 alpha-epoxy-7,8,9,10 tetrahydrobenzo[a]pyrene in the coding region of the HPRT gene, Proc Natl Acad Sci U S A 87 (1990) 8680-8684.

[21] J.L. Yang, R.H. Chen, V.M. Maher, J.J. McCormick, Kinds and location of mutations induced by (+/-)-7 beta,8 alpha-dihydroxy-9 alpha,10 alpha-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene in the coding region of the hypoxanthine (guanine) phosphoribosyltransferase gene in diploid human fibroblasts, Carcinogenesis 12 (1991) 71-75.

[22] V.M. Maher, J.J. McCormick The *HPRT* gene as a model system for mutation analysis, in: G.P. Pfeifer (Ed.), Technologies for Detection of DNA Damage and Mutations, Plenum Press, New York, 1996, pp. 381-390.

[23] R.D. Johnson, M. Jasin, Double-strand-break-induced homologous recombination in mammalian cells, Biochem Soc Trans 29 (2001) 196-201.

[24] M.E. Moynahan, A.J. Pierce, M. Jasin, BRCA2 is required for homology-directed repair of chromosomal breaks, Mol Cell 7 (2001) 263-272.

[25] M.E. Moynahan, J.W. Chiu, B.H. Koller, M. Jasin, Brca1 controls homology-directed DNA repair, Mol Cell 4 (1999) 511-518.

[26] R.M. Hofmann, C.M. Pickart, Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair, Cell 96 (1999) 645-653.

[27] S. McKenna, L. Spyracopoulos, T. Moraes, L. Pastushok, C. Ptak, W. Xiao, M.J. Ellison, Noncovalent interaction between ubiquitin and the human DNA repair protein Mms2 is required for Ubc13-mediated polyubiquitination, J Biol Chem 276 (2001) 40120-40126.

[28] H.D. Ulrich, S. Jentsch, Two RING finger proteins mediate cooperation between ubiquitin- conjugating enzymes in DNA repair, EMBO J 19 (2000) 3388-3397.

CHAPTER IV

hREV3 is essential for error-prone translesion synthesis past UV or benzo[a]pyrene diol-epoxide-induced DNA lesions in human fibroblasts

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Abstract

In *S. cerevisiae*, the *REV3* gene encodes the catalytic subunit of polymerase zeta, which is involved in translesion synthesis and is required for mutations induced by UV photoproducts and other DNA fork-blocking lesions, as well as for the majority of spontaneous mutations. To determine whether *hREV3*, the human homolog of yeast *REV3*, is similarly involved in error-prone translesion synthesis past UV photoproducts and other kinds of lesions that block DNA replication, we transfected an *hREV3* antisense construct under the control of the TetP promoter into an infinite life span human fibroblast cell strain that expresses a high level of tTAk, the activator of that promoter. Three cell stains expressing high levels of *hREV3* antisense RNA were identified and compared with the parental cell strain for their sensitivity to the cytotoxic and mutagenic effects of UV- or (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,20-tetrahydrobenzo[a]pyrene (BPDE). The latter strain gave a dose dependent increase in cell killing and induction of mutations in the single copy *HPRT* gene. In contrast, the cell strains expressing *hREV3* antisense did not exhibit significantly increased sensitivity to the cytotoxic effect of these agents, but the frequency of mutants induced by UV and by BPDE in these cell strains was reduced to ~16% and ~25% that of the parental cell strain, respectively. These data indicate that the function of hRev3 is not essential for cell survival, but is absolutely required for the induction of mutations by UV or BPDE. Whether this *in vivo* activity involves insertion of an incorrect nucleotide opposite

the fork-blocking lesion or extension beyond that position is not yet known.

***Keywords*: *hREV3*; translesion synthesis; mutagenesis; antisense RNA; UV; BPDE**

1. Introduction

DNA in prokaryotic and eukaryotic cells is continuously exposed to endogenous and exogenous DNA damaging agents. If not removed, certain types of DNA damage can impede normal DNA replication carried out by the replication complex [1]. Excision repair mechanisms physically remove DNA damage and restore the DNA to its original state. Nevertheless, cells possess damage tolerance mechanisms which enable them to continue synthesizing DNA containing fork-blocking lesions. In prokaryotes and eukaryotes, such damage tolerance mechanisms include translesion synthesis and damage avoidance. Damage avoidance is the term used to refer to various mechanisms by which cells make temporary use of an undamaged homologous DNA of the blocked sequence as a template to continue DNA replication. In mammalian cells, the undamaged copy can be either the newly synthesized daughter strand of the sister duplex [2], as suggested by Higgins et al. [3], or the homologous allelic gene. The alternative pathway for tolerating DNA fork-blocking lesions, i.e., translesion synthesis, requires specialized distributive DNA polymerases that, in contrast to the major DNA polymerases that are blocked by such damage, are

