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By

Kristin McNally

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ABSTRACT

hRev7 IS INVOLVED IN MUTAGENIC TRANSLESION SYNTHESIS PAST UV-INDUCED DNA DAMAGE IN HUMAN FIBROBLASTS

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Translesion synthesis (TLS) is a damage tolerance pathway that uses specialized DNA polymerases to insert nucleotides at or near sites of DNA damage. TLS can be an error-free or an error-prone process depending on the type of DNA damage encountered, the sequence context surrounding the damage site, and the specialized polymerase involved. The enzymes involved in TLS have been widely investigated in *E.coli* and yeast cells, and over the past five years, several human homologs of the TLS polymerases have been identified. One of the specialized DNA polymerases involved in TLS is polymerase (pol) zeta (ζ). Pol ζ in human cells is thought to be composed of hRev3, the catalytic subunit, and hRev7, a noncatalytic subunit. Previous studies in the Carcinogenesis Laboratory have shown that human cells expressing antisense against hRev3 demonstrate a significant reduction in the frequency of ultraviolet (UV_{254nm})-induced mutations, suggesting that hRev3 is critically involved in replication past UV-induced DNA damage (Gibbs et al., 1998). hRev7 was first identified in a yeast-two-hybrid assay using part of the hRev3 protein as bait, and hRev7 shares 23% identity and 53% similarity at the amino acid level with the yeast Rev7 protein. A role for hRev7 in TLS was hypothesized

based on its interaction with hRev3, as well as the significant homology hRev7 shares with yeast Rev7. Despite this evidence, however, the role of hRev7 in mutagenesis has remained uncharacterized. To determine whether hRev7 is involved in mutagenesis and TLS, RNA interference was used to establish cell strains with significantly reduced hRev7 protein. Cells with greatly reduced hRev7 were UV irradiated, and their survival and mutation frequency was compared to that of their parental cell strain and a vector control cell strain. Cell strains with reduced hRev7 were more sensitive to the cytotoxic effects of UV radiation than parental or vector control cells. In addition, the frequency of UV-induced mutations in the HPRT gene in cells with reduced hRev7 was significantly decreased compared to parental and vector control cells. These data strongly suggest that hRev7 is involved in UV-induced mutagenesis in human cells. The kinds of UV-induced base substitutions in cells with decreased hRev7 differed from those induced in parental and vector control cells, also suggesting an important role for hRev7 in bypass of UV-induced DNA damage. Furthermore, cells with decreased hRev7 exhibited a UV-induced delay in S-phase progression compared to control cells. Taken together, these data indicate that hRev7 plays an important role during replication past UV-induced DNA damage in human cells, and strongly suggest that hRev7 interacts with hRev3 to constitute human polymerase ζ.

DEDICATION

I dedicate my dissertation to my Mom,

Karen, Tim, Sammo and Telula.

I love you all very much.

"Consistency is a highly overrated virtue. I'm not ashamed to admit that I no longer believe half of what I was sure of 10 years ago. You make mistakes, you get new information, you change your mind along the way. It's a natural process."

-William Faulk in *The Week*

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ABBREVIATIONS

A	adenine
AAF	N-2-acetylaminofluorene
ADAM	a disintegrin and metalloprotease domain
ADP	adenovirus death protein
AP	apurinic/apyrimidinic site
APC	anaphase promoting complex
BER	base excision repair
BPDE	(+)-7B, 8α-dihydroxy-9α, 10α-epoxy-7,8,9,10-tetrahydro-benzo[a]pyrene or benzo [a]pyrene diol-epoxide
BRCT	BRCA1 carboxyl-terminal domain
C	cytosine
cDNA	complementary DNA
CPD	*cis-syn* cyclobutane pyrimidine dimer
CMV	cytomegalovirus
DA	damage avoidance
DNA	deoxyribonucleic acid
DSB's	double-strand breaks
DT	damage tolerance
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycoltetraacetic acid
ENU	N-ethyl-N-nitrosourea

G	guanine
HEK	human embryonic kidney
HEPES	4-(2-Hydroxyethyl)-1-piperazine-ethanesulphonic acid
HNPCC	hereditary non-polyposis colorectal cancer
HPRT	hypoxanthine phosphoribosyl transferase
HR	homologous recombination
Hyg	hygromycin
IR	ionizing radiation
LB	Luria broth
MAD	mitotic arrest deficient
MMR	mismatch repair
NER	nucleotide excision repair
NHEJ	non-homologous end joining
ORF	open reading frame
PAD	polymerase associated domain
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
Pfu	*Pyrococcus furiosus*
6-4 PP	6-4 pyrimidine-pyrimidone photoproduct
PMSF	phenylmethylsulphonyl fluoride
pol	polymerase
PCR	polymerase chain reaction
PRCC	papillary renal cell carcinoma

RB	retinoblastoma gene
Rb	retinoblastoma protein
RPM	revolutions per minute
ss	single-strand
SSB	single-strand binding protein
T	thymine
TCS	trichosanthin
TG	6-thioguanine
TG^r	6-thioguanine-resistant
TLS	translesion synthesis
UTM	untargeted mutagenesis
UTR	untranslated region
UV	ultraviolet radiation$_{254nm}$
XP	xeroderma pigmentosum
XPA	xeroderma pigmentosum complementation group A
XPV	xeroderma pigmentosum variant

INTRODUCTION

DNA damage is ubiquitous in nature, and is potentially lethal to cells if left unrepaired. Many forms of damage distort the DNA structure and block DNA synthesis. In order to overcome this barrier, cells have evolved different mechanisms to deal with DNA damage. Cells have several repair pathways that remove or repair damaged DNA, which are critical to ensure faithful replication of DNA (for review see Marti et al., 2002; Sinha and Hader, 2002; Charames and Bapat, 2003). One of the major classes of genes known to be involved in cancer comprise DNA repair proteins, underscoring the importance of these pathways in preventing cancer (Cotran et al., 1999). In addition to repair pathways, cells may activate checkpoints to delay cell cycle progression, thereby allowing more time for repair of the damage before replication proceeds.

In addition to DNA repair pathways and cell cycle checkpoints, cells have damage tolerance pathways to temporarily cope with DNA damage. One damage tolerance pathway, called damage avoidance (DA), allows cells to use a copy of the region of damaged DNA, such as the newly replicated daughter strand, as an undamaged template for replication (for review see Broomfield et al., 2001; Smirnova and Klein, 2003). Because an undamaged copy is used as the template, damage avoidance is considered to be an error-free process. Translesion synthesis (TLS), another damage tolerance pathway, uses specialized DNA polymerases to incorporate nucleotides across from DNA damage. Most of these specialized polymerases belong to the Y family of DNA

polymerases and have characteristics that give them the unique ability to insert nucleotides across from DNA damage, unlike replicative polymerases. These characteristics include being poorly processive, and lacking a 3' to 5' exonuclease proofreading activity. Like classical DNA polymerases, Y family polymerases have an overall right hand three-dimensional structure. However, Y family polymerases have wider active sites, which cause them to be less discriminating when incorporating nucleotides (Trincao et al., 2001; Ling et al., 2001; Zhou et al., 2001; Silvian et al., 2001). Because of this ability to be less discriminating, TLS is frequently an error-prone process; therefore, although TLS polymerases potentially enhance survival, the risk of generating mutations also increases.