capable of incorporating nucleotides opposite the damaged template [4]. After distributive incorporation of nucleotides, followed by extension of a limited number of nucleotides, the major DNA replication complex is considered to resume replication activity by using the original strand as the template. Translesion synthesis can be either "error-prone" or "error-free" depending upon such factors as the type of lesion, its sequence context in the DNA, the availability of specialized distributive translesion synthesis polymerases in the cell, and the characteristic of the polymerases used. At this time, polymerase zeta, eta, iota, and kappa are considered to be the major DNA polymerases carrying out translesion synthesis past different kinds of DNA lesions in eukaryotes [5-10].

In *S. cerevisiae* yeast, *REV3* encodes the catalytic subunit of polymerase zeta (pol ζ), and *REV7* encodes the non-catalytic subunit. Compared to the wild type, yeast *rev3* mutants displayed only slightly increased sensitivity to the cytotoxic effect of UV [11], but loss of function of Rev3 eliminated almost all of the damage-induced mutations and a large portion of spontaneous mutations [12]. These studies indicate that Rev3 protein in yeast is not essential for preventing cell death induced by UV, but is essential for error-prone translesion synthesis past a variety of fork-blocking lesions. Lawrence and colleagues [5] showed that when tested *in vitro* using primer extension assays, yeast Rev3 and Rev7 proteins form functional pol ζ which is capable of inserting nucleotides opposite a thymine-thymine (T-T) dimer. Yeast pol ζ *in vitro* also performs error-prone

translesion synthesis past a T-T (6-4) photoproduct or an acetylaminofluorene-adducted guanine [13]. In addition, yeast pol ζ extends mismatches *in vitro* with a high efficiency [13, 14].

The human homolog of the yeast *REV3* gene, *hREV3*, was independently cloned by three groups [6, 7, 15]. Studies using the yeast two-hybrid analysis [16] indicates that the protein coded by *hREV3* interacts with that coded by a putative *hREV7*. One of these reports of the identification of *hREV3* included results from a pilot study of role of hRev3 in the induction of mutation in intact human cells by UV irradiation. The data showed that in a cell strain expressing *hREV3* antisense the frequency of mutants induced by UV was significantly reduced [7]. These results suggest that the function of *hREV3* is similar to that of its yeast counterpart. To examine the role of *hREV3 in vivo* more thoroughly and expand the study to include additional types of fork-blocking lesions, in addition to photoproducts of UV_{254nm} irradiation, we transfected *hREV3* antisense construct under the control of TetP promoter into an infinite life span human fibroblast cell strain that expresses a high level of tTAk which is required for the activation of that promoter. Three transfectants expressing high levels of *hREV3* antisense RNA were identified. These cell strains were compared with their parental strain for sensitivity to the cytotoxic and mutagenic effect of UV or BPDE. Our data show that, compared to their parental strain, the cell strains expressing high levels of *hREV3* antisense did not exhibit significantly increased sensitivity to the cytotoxic effect of UV or BPDE, but exhibited ~84% and ~75%

reduction of the frequency of *HPRT* mutants induced by UV and by BPDE, respectively. Our data indicate that the function of hRev3 is not essential for cell survival, but is required for UV- and for BPDE-induced mutations.

2. Material and methods

2.1. Cell culture

Cells used for cytotoxicity and 6-thioguanine (TG, Sigma) assays were cultured in Eagle's minimal medium supplemented with L-aspartic acid (0.2 mM), L-serine (0.2 mM), sodium pyruvate (1 mM), hydrocortisone (1 μg/ml), supplemented calf serum (Hyclone, Logan, UT) (10% vol/vol), penicillin (100 U/ml), and streptomycin (100 μg/ml) (culture medium). This medium containing TG (40 μM) was used for selection of *HPRT* mutants. When these cell strains were removed from storage in liquid nitrogen to be used in biological experiments, they were cultured for several days in McM medium lacking histidine containing histidinol or histidinol plus puromycin as described previously [2] to be sure that they retained the transfected genes coding for these selectable markers.

2.2. Transfection

The *hREV3* antisense construct was transfected into the parental cell strain using LipofectAMINE (Life Technologies, GibcoBRL). The same construct but not containing the antisense DNA, was also transfected into the parental strain to generate vector control transfectants. After 48 h, transfectants were selected in McM medium lacking histidine but containing histidinol plus 1 μg/ml puromycin.

This medium was renewed every five days. When the clones had formed, they were isolated, expanded in the selection medium, and stored frozen until needed.

2.3. Northern analysis

RNA was extracted from cells using RNAzol^{TM}B (TEL-TEST, INC.) according to the manufacture's recommendations. RNA was quantified and equal amount (15 μg) of total RNA was electrophoresed in 1.2% formaldehyde agrose gel. The gel was washed and transferred to Hybond-N^{+} (Amersham) nylon membranes. Hybridization was carried out according to the standard procedures using a sequence-specific probe labeled by PCR to determine the level of antisense *hREV3* expression. The membrane was stripped and re-probed with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) to compare the relative amount of total RNA loaded for each sample.

2.4. Western analysis for hRev3 protein

Nuclear proteins from cell strains were extracted as described [17]. Standard western analysis was carried out by running 4% acrylamide gel using 100 μg nuclear protein for each sample, followed by transfer in 0.1 M CAPS (pH=11) (Sigma) overnight and probing with five different antibodies against hRev3.

2.5. UV treatment and BPDE treatment

Exposure of the cells to UV and to BPDE was carried out as described [18] Briefly, cells in exponential growth were detached from the dishes using trypsin,

diluted in culture medium, plated at densities ranging from 0.5×10^6 to 1.5×10^6 per 150mm diameter dish or at cloning density for UV irradiation or plated at 10^4 cells/cm^2 for BPDE treatment. They were allowed ~12 h to attach before being exposed to the agent. The medium was removed from the dishes, and cells were rinsed twice with phosphate-buffered saline (PBS) before the treatment. For UV, cells were irradiated in a film of PBS. Immediately following irradiation the medium was added to each dish. For BPDE, Eagle's minimal essential medium lacking serum was added to each dish after PBS was removed. BPDE was dissolved in anhydrous dimethylsulfoxide (DMSO) immediately before treatment and delivered to each dish by micropipette to yield the desired concentrations. An appropriate amount of DMSO was added to the dishes to ensure that all the cells were exposed to the same concentration of DMSO (<0.5%). After 1 h incubation at 37°C, in a humidified 5% CO_2 incubator, the medium containing BPDE was removed, and replaced with fresh cultural medium.

2.6. UV- and BPDE-induced cytotoxicity assay

The UV_{254nm}-induced and BPDE-induced cytotoxicity was assayed as described [18]. Briefly, for UV-induced cytotoxicity, the cells were plated cloning densities and irradiated as above. The medium was renewed one day and seven days after UV-irradiation. The cells were stained ~14 days after UV treatment. The survival was determined by comparing the cloning efficiency of the irradiated cells to that of the sham-irradiated control cells, and the value was expressed as percent of that of those control cells. For determining BPDE-induced cytotoxicity,

the cells were plated and treated as above. After 1 h BPDE treatment, the cells were washed with PBS, trypsinized, diluted in the culture medium, and plated at desired cloning densities. The culture medium was renewed 7 days after plating, and cells were stained ~7 days later. The survival was calculated from the relative colony-forming ability of the BPDE-treated cells compared to the mock-treated control.

2.7. UV-induced and BPDE-induced HPRT mutation assay

The method used to determine the frequency of UV or BPDE-induced *HPRT* mutants has been described [19]. Briefly, for UV experiments, sufficient cells were plated at 0.5×10^6 to 2×10^6 per 150-mm diameter dish ensure to have at least 1×10^6 surviving founder cells per dose. For BPDE experiments, cells were plated into a series of 150mm diameter dishes at 10^4 cells/cm^2. After treatment, the cells were allowed an 8-day expression period to deplete the wild type Hprt protein before being assayed for the frequency of TG resistant cells. A separate set of cells plated at cloning density with medium lacking TG was used to determine the cloning efficiency of the cells at the time of selection,. The frequency of mutants was calculated as follows: total TG resistant clones divided by the total number of clonable cells selected. The induced frequencies were calculated by subtracting the background frequencies in the control population.

3. Results and discussion

3.1. Generation and characterization of cell strains expressing high levels of hREV3 antisense RNA

To generate cell strains expressing high levels of antisense *hREV3* RNA, we first generated an immortal, fibroblast-derived cell strain, designated 9N, by transfecting MSU-1.2-E7.2 cell strain [20] with a tetracycline transactivator (tTAk) construct under the control of TetP promoter [21, 22]. Western analysis indicated that the 9N cells express a high level of tTAk protein (data not shown) which is required for the activation of TetP promoter. An expression vector designated pTet-Puro contains a TetP promoter and a gene for puromycin resistance was designed. The antisense *hRev3* DNA, a 1,506-bp *Sal*I fragment of the *hREV3* cDNA, containing 48 bp of 5'-untranslated sequence, 1,415 bp of coding sequence, and polylinker sequences at each end, was cloned into pTet-Puro in the antisense orientation under the control of the TetP promoter to generate the plasmid pKS-2. pKS-2 was used to transfect the 9N parental cells to generate transfectants expressing *hREV3* antisense. A pTet-Puro vector lacking the antisense DNA was used to transfect the 9N cell strain to obtain vector control transfectants. At least 100 *hREV3* transfectants and 10 vector control transfectants were isolated and expanded for future study. RNA from these strains and the parental cell strain, 9N, was isolated and subjected to Northern analysis. Three transfectants were identified to express high levels of *hREV3* antisense. As shown in Figure 1, as expected, the parental cell strain and vector control transfectants (V1 and V2) do not express *hREV3* antisense RNA, whereas cell strains 6I, 8B, and 12C expressed high levels of antisense.