Homologs of TLS enzymes have been found in many organisms, suggesting that this phenomenon has been conserved throughout evolution (Gibbs et al., 1998; Han et al., 1998; Van Sloun et al., 1999; Gibbs et al., 2000; Murakumo et al., 2000; Eeken et al., 2001; Masuda et al., 2002; Sakai et al., 2002; Sakamoto et al., 2003; Sonoda et al., 2003; Simpson and Sale, 2003). In human cells, there are five enzymes known to be involved in TLS: polymerase (pol) eta (η), pol iota (ι), pol kappa (κ), pol zeta (ζ), and Rev1. Pol ζ is composed of two proteins; Rev3 and Rev7. Based on yeast studies, pol ζ is an error-prone TLS polymerase involved in the tolerance of many kinds of DNA damage, including UV radiation, ionizing radiation, and several chemical carcinogens (Lemontt 1971; Lemontt 1972; Prakash 1976; Ruhland and Brendel, 1979; Henriques and Moustacchi,

1980; Lawrence et al., 1984; Lawrence et al., 1985). Much less is known about pol ζ in human cells. Human Rev3 (hRev3) is a member of the B family of DNA polymerases because it contains classical DNA polymerase motifs (Morrison et al., 1989; Gibbs et al., 1998; Morelli et al., 1998; Xiao et al., 1998). Rev3 is unique, then, as a TLS polymerase because all other polymerases involved in TLS belong to the Y family of DNA polymerases (for review see Lawrence, 2004; Vaisman et al., 2004; Ohmori et al., 2004; Yang, 2005). Studies in the Carcinogenesis Laboratory have shown that cells expressing high levels of antisense against hRev3 have significantly reduced UV- and BPDE-induced mutation frequencies, indicating that hRev3 is important for the tolerance of DNA damage induced by these agents in human cells (Gibbs et al, 1998). To date, human Rev3 has not been isolated, presumably because of the very low cellular levels of hRev3. Because of this limitation, *in vitro* data about the abilities and preferences of hRev3 in bypassing different types of DNA lesions is currently lacking.

Rev7 forms a heterodimer with Rev3 and is a non-catalytic subunit of pol ζ in yeast. Human Rev7 shares high homology with the yeast protein and it also interacts with hRev3 in yeast-two-hybrid assays and *in vitro* binding assays (Murakumo et al., 2000; Murakumo et al., 2001). Taken together, these data strongly support the hypothesis that hRev7 is the noncatalytic subunit of human pol ζ. However, the role of hRev7 in mutagenesis remained uncharacterized. The purpose of this dissertation research has been to elucidate the role of hRev7

in mutagenesis. I hypothesized that hRev7 is the human homolog of the yeast Rev7 protein, such that, it plays a role in mutagenic TLS as a subunit of pol ζ. The results of my studies have shown that hRev7 is involved in mutagenic translesion synthesis past UV-induced DNA damage.

This dissertation is organized into four parts. **Chapter I** is a comprehensive review of the literature, focusing on the process of TLS in the three most widely studied systems to date: *E.coli*, *Saccharomyces cerevisiae*, and humans. **Chapter II** describes experiments that elucidate the effects of reduced hRev7 in human fibroblast cells. This chapter contains results of the experiments using UV radiation as the DNA damaging agent, which will be submitted as a manuscript. **Chapter III** describes the results of research carried out on cells that overexpress hRev7. **Chapter IV** is the final chapter, which discusses the areas of important future research involving hRev7.

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Chapter I

LITERATURE REVIEW

A. DNA DAMAGE AND REPAIR

Damage to DNA can occur from physical agents, such as UV radiation, and chemical agents, such as benzo [a] pyrene diol-epoxide (BPDE). DNA damage can also occur from endogenous sources such as oxidative agents. If left unrepaired, DNA damage can lead to mutations in the genetic code. A major way that DNA damage can lead to a mutation is through the action of specialized DNA polymerases during translesion synthesis (TLS) (discussed in detail later). Removing DNA damage is important for maintaining cellular survival because DNA damage blocks replication by pol delta. Blocked replication forks can break down and lead to decreased cellular survival. Cells have several sophisticated pathways to repair DNA damage so that the polymerase delta replication complex can accurately replicate the DNA. These repair pathways include base excision repair, nucleotide excision repair, and mismatch repair.

Base excision repair (BER)

BER is an important pathway responsible for repairing apurinic/apyrimidinic (AP) sites, oxidative damage, and single strand breaks present in DNA (Mitra et al., 1997; Wilson, 1998). The process of BER is conserved from *E.coli* to humans, and includes recognition of damaged DNA, excision of the damaged base from

phosphor-ribose backbone by DNA glycosylases, conversion of the 3' termini to a 3'-OH by AP endonucleases, incorporation of nucleotides by pol β, and sealing of the patch by DNA ligase (Nash et al., 1996; Piersen et al., 1996; Srivastava et al., 1998; Wilson, 1998; Tomkinson and Mackey, 1998; Pearl, 2000; Zharkov et al., 2002). Gross defects in BER are thought to be lethal, based on the observation that mice engineered to have a major defect in BER do not survive embryogenesis (Xanthoudakis et al., 1996; Gu et al., 1994). More recently, however, minor defects in BER have been linked to colorectal and lung cancer demonstrating the importance of repairing DNA damage via the BER pathway (Chow et al., 2004; Frosina, 2004).

Nucleotide excision repair (NER)

Another repair pathway, NER, efficiently removes bulky lesions that severely distort the DNA structure, such as those caused by UV radiation and BPDE. Numerous proteins are required for NER, and each protein is involved in a different step of recognizing DNA damage, recruiting additional factors, excising the damaged region, and filling of the gap (for review see Sancar, 1996; Wood, 1997; Sancar et al., 2004). After recognition of damage, a strand of approximately 23-32 nucleotides containing bulky DNA damage is excised (Huang et al., 1992). Once the damaged region has been removed, the resulting gap in the DNA is filled by the combined efforts of DNA pol delta, proliferating cell nuclear antigen (PCNA), single-strand (ss) binding protein, and DNA ligase.