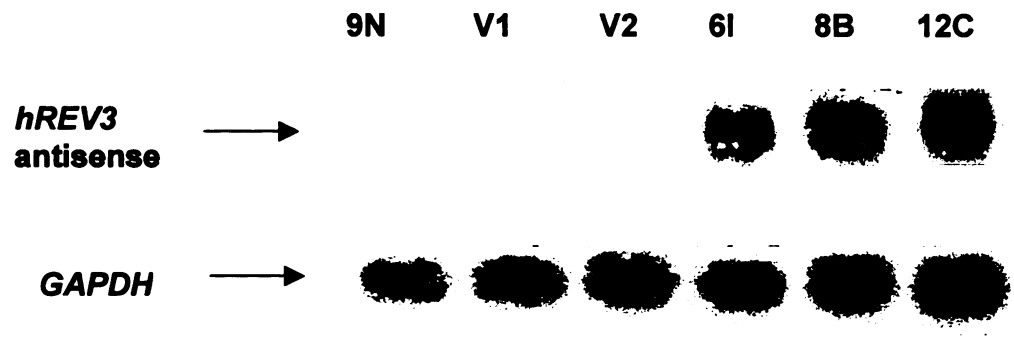

Figure 1

Figure 1. Northern blot analysis for detecting the expression of *hREV3* antisense. The parental cell strain 9N and two vector control transfectants V1 and V2 are not expressing antisense. Three transfectant cell strains 6I, 8B, and 12C express high levels of *hREV3* antisense. *GAPDH* was used as a loading control.

Expression of endogenous *hREV3* mRNA in the parental cell strain or the transfectant cell strains was not detected, indicating that the mRNA level is very low in human fibroblasts.

3.2. Western analysis of hRev3 protein

We attempted to determine that cell strains 6I, 8B, and 12C have a significantly lower amount of hRev3 protein than does the parental cell strain 9N. To do this, we raised independent anti-rabbit polyclonal antibodies against three peptide sequences in hRev3 and addition purchased two independent anti-goat polyclonal antibodies against hRev3 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Compared to the results from the pre-immune sera, no additional band was detected between 250 kDa and 450 kDa with these polyclonal antibodies against hRev3 which has a putative molecular weight about 350 kDa (data not shown). These data indicate the level of hRev3 protein is too low to be detected by these antibodies. Similar results were obtained using cell lysates instead of nuclear extracts. Three possibilities could explain these results: 1) none of these five different antibodies is sensitive enough to detect hRev3 protein; 2) the level of hRev3 protein level is too low to be detected; 3) the combination of these two. According to our Northern results, the endogenous mRNA of *hREV3* was virtually undetectable. In addition, *hREV3* mRNA has a translation false start site [7] which may reduce the efficiency of translation. Therefore, it is very possible that the level of hRev3 protein is very low in these human fibroblast strains and is beyond the limit of detection using these available

antibodies. In any event, we are not able to compare the hRev3 protein level directly between the parental cell strain and cell strains expressing *hREV3* antisense.

3.3. The effect of hREV3 antisense expression on UV-induced cytotoxicity

Cell strains 6I, 8B, and 12C, expressing high levels of *hREV3* antisense, were compared with their parental cell strain 9N for UV-induced cytotoxicity assays. Strains 6I, 8B, and 12C were not more sensitive to the cytotoxic effect of UV than the parental cell strain, (Fig. 2A), which is consistent with the results of the preliminary study involving a separate set of cell strains [7]. In addition, two vector control transfectants exhibited sensitivity to the cytotoxic effect of UV that was similar to the parental cell strain 9N (data not shown). These results suggest that hRev3 protein is not essential for preventing UV-induced cell killing, which is consistent with what is found in *S. cerevisiae* yeast.

3.4. The effect of hREV3 antisense expression on UV-induced mutagenesis

As expected, the parental cell strain 9N showed a dose-dependent increase of UV-induced *HPRT* mutant frequency. 6I, 8B, and 12C cell strains also showed a dose-dependent increase of UV-induced *HPRT* mutant frequency, but they exhibited >84% reduction of the frequency at each dose (Fig. 2B), which is consistent with the results found in the pilot study [7]. The two vector control transfectants exhibited a frequency of UV-induced *HPRT* mutants similar to that of the parental strain (data not shown), suggesting that these biological effects

Figure 2. The effect of expression of antisense *hREV3* on the survival and induced frequency of mutations observed with cell strains exposed to UV. Parental cell strain 9N (open circle), cell strains 6I (closed circle), 8B (closed triangle), and 12C (closed square). Some points have been offset slightly in order to be visible. (A) The UV-induced cytotoxicity in the parental cell strain and cell strains expressing antisense *hREV3*. The solid line represent the least square line for all the data points. (B) The UV-induced frequency of *HPRT* mutants in the parental cell strain and cell strains expressing antisense *hREV3*. The observed mutant frequency was corrected by the cloning efficiency of the cells at the time of selection, and the induced mutation frequency was obtained by subtracting the background frequencies, which for the most part were less than 10 per million clonable cells. For the majority of the data points, the cloning efficiency of the cells at the time of selection ranged from 40-54%, so the correction factor was less than 3-fold. In panel B, the solid lines represent the least-squares lines for the data obtained from the parental cell strain and cell strains expressing *hREV3* antisense, respectively.

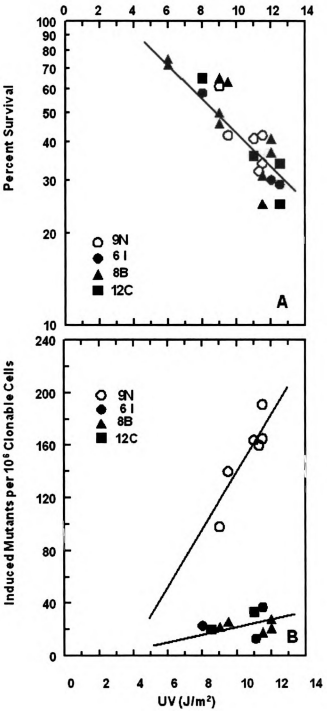
Percent Survival
Induced Mutants per 10^6 Clonable Cells
UV (J/m²)
9N
6 I
8B
12C
A
B

Figure 2

observed in 6I, 8B, and 12C were caused by the expression of antisense *hREV3* RNA and presumably caused by the loss of expression of hRev3 protein. Moreover, studies involving growth curve to compare rate of replication of the parental cell strain and vector control transfectants with that of cell strains, 6I, 8B, and 12C, did not show any evidence of a growth disadvantage (data not shown). This indicated that the biological effects were not caused by cells being sick or growing too slowly. Flow cytometry analysis indicated that 6I, 8B, and 12C cell strains are completely diploid (data not shown). Taken together, these data suggest that 6I, 8B, and 12C have a significantly reduced level of hRev3 protein, and indicate that hRev3 protein is essential for the error-prone translesion synthesis past UV-induced lesions in human cells.

3.5. The effect of hREV3 antisense expression on BPDE-induced cytotoxicity

Cell strains 6I and 12C were compared with the parental cell strain 9N for BPDE-induced cytotoxicity. Because in BPDE-induced cytotoxicity experiments, the survival is related to the amount of BPDE bound in the cell, and it was difficult to be certain that the amount of BPDE weighed out each time was equal because so little was used (20-30μg), the dose we gave was not consistent each time due to weighing error. In addition, BPDE is chemically unstable, and the plated cell density affects the cytotoxicity. Therefore, it is very difficult to compare the cell survival between the experiments. However, within one experiment, we did not observe a significant difference in BPDE-induced cytotoxicity between 9N, 6I, and 12C cell strains. For example, in one experiment, the survival at 0.06μM of

BPDE for 9N, 6I, and 12C was 68%, 71%, and 68%, respectively. The survival at 0.1μM BPDE for 9N, 6I, and 12C was 47%, 50%, and 50%, respectively (Fig. 3A).

3.6. The effect of hREV3 antisense expression on BPDE-induced mutagenicity

As expected, the parental cell strain 9N showed a dose-dependent increase of BPDE-induced *HPRT* mutant frequency. Cell strains 6I and 12C also showed a dose-dependent increase of BPDE-induced *HPRT* mutant frequency, but at each dose they exhibited >75% reduction of the frequency. Because the cell survival is not always consistent between experiments, and the mutant frequency is correlated to the cell survival, we plotted mutation frequency against the percentage of survival. Our data showed that at the same survival, the BPDE-induced mutation frequency in cell strains 6I and 12C was reduced to ~17% and ~23%, respectively, compared to the parental cell strain 9N (Fig. 4). These data support the hypothesis that hRev3 protein is also essential for the error-prone translesion synthesis past BPDE-induced fork-blocking lesions in human cells.