The importance of NER is clearly demonstrated by the xeroderma pigmentosum (XP) phenotype. XP is an autosomal recessive disease occurring in humans at a frequency of 1:250,000 in the US (Bootsma et al., 1998). Cells from XP patients are sensitive to the cytotoxic effects of UV radiation and are hypermutable after exposure to UV radiation. Patients with XP have a defect in one of the eight major proteins involved in NER (Cleaver, 1968). These different complementation groups vary in the severity of their phenotype, but all XP patients have a high rate of skin cancer directly related to their inability to repair UV-induced DNA damage (reviewed in Berneburg and Lehman, 2001). Specifically, UV-induced DNA damage is excised at significantly reduced rates in cells from XP patients compared to normal cells, leading to large amounts of unrepaired DNA damage (Cleaver, 1968). The presence of damage at the time of DNA replication causes a high frequency of mutations, which leads to an increased incidence of cancer. Overall, the frequency of cancer in XP patients is approximately 1000 times that seen in the general population under 20 years of age (Cleaver, 1999).

Mismatch Repair (MMR)

MMR is responsible for repairing single base pair mismatches and short mismatched loops in newly synthesized DNA, which are often a result of replicating microsatellite regions (reviewed in Bronner et al., 1994; Peltomaki, 1997; Peltomaki and de la Chapelle, 1997). Microsatellites are regions of DNA where single nucleotide or short DNA sequences are repeated. Regions of

microsatellites are prone to replication errors because replication of these regions can cause the replication complex to slip, resulting in inaccurate replication. This phenomenon is known as microsatellite instability (MSI). A link to cancer was discovered when MSI was determined to be the genetic basis for tumorigenicity in hereditary nonpolyposis colorectal cancer (HPNCC) (Boland and Goel, 1998; Lynch et al., 2003; Colombino et al., 2003). HPNCC is an autosomal dominant disease accounting for 5-10% of the total number of colon cancer cases (Thorson et al., 1999). Defects in MMR genes were found to be responsible for the high frequency of mutations found in HPNCC, ultimately leading to tumor formation (Fishel et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993; Bronner et al., 1994). To date, greater than 400 mutations in MMR genes have been found in this type of cancer (Peltomaki, 2003).

The DNA repair pathways are very important for repairing damaged DNA because DNA damage plays a critical role in the process of mutagenesis. DNA damage is often miscoding, which can cause the incorrect nucleotide to be inserted during replication, ultimately leading to a mutation. The process of DNA damage being converted into a mutation is called translesion synthesis and is discussed in detail in this literature review. To summarize, DNA damage can lead to mutations in the genetic code, which in turn, play a causal role in the development of cancer.

B. MUTAGENESIS LEADS TO CARCINOGENESIS

Mutations in the DNA can lead to cancer. In most cases, this process requires, first, that a mutation give rise to a selective growth advantage for a cell and for that cell to undergo clonal expansion. Then, another mutation may occur in this population giving rise to another selective advantage, and as this process continues, the accumulated changes allow for transformation of the cells, leading to cancer. Hanahan and Weinberg (2000) define seven categories of changes that cells can acquire to become malignant including self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, limitless replicative potential, evasion of apoptosis, sustained angiogenesis, tissue invasion, and metastasis. There are, however, some interesting and well investigated examples of a mutation in a tumor suppressor gene leading to many of the aforementioned changes and, ultimately, to cancer. Tumor suppressor genes, including p53 and RB, are genes that lead to cancer when they become inactivated.

p53 is a transcription factor that plays a role in many cellular functions including cell cycle regulation, transcriptional regulation, and apoptosis. Over 50% of human cancers have a mutation in the p53 gene, underscoring the importance of this protein in preventing human cancer (Soussi and Beroud, 2001). Specifically, thousands of different mutations in p53 have been reported (Soussi et al., 2005). Many of the mutations are located in the DNA binding domain of p53, and these mutations create either loss of function and gain of function mutations. Often, these mutations function as dominant negative mutations, sequestering normal p53 from its cellular activities. In addition, mutant p53 may interact with p63 and

p73 proteins, homologs of p53, which are important for inducing apoptosis (Melino et al., 2002). Depending on the mutation, the normal cellular role of p53 in apoptosis, transcription, and induction of cell cycle checkpoints may become deregulated, leading to the induction of mutations and cancer. The high frequency of p53 mutations in human cancers is one reason why p53 has been so widely investigated, and why it may one day be an important therapeutic target for cancer treatment.

A second example of a tumor suppressor gene is the Retinoblastoma (RB) gene, which when mutated, causes retinoblastoma, an autosomal dominant disorder manifested as retinal tumors, occurring in 1/20,000 live births (Suckling et al., 1982). There are two forms of retinoblastoma, heritable and nonheritable, both resulting from what Knudson defined as the two-hit hypothesis (Knudson, 1971). The heritable form occurs when a mutation in RB is first inherited from a parent in germinal cells, and then a second mutation in RB occurs in somatic retinal cells. This type of Retinoblastoma results in tumor formation in one or both eyes. The nonheritable form arises when two mutations occur in somatic retinal cells. Because of the low frequency of such a mutational event, tumors only form in one eye in this condition. In 1986, the RB gene was cloned from a fragment of genomic DNA that was known to be homozygously deleted in retinoblastoma (Dryja et al.,1986; Friend et al., 1986). The RB gene encodes pRb, a 928 amino acid protein, which is part of a family of nuclear proteins that bind to the E2F family of transcription factors (Umen and Goodenough, 2001). pRb is regulated

by phosphorylation, and its most prominent role is regulating cell cycle progression (Mittnact, 1998). During the G1 phase of the cell cycle, hypophosphorylated pRb binds to E2F and represses E2F-mediated transcription. In late G1, pRb becomes phosphorylated and releases E2F, allowing transcription of E2F-regulated genes required for cell division. Overall then, pRb functions as a tumor suppressor by controlling cell cycle phase transition by transcriptional repression. In addition to cell cycle regulation, pRb has other tumor suppressive roles in apoptosis and differentiation (for review see DiCiommo et al., 2000; Classon and Harlow, 2002). When Rb becomes mutated, these functions become deregulated, leading to transcriptional activation of many different genes. Mutations in Rb have been identified in over 1000 patients with retinoblastoma, demonstrating the important tumor suppressive function of pRb (Lohmann, 1999). Taken together, the previous examples demonstrate the importance of DNA repair systems in the prevention of mutations because mutations play a causal role in the development of cancer.

C. TRANSLESION DNA SYNTHESIS IN PROKARYOTES

1. The SOS response in *E.coli*

The SOS system is a cellular response to DNA damage in *E.coli* (Radman, 1975; Witkin, 1976; Walker, 1985). After exposure to DNA damaging agents, the SOS response results in the induction of over 40 genes that are involved in DNA repair

and damage tolerance (reviewed in Friedberg et al., 1995; Fernandez et al., 2000; Sutton et al., 2000). The SOS system ensures that *E.coli* cells adequately respond to DNA damage, which helps to enhance their survival. Several proteins play pivotal roles in the SOS response, including LexA and RecA.