Figure 3. The biological effects of antisense *hREV3* expression in the parental cell strain 9N (open circle), and cell strains expressing *hREV3* antisense 6I (closed circle) and 12C (closed square). Some points are offset slightly in order to be visible. (A) The BPDE-induced cytotoxicity in the parental cell strain and cell strains expressing antisense *hREV3*. The solid line represents the least square line for all the data points. (B) The BPDE-induced frequency of *HPRT* mutants in the parental cell strain and cell strains expressing antisense *hREV3*. The observed mutant frequency was corrected by the cloning efficiency of the cells at the time of selection, and the induced mutation frequency was obtained by subtracting the background frequencies, which mostly are less than 10 per million clonable cells. For the majority of the data points, the cloning efficiency of the cells at the time of selection ranged from 39-55%, so the correction factor was less than 3-fold. The solid lines represent the least-squares lines for the data obtained from the parental cell strain and cell strains expressing *hREV3* antisense, respectively.

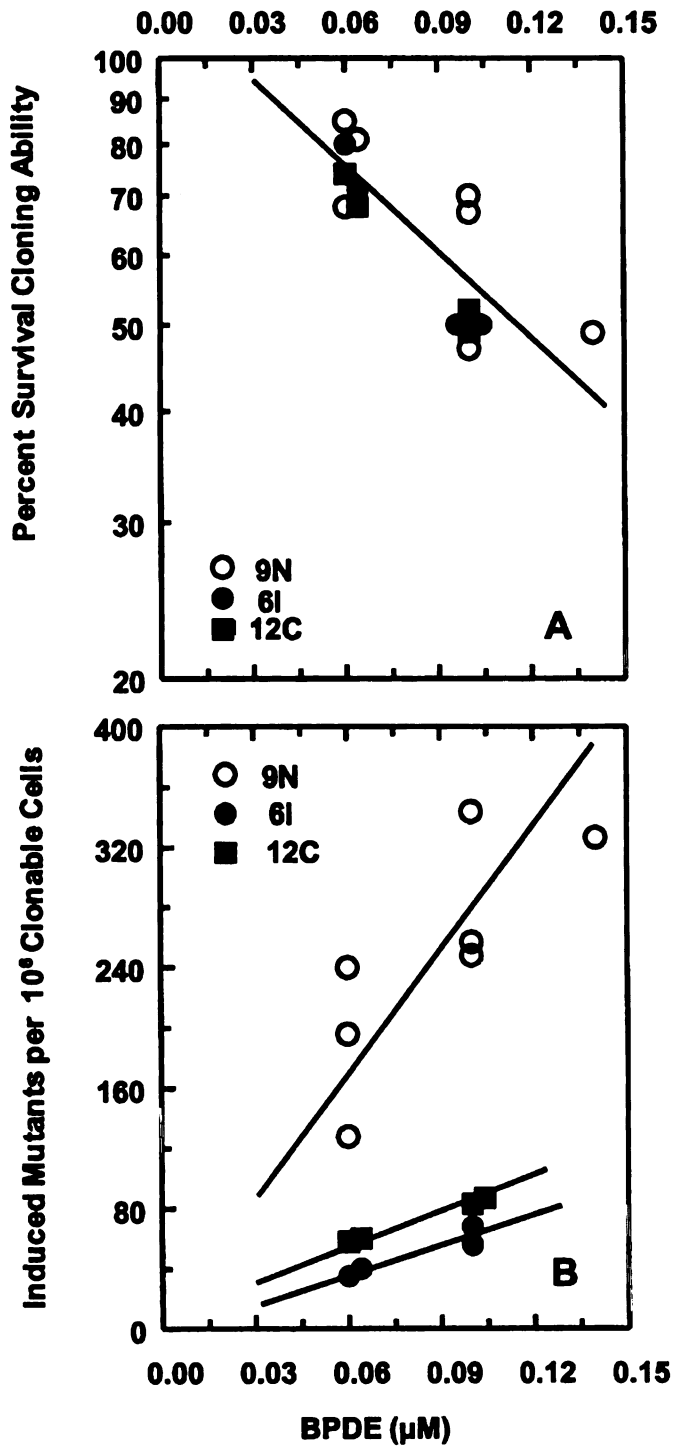
0.00 0.03 0.06 0.09 0.12 0.15
Percent Survival Cloning Ability
100
90
80
70
60
50
40
30
20
9N
6I
12C
A
Induced Mutants per 10⁸ Clonable Cells
400
320
240
160
80
0
9N
6I
12C
B
0.00 0.03 0.06 0.09 0.12 0.15
BPDE (μM)