LexA is a repressor protein responsible for keeping the SOS-responsive genes down-regulated until their functions are required (Brent and Ptashne, 1981; Little et al., 1981). LexA binds to the SOS box located in or near the promoters of SOS responsive genes. Binding of LexA to the SOS box interferes with the binding of RNA polymerase and subsequently prevents transcription of these genes. Thus, the repressor function of LexA is a critical regulatory mechanism underlying the SOS response.

Another key protein required for the SOS response is RecA (Miura and Tomizawa, 1968; Walker, 1985). When DNA damage blocks the replicative polymerase, it is believed that replication is aborted, exposing single-strand (ss) regions of DNA (Craig and Roberts, 1981; Sassanfar and Roberts, 1990). RecA becomes activated by binding to these ssDNA regions, creating a nucleoprotein filament. The activated form of RecA/ssDNA stimulates LexA to cleave itself via autodigestion (Little, 1984; Little, 1993). This results in a decrease in the cellular levels of the LexA repressor protein and an increase in SOS proteins through an induction of the SOS regulon. The SOS-induced proteins then perform repair or tolerance functions, allowing the cells to cope with damage. As cells recover

from DNA damage, the amount of ssDNA decreases, reducing the amount of the activated RecA/ssDNA nucleoprotein filament. This, in turn, results in reaccumulation of the LexA repressor protein and shutdown of the SOS response. The SOS system is, therefore, a complex and critical response to DNA damage in *E.coli* cells.

2. TLS polymerases: Pol IV and Pol V

Based on genetic evidence using a temperature sensitive pol III alpha subunit mutant, it was initially thought that pol III, the major replicative polymerase, was responsible for SOS mutagenesis in *E.coli* (Bridges and Mottershead, 1976; Hagensee et al., 1987; Bridges and Bates, 1990). More recently, however, two SOS inducible proteins, pol V (umuD'2C) and pol IV (DinB), were found to play a role in damage-induced mutagenesis via TLS. These proteins belong to the Y family of DNA polymerases and are required for TLS in *E.coli* (for review see Fuchs et al., 2004; Yang, 2005). TLS is the basic mechanism of SOS mutagenesis, allowing bypass of DNA damage that blocks the normal replicative polymerase (Friedberg et al., 1995). This tolerance, however, comes with the risk of generating mutations.

2.1 DNA polymerase V ($umuD_2'C$)

UV-induced mutagenesis in *E.coli* requires both the umuC protein and the umuD' protein (Burchkhard et al., 1988; Nohmi et al., 1988; Shinagawa et al., 1988). The umuDC operon was initially identified using a genetic screen for mutant

E.coli cells that were deficient in induced mutagenesis (Steinborn, 1978). umuD' is a proteolytically cleaved fragment of the umuD precursor protein. Upon interaction with the RecA nucleoprotein filament, umuD undergoes autodigestion resulting in cleavage of the N-terminal 24 amino acids (Walker, 1985). Autodigestion generates the umuD' protein, which can bind to umuC and form the umuD'$_2$C complex (Bruck, 1996). It was initially believed that the umuD'$_2$C complex assisted pol III in bypass of DNA damage *in vivo* (Bridges and Mottershead, 1976). *In vitro* evidence was lacking because of the inability to purify active umuC. More recently, however, two independent groups have reconstituted TLS *in vitro* using purified *E.coli* proteins. Goodman and colleagues (Tang et al., 1998; Tang et al., 1999) and Livneh and colleagues (Reuven et al., 1998; Reuven et al., 1999) found that umuD'$_2$C alone was unable to promote TLS *in vitro*, however, the addition of RecA and single-strand binding protein (SSB) stimulated the polymerase activity of umuD'$_2$C (Tang et al., 1998; Reuven et al., 1998). The absence of pol III in this system provided solid evidence that umuD'$_2$C has intrinsic polymerase activity. This polymerase activity was further stimulated by the addition of the β clamp and the γ clamp loader (from the pol III complex), suggesting *in vivo* roles for these proteins during TLS. Because of this polymerase activity, umuD'2C is now called *E.coli* DNA pol V (Tang et al., 1998; Tang et al., 1999). The observation that umuC alone was capable of minimal DNA synthesis on undamaged templates suggested that the catalytic activity of pol V resides within umuC (Reuven et al., 1999). In addition, a mutant form of pol V that lacked polymerase activity was

unable to catalyze TLS, linking the polymerase activity of pol V to TLS (Tang et al., 1999).

In vitro experiments using templates containing different types of DNA lesions have provided information about the translesion bypass abilities of pol V. Pol V efficiently bypasses synthetic abasic sites, TT cis-syn cyclobutane pyrimidine dimers (CPD), and TT 6-4 pyrimidine-pyrimidone photoproducts (PP), in contrast to the pol III holoenzyme, which is strongly inhibited by these lesions (Tang et al., 1999; Tang et al., 2000; Fujii et al., 2004). In addition, the nucleotide incorporation specificities for pol V *in vitro* correlated with *in vivo* data, indicating a physiological role for pol V in DNA damage bypass (Lawrence et al., 1990a; Lawrence et al., 1990b; LeClerc et al., 1991; Smith et al., 1996). We now know that umuC is the founding member of the Y family of DNA polymerases. Since then, several homologs of umuC have been found in higher eukaryotes (Nelson et al., 1996b; Johnson et al., 1999a; Masutani et al., 1999; Lin et al., 1999a; Lin et al, 1999b; McDonald et al., 1999; Gerlach et al., 1999).

Pol V is also believed to play a regulatory role in cells by eliciting a cell cycle checkpoint response to DNA damage. Opperman and colleagues have shown that umuC and uncleaved umuD inhibit the recovery of replication after exposure to DNA damaging agents (Opperman et al., 1999). Because uncleaved umuD has a cellular function distinct from umuD', it is hypothesized that the two proteins act as a molecular switch, telling the cell when to activate TLS.

Opperman proposed the following model: the presence of DNA damage leads to the upregulation of umuD and umuC through the SOS response. Together these proteins act to inhibit DNA replication, which allows DNA repair to occur. If the damage persists, there is an increase in RecA/ssDNA nucleoprotein filaments, which interact with umuD to stimulate its autodigestion to umuD'. This interaction shifts the cellular pool of umuD to umuD', inactivating the checkpoint and activating TLS. This response ensures the most efficient repair of DNA damage, followed by TLS of any remaining damage, keeping the risk of mutations minimal, but allowing mutagenesis when necessary.

2.2 DNA polymerase IV (dinB)

In 1995, Ohmori and colleagues identified an open reading frame (ORF) that contained a LexA repressor binding site in the promoter region (Ohmori et al., 1995). This ORF was identified as dinB, or the damage inducible locus, which was previously shown to be up-regulated as part of the SOS response (Kenyon and Walker, 1980). The dinB protein shares strong sequence homology with umuC-like proteins, members of the Y family of DNA polymerases.

After exposure to DNA damaging agents, *E.coli* cells that have a mutant dinB are not different from wildtype cells in induced mutagenesis *in vivo*. In contrast, dinB mutant *E coli* cells no longer demonstrate untargeted mutagenesis (UTM) of lambda phage (Brotcorne-Lannoye and Maenhaut-Michel, 1986). UTM is the process of mutagenesis on undamaged lambda phage as a result of being

replicated in a UV-irradiated host cell. In addition, *E.coli* cells that overexpress dinB exhibit an increased spontaneous mutation frequency (Kim et al., 1997). Taken together, this evidence suggested that dinB is involved in mutagenesis in *E.coli*.

When Wagner and colleagues used a His-tagged dinB fusion protein in primer extension reactions, they found that it had DNA polymerase activity (Wagner et al., 1999). Mutating conserved residues in dinB abolished the catalytic activity *in vitro*, and also abolished the lamba phage UTM *in vivo*, directly linking the polymerase activity of dinB to mutagenesis (Kim et al., 1997; Wagner et al., 1999). dinB is now called *E.coli* DNA polymerase IV (Nohmi et al., 1988; Wagner et al., 1999).

Primer extension reactions further demonstrated that pol IV is a poorly processive enzyme (Wagner et al., 1999). Like pol V, the processivity of pol IV was stimulated by the addition of the pol III β clamp *in vitro*; however, whether this occurs *in vivo* remains to be determined (Tang et al., 2000; Wagner and Nohmi, 2000). Like other Y family polymerases, pol IV lacks a 3' to 5' exonuclease activity, although on undamaged templates, pol IV preferentially catalyzes the incorporation of the correct nucleotide in primer extension reactions (Wagner et al., 1999; Kobayashi et al., 2002; Tang et al., 2000). These results demonstrate a relatively high fidelity for pol IV, at least at the incorporation step, and suggest that the role of pol IV in mutagenesis is primarily through extension

of mismatched primer termini. The relative contribution to mutagenesis for pol IV insertion compared to extension has not been shown *in vivo*. However, pol IV is able to efficiently extend mismatched primer termini *in vitro*, supporting the hypothesis that the major role of pol IV in mutagenesis is at the extension step (Kobayashi et al, 2002).

Base substitutions are greatly increased during pol IV mutagenesis *in vitro*, with 70% of base substitutions representing changes at guanine residues (Kobayashi et al., 2002). Overexpression of pol IV demonstrated a similar spectrum *in vivo*, with the majority of base substitutions at G:C base pairs, suggesting that pol IV is primarily responsible for tolerating damage on guanine residues both *in vitro* and *in vivo* (Wagner and Nohmi, 2000).

The ability of pol IV to bypass different types of DNA damage has been investigated *in vitro* as well as *in vivo*. *In vitro* bypass by pol IV has varied efficiencies, but includes the following lesions: TT cis-syn CPD's, TT 6-4 PP's, abasic sites, 8-oxoguanines, O_6-methylguanines, N-2-AAF adducted guanines, and BPDE-adducted guanines (Suzuki et al., 2002; Shen et al., 2002; Maor-Shoshani et al., 2003). Bypass of BPDE adducts are especially efficient *in vitro*, which suggested a primary role for pol IV in bypass of BPDE-induced DNA damage *in vivo* (Napalitano et al., 2000; Shen et al., 2002; Maor-Shoshani et al., 2003).

In vivo data indicates that pol IV efficiently bypasses a BPDE-adducted guanine, suggesting a primary role for bypass of this type of damage. Pol IV can bypass, with less efficiency, a 4-nitroquinoline N-oxide (4-NQO) lesion and oxidative damage (Napolitano et al., 2000; Kim et al., 2001; Wagner et al., 2002). The discrepancies between the *in vitro* and *in vivo* capabilities of pol IV bypass are presumably a result of sequence context and different requirements for TLS *in vitro* and *in vivo* (Lenne-Samuel et al., 2000; Napalitano et al., 2000).

While our understanding of the Y family polymerases in *E.coli* has greatly advanced over the last decade, additional *in vivo* studies are needed to clearly establish the process of TLS within *E.coli* cells. In addition, studies in higher eukaryotes need to be performed and compared with the current understanding of the *E.coli* system to generate a clear picture of the specific roles of each polymerase during TLS.

D. TLS IN EUKARYOTES

1. TLS in Yeast (*Saccharomyces cerevisiae*)

Studies in yeast cells have provided some of the best information regarding TLS polymerases. Rev1 was discovered in yeast and shares high homology at the amino acid level with the umuC protein of *E.coli*, providing some indication that TLS occurs in yeast as well as in *E.coli*, and suggesting that the process of TLS is conserved in higher eukaryotes. TLS occurs in yeast cells in the absence of

UV irradiation, suggesting that a DNA damage-inducible SOS-like mechanism does not exist in yeast cells.

1.1 The REV proteins

The Rev proteins were first identified using a screen for yeast mutants with reduced frequencies of UV-induced mutations (Lemontt 1971; Larimer et al., 1989; Morrison et al., 1989). The *REV3* gene and the *REV1* gene were identified in these studies. Lawrence later identified the *REV7* gene using a similar approach (Lawrence et al., 1985a; Torpey et al., 1994). Characterization of the Rev genes demonstrated that they were important for mutagenesis induced by several DNA damaging agents including ionizing radiation, 4-nitroquinoline-1-oxide, ethyl methane sulfonate, and several alkylating agents (Lemontt, 1972; Prakash, 1976; McKee and Lawrence, 1979a; McKee and Lawrence, 1979b; Ruhland and Brendel, 1979; Lawrence et al., 1985b; Lawrence et al., 1985c). In addition, Rev1 and Rev3 mutants demonstrated a significant reduction in the rate of spontaneous mutations, suggesting that Rev1 and Rev3 are involved in spontaneous mutagenesis (Quah et al., 1980; Glassner et al., 1998). These initial studies clearly demonstrate an important role for the Rev proteins in induced mutagenesis in yeast cells.

Rev1 shares sequence similarity in certain regions with the umuC protein of *E.coli* (Larimer et al., 1989). Because of this homology, Rev1 is classified as a

member of the Y family of DNA polymerases. The Rev1 protein contains 985 amino acids with a mass of 112 kDa.

Yeast Rev1 has two distinct functions in cells. First, Rev1 was shown to possess a template-dependent deoxycytidyl transferase activity (Nelson et al., 1996b). Using *in vitro* primer extension reactions with an abasic site as the lesion, Rev1 was found to preferentially insert a C residue opposite the lesion. Because it was previously known that the majority of bypass events at abasic sites *in vivo* result from insertion of a C residue, the deoxycytidyl transferase activity of Rev1 gained physiological relevance (Gibbs and Lawrence, 1995). Otsuka and colleagues demonstrated, however, that the deoxycytidyl transferase activity of Rev1 was not completely essential for bypass of an abasic site. The investigators mutated specific residues in Rev1 that abolished tranferase activity and showed that the mutant Rev1 was able to bypass some abasic sites, suggesting that Rev1 has another function important for TLS that is distinct from the deoxycytidyl tranferase (Otsuka et al., 2002).