Figure 3

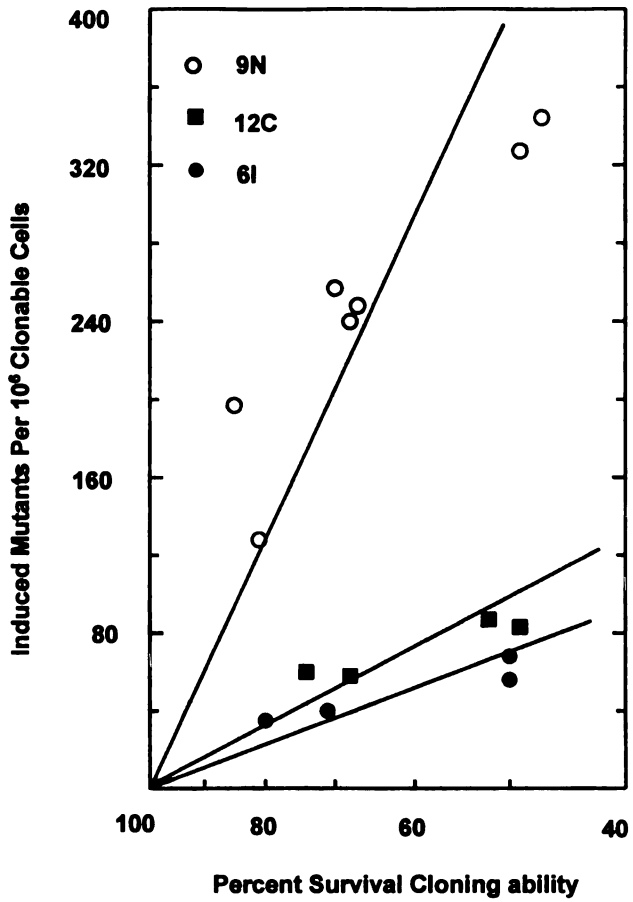

Figure 4. BPDE-induced mutagenesis versus percent cell survival in the parental cell strain 9N (open circle), and cell strains 6I (closed circle) and 12C (closed square)

3.7. Significance

The UV- and BPDE-induced mutagenesis data (Fig. 2B and 3B) suggest that the cell strains expressing *hREV3* antisense have a significantly reduced level of hRev3 protein compared to the parental cell strain, and that hRev3 protein is essential for UV- and BPDE-induced error-prone translesion synthesis. Human polymerase eta (pol η), which is able to carry out the error-free translesion synthesis for UV-induced T-T dimers [23] and is mutated in xeroderma pigmentosum variant patients [8, 24], is functional in these cell strains expressing *hREV3* antisense. pol η was shown to insert G across from T-T (6-4)s *in vitro* [26] and may have contributed mutations. Polymerase iota (pol ι), which is a remarkable error-prone DNA polymerase *in vitro* for T-T dimers and T-T (6, 4s) photoproducts [9], is presumably functional in these cells as well. Therefore, the UV-induced mutation frequency observed in these cell strains expressing antisense (~16% of the parental cell strain) could be due to these DNA polymerases or due to the remaining hRev3 protein. Pol η was also shown *in vitro* to be able to insert A across from BPDE-adducted guanine [26, 27]. It might play a role in generating the BPDE-induced mutants in the cell strains expressing *hREV3* antisense.

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References

[1] E.C. Friedberg, G.C. Walker, W. Siede, DNA Repair and Mutagenesis, Am. Soc. Microbiol. Press, Washington, D.C., 1995.

[2] Z. Li, W. Xiao, J.J. McCormick, V.M. Maher, Identification of a protein essential for a major pathway used by human cells to avoid UV- induced DNA damage, Proc Natl Acad Sci U S A 99 (2002) 4459-4464.

[3] N.P. Higgins, K. Kato, B. Strauss, A model for replication repair in mammalian cells, J Mol Biol 101 (1976) 417-425.

[4] C.W. Lawrence, V.M. Maher, Eukaryotic mutagenesis and translesion replication dependent on DNA polymerase zeta and Rev1 protein, Biochem Soc Trans 29 (2001) 187-191.

[5] J.R. Nelson, C.W. Lawrence, D.C. Hinkle, Thymine-thymine dimer bypass by yeast DNA polymerase zeta, Science 272 (1996) 1646-1649.

[6] W. Xiao, T. Lechler, B.L. Chow, T. Fontanie, M. Agustus, K.C. Carter, Y.F. Wei, Identification, chromosomal mapping and tissue-specific expression

of hREV3 encoding a putative human DNA polymerase zeta, Carcinogenesis 19 (1998) 945-949.

[7] P.E. Gibbs, W.G. McGregor, V.M. Maher, P. Nisson, C.W. Lawrence, A human homolog of the Saccharomyces cerevisiae REV3 gene, which encodes the catalytic subunit of DNA polymerase zeta, Proc Natl Acad Sci U S A 95 (1998) 6876-6880.