The as-yet unidentified second function of Rev1 appears to be important during TLS. Because the phenotype for Rev1 mutant cells was similar to that seen with Rev3 or Rev7, it is hypothesized that Rev1 is required for pol ζ activity during TLS. For example, Rev1 function was found to be required for bypass of a TT 6-4 PP (Nelson et al., 2000). Cytosine incorporation occurs only very rarely opposite this type of lesion, suggesting that the role of Rev1 in bypass of the TT

6-4 PP requires a function different from the deoxycytidyl transferase. The exact role of Rev1 during TLS, however, has yet to be established.

The *REV3* gene encodes a protein of 1504 amino acids with a mass of 173 kDa. Sequence analysis of the *REV3* gene revealed a protein distantly related to DNA polymerase delta, containing polymerase motifs characteristic of B family DNA polymerases (Morrison et al., 1989). The polymerase activity of Rev3 was first demonstrated using *in vitro* primer extension assays with a GST-Rev3 fusion protein. Rev3 was found to interact with Rev7 in a yeast-two-hybrid assay, and the addition of purified Rev7 to the primer extension reaction enhanced the polymerase activity of Rev3 over 20-fold, suggesting that the interaction between Rev3 and Rev7 is functional (Nelson et al., 1996a). The Rev7 protein contains 245 amino acids with a molecular weight of 29 kDa. Rev7 contains a HORMA domain, thought to be important for protein-protein interactions and recognition of chromatin (Aravind and Koonin, 1998). Together, Rev3 and Rev7 form the sixth DNA polymerase discovered in eukaryotic cells, called pol zeta (ζ). All *in vitro* experiments have subsequently used both subunits of pol ζ.

Rev3, the catalytic subunit of pol ζ, is expressed at very low levels in yeast cells. This is presumably due to an upstream ATG in the 5' untranslated region (UTR), reducing the translational efficiency of the correct ATG. Because of the low cellular levels of Rev3, it is thought to be the limiting factor for pol ζ activity. However, because native pol ζ has never been isolated from cells, it is unclear if

other proteins are involved in the pol ζ complex. Pol ζ is a poorly processive enzyme lacking a 3' to 5' exonuclease activity, similar to Y family polymerases (Nelson et al., 1996a). Pol ζ is unique because it is a member of the B family of DNA polymerases, but participates in TLS and has many characteristics of Y family polymerases.

In vitro data have provided insight into the specific functions of Rev3 as the catalytic subunit of pol ζ during TLS. Pol ζ is extremely efficient at extending mismatched primer termini (Lawrence and Hinkle, 1996; Johnson et al., 2000; Lawrence et al., 2000a). For example, when a primer terminal G was mispaired with a template T, pol ζ had an extension efficiency of 54% for this particular mismatch (Lawrence et al., 2000a). Other mismatches were less efficient, but pol ζ was consistently and significantly more efficient at extending mismatches than pol α, which also lacks a 3' to 5' exonclease proofreading activity (Mendelmen et al., 1990). Pol ζ can also incorporate nucleotides, although only with moderate efficiency and fidelity. From these *in vitro* data, a model has been proposed that yeast pol ζ does not frequently insert nucleotides opposite DNA damage. Instead, pol ζ proficiently extends from termini resulting from insertions done by other polymerases. This model suggests that the major role of pol ζ is during the extension step of TLS. This model is supported by additional *in vitro* experiments using multiple polymerases indicating that pol ζ is able to extend from a mismatch inserted by other specialized polymerases (Johnson et al., 2000a; Prakash and Prakash, 2002). While the *in vitro* data suggest such a

model, *in vivo* evidence to define the exact insertion or extension role for pol ζ in TLS is lacking.

In addition to demonstrating the polymerase activity and transferase activity of the Rev proteins, *in vitro* experiments using templates containing different kinds of DNA lesions have provided information about the bypass abilities of the Rev proteins. Studies using yeast pol ζ demonstrated varied bypass abilities depending on the type of DNA damage. For example, pol ζ alone inefficiently bypassed an abasic site, but in an experiment using both pol ζ and Rev1, pol ζ efficiently extended from a C residue incorporated opposite the abasic site by Rev1 (Nelson et al., 1996b). Pol ζ alone can bypass a TT CPD with low efficiency, however, pol η's ability to efficiently and accurately bypass this type of damage indicates that it is probably the major polymerase responsible for bypass of TT CPD's (Johnson et al., 1999a; Gibbs et al., 2005). The literature is inconsistent about the ability of pol ζ to bypass a TT 6-4 PP. Johnson and colleagues (Johnson et al., 2001) demonstrated that pol ζ was unable to insert a nucleotide opposite the 3' T of the photoproduct, but while complete bypass was inhibited, pol ζ was able to extend after a nucleotide was inserted across from the lesion by another polymerase. In contrast, Guo and colleagues demonstrated that pol ζ could insert opposite both the 5' T and the 3' T of a 6-4 photoproduct (Guo et al., 2001). The difference in results obtained here are probably due to different experimental conditions, but also demonstrate the variability that *in vitro* systems can produce.

Additionally, pol ζ can bypass an AAF-adducted guanine lesion with limited efficiency, but can efficiently extend from a mispaired nucleotide inserted opposite the lesion by another polymerase (Guo et al., 2001). Pol ζ can accurately bypass BPDE-adducted guanine lesions, inserting a C residue opposite the damage (Simhadri et al., 2002). Pol ζ can insert nucleotides opposite a thymine-glycol lesion as well as extend from the inserted nucleotides for complete bypass (Johnson et al., 2003). Taken together, the *in vitro* data demonstrate that yeast pol ζ plays a role in mutagenesis at both the insertion and extension steps of TLS.

In summary, polymerase ζ in yeast cells is composed of Rev3 and Rev7. Rev1 appears to play a role in a similar or the same pathway as Rev3 and Rev7, but a complex of the three proteins has not been isolated and Rev1 does not bind to Rev3 or Rev7 in yeast-two-hybrid assays. While the previous studies helped to determine that the Rev proteins are important for induced, and often spontaneous mutagenesis, the exact roles of these proteins in the process of TLS in yeast remains unknown.

1.2 Polymerase η

Yeast Rad30 was first identified when the *Saccharomyces cerevisiae* genome was searched for proteins homologous to *E.coli* proteins dinB and umuC (McDonald et al., 1997; Roush et al., 1998). Shortly thereafter, Rad30 was

purified and shown to possess a template-dependent, DNA polymerase activity. Because of this inherent polymerase activity, Rad30 is called polymerase eta (η). Further biochemical characterization provided a glimpse into the physiological role of pol η, that is, the ability to efficiently and accurately bypass a UV-induced TT CPD (Johnson et al., 1999a).

Like other Y family polmerases, pol η has low fidelity because it lacks a 3' to 5' exonuclease activity and is poorly processive, inserting only a few nucleotides per binding event. Pol η contributes to mutagenesis by both misinserting a nucleotide and extending mismatches during TLS. However, mismatch extension by pol η is less efficient than incorporation of an incorrect nucleotide (Washington et al., 2001a; Washington et al., 2001b). Misinsertion and mismatch extension rates by pol η are affected by the sequence context surrounding the damage site and the composition of the mismatch (Mendelman et al., 1989; Goodman et al., 1993; Goodman and Fygensen, 1998).

The crystal structure of the catalytic core of Y family polymerases, which was determined using yeast pol η as an example, has provided insight into the tolerance of DNA damage by specialized polymerases. Y family polymerases contain five conserved motifs, I-V, important for their TLS function (Kulaeva et al., 1996). Even though there is almost no direct homology at the amino acid level with B family DNA polymerases, the crystal structure of yeast pol η revealed that the three-dimensional structure resembles the right hand characteristic of B

family DNA polymerases (Kulaeva et al., 1996; Ling et al., 2001; Silvian et al., 2001; Trincao et al., 2001; Zhou et al., 2001). Analagous to the structure of B family polymerases, pol η contains a thumb domain involved in DNA binding and processivity, a fingers domain important for nucleoside triphoshate selection, and a palm domain containing conserved residues important for the nucleotide transfer reaction. The palm domain of pol η contains the same three conserved residues that are important for coordinating metal ions found in classical DNA polymerases, indicating similarity between Y family polymerases and classical DNA polymerase at the amino acid level. In contrast, the thumb and finger domains of pol η were observed to be short compared to classical DNA polymerase domains. The short fingers of pol η are missing alpha-helix domains that are critical for fidelity in classical DNA polymerases, suggesting a mechanism for tolerance by specialized polymerases. In addition, pol η contains an extra domain called the polymerase-associated domain (PAD), which mimics an extra set of fingers allowing pol η to increase the surface area of the catalytic site as well as increase processivity. The PAD domain is not present in classical DNA polymerases, but is found in other Y family polymerases.

By fitting the crystal structure of pol η with the template-primer ddNTP ternary complex of T7 polymerase, Trincao and colleagues (Trincao et al., 2001) demonstrated that the binding pocket is fairly open, providing a possible explanation for the low fidelity of pol η. For example, when a thymine-thymine dimer was modeled into the active site of pol η, they observed that it is much

more open than seen with classical DNA polymerases – potentially able to accommodate both thymine template residues. This wide active site is much less discriminating, and the ability to bind two nucleotides simultaneously provides a mechanism for error. Kinetic analysis supports the model of pol η binding both thymine bases of a CPD (Washington et al., 2003a). However, several studies demonstrate that correct Watson-Crick base pairing is an important factor for determining the efficiency and fidelity of pol η (Haracska et al., 2000; Ling et al., 2003; Washington et al., 2003b). Therefore, while the relaxed catalytic site allows for less discrimination, correct Watson-Crick base pairing also plays a role in the fidelity of pol η.

In vitro assays investigating the ability of pol η to bypass a variety of DNA lesions have provided insight into the TLS abilities of pol η *in vivo*. It should be noted, however, that while in vitro experiments have provided information about the basic properties of these enzymes, it is possible that *in vitro* experiments do no fully recapitulate what happens in cells at sites of DNA damage when all necessary factors are present. Pol η's major role is most likely to accurately insert two A residues across from a UV-induced TT CPD, leading to error-free TLS of this particular lesion almost 99% of the time (Johnson et al., 1999b; Johnson et al., 2000b). Because of the high efficiency and accuracy of bypass of this type of lesion, pol η is thought to be solely responsible for TLS past TT CPD's. In contrast, pol η is not able to fully bypass a TT 6-4 PP, but can inefficiently insert a nucleotide opposite the 3' T of this lesion (Johnson et al.,

2001). Pol η is also capable of bypassing an 8-oxoguanine lesion, preferentially and accurately inserting a C residue opposite the lesion (Yuan et al., 2000; Haracska et al., 2000; Boitex et al., 2002; Carlson et al., 2005). Pol η can only inefficiently insert nucleotides opposite an abasic site, preferentially inserting a G residue (Yuan et al., 2000; Haracska et al., 2001). Pol η can also inefficiently insert nucleotides opposite an AAF adducted guanine, preferentially incorporating the correct C residue. Complete bypass of an AAF adduct and an abasic site by pol η is inhibited, suggesting that another polymerase must perform extension (Yuan et al., 2000; Kusumoto et al., 2002;).

In vivo studies using yeast strains mutant for pol η transformed with plasmid DNA containing different DNA lesions confirm the bypass abilities seen for pol η *in vitro*. Pol η was found to be essential for bypassing the TT CPD *in vivo*, as expected by the high efficiency and accuracy with which pol η bypasses this lesion *in vitro* (Bresson and Fuchs, 2002; Gibbs et al., 2005). In addition, pol η is involved in bypass of an 8-oxoguanine lesion *in vivo*, which correlates with *in vitro* data (Stanislav et al., 2003). In contrast, pol η is only rarely involved in bypass of a 6-4 PP and an abasic site *in vivo*, also consistent with *in vitro* data (Bresson and Fuchs, 2002; Gibbs et al., 2005).

To date, yeast cells have been found to contain three enzymes involved in TLS: Pol ζ, Rev1 and pol η. Although the capabilities of these enzymes have been actively investigated *in vitro* and *in vivo*, and we have a better understanding of

TLS in yeast, studying these enzymes in human cells will provide us with a better understanding of TLS in humans because yeast cells do not contain all of the specialized polymerases that are present in human cells.

2. TLS in Human Cells

Specialized DNA polymerases capable of performing TLS past sites of DNA damage have recently been discovered in human cells. Four of the five human polymerases implicated in TLS belong to the Y family of DNA polymerases. The major role of Y family polymerases is to rescue stalled replications forks in order to enhance survival. A critical common feature of Y family polymerases is that they lack a 3' to 5' exonuclease activity. This lack of proofreading, combined with relaxed catalytic sites, enables these polymerases to bypass DNA damage with varied efficiencies. These same features, however, cause the specialized DNA polymerases to have high error-rates when copying undamaged DNA. Sequence analysis revealed strong homology within the Y family of DNA polymerases. Specifically, there are five conserved motifs, I-V, found in most Y family polymerases (Figure 1). There is no direct homology between specialized DNA polymerases and classical DNA polymerases at the nucleotide level. However, conserved residues found in motifs I, II, and III are the same as critical conserved residues found in motifs A, B, and C of classical DNA polymerases (Delarue et al., 1990).