[8] C. Masutani, R. Kusumoto, A. Yamada, N. Dohmae, M. Yokoi, M. Yuasa, M. Araki, S. Iwai, K. Takio, F. Hanaoka, The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta. Nature 399 (1999) 700-704.

[9] A. Tissier, E.G. Frank, J.P. McDonald, S. Iwai, F. Hanaoka, R. Woodgate, Misinsertion and bypass of thymine-thymine dimers by human DNA polymerase iota, EMBO J 19 (2000) 5259-5266.

[10] V.L. Gerlach, W.J. Feaver, P.L. Fischhaber, E.C. Friedberg, Purification and characterization of pol kappa, a DNA polymerase encoded by the human DINB1 gene, J Biol Chem 276 (2001) 92-98.

[11] J.F. Lemontt, Pathways of ultraviolet mutability in Saccharomyces cerevisiae. I. Some properties of double mutants involving uvs9 and rev, Mutat Res 13 (1971) 311-317.

[12] C.W. Lawrence, D.C. Hinkle, DNA polymerase zeta and the control of DNA damage induced mutagenesis in eukaryotes, Cancer Surv 28 (1996) 21-31.

[13] D. Guo, X. Wu, D.K. Rajpal, J.S. Taylor, Z. Wang, translesion synthesis by

yeast DNA polymerase zeta from templates containing lesions of ultraviolet radiation and acetylaminoflurene, Nucleic Acids Res 29 (2001) 2875-2883.

[14] R.E. Johnson, M.T. Washington, L. Haracska, S. Prakash, L. Prakash, Eukaryotic polymerases iota and zeta act sequentially to bypass DNA lesions, Nature 406 (2000) 1015-1019.

[15] W. Lin, X. Wu, Z. Wang, A full-length cDNA of hREV3 is predicted to encode DNA polymerase zeta for damage-induced mutagenesis in humans, Mutat Res 433 (1999) 89-98.

[16] Y. Murakumo, Y. Ogura, H. Ishii, S. Numata, M. Ichihara, C.M. Croce, R. M. Fishel, Takahashi, Interactions in the error-prone postreplication repair proteins hREV1, hREV3, and hREV7, J Biol Chem 276 (2001) 35644-35651.

[17] E. Schreiber, P. Matthias, M.M. Muller, W. Schaffner, Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells, Nucleic Acids Res 17 (1989) 6419.

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

[19] V.M. Maher, J.J. McCormick The *HPRT* gene as a model system for mutation analysis, in: G.P. Pfeifer (Ed.), Technologies for Detection of DNA Damage and Mutations, Plenum Press, New York, 1996, pp. 381-390.

[20] H. Zhang, T. Tsujimura, N.P. Bhattacharyya, V.M. Maher, J.J. McCormick, O6-methylguanine induces intrachromosomal homologous recombination in human cells, Carcinogenesis 17 (1996) 2229-2235.

[21] M. Gossen, H. Bujard, Tight control of gene expression in mammalian cells by tetracycline-responsive promoters, Proc Natl Acad Sci U S A 89 (1992) 5547-5551.

[22] P. Shockett, M. Difilippantonio, N. Hellman, D.G. Schatz, A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice, Proc Natl Acad Sci U S A 92 (1995) 6522-6526.

[23] C. Masutani, M. Araki, A. Yamada, R. Kusumoto, T. Nogimori, T. Maekawa, S. Iwai, F. Hanaoka, Xeroderma pigmentosum variant (XP-V) correcting protein from HeLa cells has a thymine dimer bypass DNA polymerase activity, EMBO J. 18 (1999) 3491-3501.

[24] R.E. Johnson, C.M. Kondratick, S. Prakash, L. Prakash, hRAD30 mutations in the variant form of xeroderma pigmentosum, Science 285 (1999) 263-265.

[25] R.E.Johnson, L. Haracska, S. Prakash, L. Prakash, Role of DNA polymerase zeta in the bypass of a (6-4) TT photoproduct, Mol Cell Biol 21 (2001), 3558-3563.

[26] D. Chiapperino, H. Kroth, I.H. Kramarczuk, J.M. Sayer, C. Masutani, F. Hanaoka, D.M. Jerina, A.M. Cheh, Preferential misincorporation of purine nucleotides by human DNA polymerase eta opposite benzo[a]pyrene 7,8-

diol 9,10-epoxide deoxyguanosine adducts, J Biol Chem 277 (2002) 11765-11771.

[27] Y. Zhang, F. Yuan, X. Wu, O. Rechkoblit, J.S. Taylor, N.E. Geacintov, Z. Wang, Error-prone lesion bypass by human DNA polymerase eta, Nucleic Acids Res 28 (2000) 4717-4724.