Figure 1: Conserved structure of human specialized DNA polymerases.

A. Members of the Y family of DNA polymerases share five conserved sequence motifs (green boxes). In addition, these polymerases all contain nuclear localization signals (NLS, red rectangle) and PCNA binding motifs (yellow square). Some of these proteins also contain C_2H_2 motifs (blue box), but the function of this motif is unknown. **B.** Structure of the proteins that compose human polymerase zeta. hRev3 contains the polymerase motifs characteristic of B family polymerases (gray boxes). hRev7 contains a HORMA domain (gray rectangle). Polymerase zeta is unique because it is not a member of the Y family of DNA polymerases, but it is involved in TLS. Images in this dissertation are presented in color.

Conserved structure of human specialized DNA polymerases

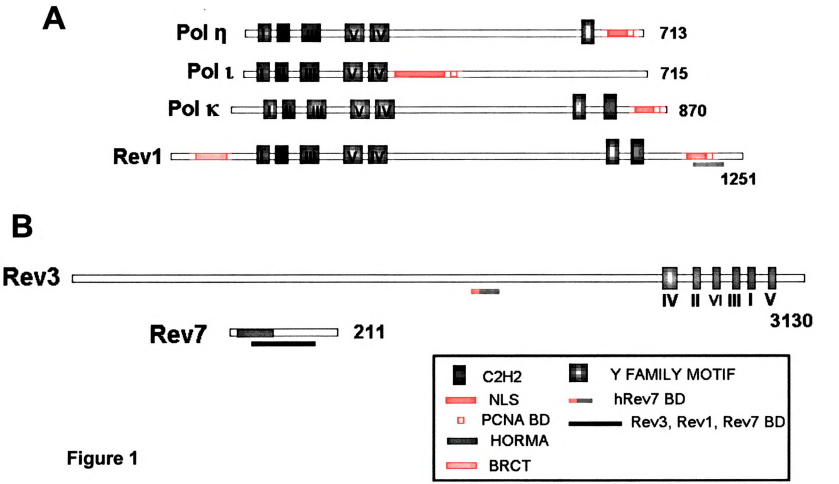

Figure 1

Interest in the specialized DNA polymerases grew rapidly because of their ability to replicate damaged DNA in an error-prone manner, frequently leading to mutations. This interest was further stimulated when mutations in polymerase η were found to cause the xeroderma pigmentosum variant (XPV) phenotype (Masutani et al., 1999; Johnson et al., 1999b). Patients with XPV are sensitive to sunlight and have a very high incidence of skin cancer, because of an inability to carry out TLS past UV-induced DNA damage. Perhaps even more interesting are recent studies implicating other TLS polymerases in different human cancer phenotypes. These discoveries underscore the importance of the TLS polymerases in preventing as well as creating mutations.

2.1 Polymerase η

Since its discovery as the protein responsible for the XPV disease phenotype (Masutani et al., 1999; Johnson et al., 1999b), Rad30A has been the most widely studied Y family polymerase both *in vitro* and *in vivo*. Human Rad30A is homologous to the yeast Rad30 protein, both in structure and function. Homologs of Rad30 have been found in yeast (McDonald et al., 1997), mouse (McDonald et al., 1999), *Drosophila* (Ishikawa et al., 2001), and humans (Masutani et al., 1999; Johnson et al., 1999b). Results from *in vitro* primer extension reactions demonstrated that Rad30A has DNA polymerase activity and uses all four nucleotides in a template-dependent manner (Johnson et al., 1999b). Rad30A was subsequently called DNA polymerase η.

The gene encoding human DNA pol η is located on chromosome 6p.21 and is ubiquitously expressed in all tissues. Unlike yeast pol η, the human protein is not induced after cells are treated with UV radiation (Yamada et al., 2000). Pol η is a poorly processive enzyme and lacks a 3' to 5' exonuclease activity, like other members of the Y family. The catalytic activity of pol η resides in the N-terminal region, containing the motifs I-V, found in other Y family polymerases.

Studies involving the structure of pol η have provided useful information about the conserved structure of Y family polymerases (Ling et al., 2001; Silvian et al., 2001; Trincao et al., 2001; Zhou et al., 2001). The crystal structure of pol η is similar to other DNA polymerases, such that it resembles the characteristic right hand with the catalytic domains contained in the thumb region. The studies elucidating the crystal structure of Y family polymerases have been performed using *S. cerevisae* pol η or *Sulfolobus solfataricus* Dpo4. Nevertheless, they provide useful information about the similarities and differences between classical DNA polymerases and Y family polymerases. Briefly, Y family polymerases resemble a right hand containing thumb, palm, and finger domains similar to classical DNA polymerases. The palm domain contains three conserved residues, Asp30, Asp155, Glu156, strikingly similar to the catalytic site of classical DNA polymerases, suggesting the basic polymerase mechanisms are conserved. In contrast, the thumb and fingers domains are short compared to classical DNA polymerases. To compensate for this, pol η contains an extra domain, the PAD, not found in polymerases outside of the Y family (Trincao et

al., 2001). This PAD domain is thought to be critical for increasing the area of the catalytic site (Trincao et al., 2001; Boudsocq et al., 2004). This mechanism for tolerance through relaxed discrimination also provides a mechanism for error. The Y family polymerases have a three-dimensional structure similar to classical DNA polymerases, but contain some modifications, which allow them to be less discriminating, suggesting that these polymerases have evolved necessary modifications specifically for lesion bypass.

Using purified pol η and DNA templates containing different types of DNA damage, *in vitro* experiments have provided useful information about the biochemical properties of human pol η. The most well characterized property of human pol η is that it efficiently replicates a cis-syn TT CPD, inserting two A residues across from the damage site, leading to accurate replication of this type of DNA damage (Johnson et al., 2000b; Masutani et al., 2000; McCullogh et al., 2004). Replication of the TT CPD is so efficient and accurate that pol η is widely believed to be solely responsible for bypassing this type of lesion. In contrast to the CPD lesion, pol η is unable to fully replicate a 6-4 PP. However, pol η is able to insert a nucleotide opposite the 3' T of the photoproduct, preferentially inserting a guanine residue (Masutani et al., 2000; Zhang et al., 2000a; Johnson et al., 2001). Pol η can efficiently bypass cisplatin-induced DNA damage, but whether this bypass is error-free or error-prone is inconsistent in the literature (Vaisman et al., 2000; Masutani et al., 2000). Pol η preferentially inserts the correct C opposite an 8-oxoguanine lesion, and extends for complete bypass of

this type of lesion (Zhang et al., 2000a; Haracska et al., 2000). Additionally, pol η is able to accurately insert the correct nucleotide opposite an N-2-acetylaminofluorene guanine lesion, but is unable to fully bypass this lesion (Masutani et al., 2000; Haracska et al., 2000; Kusumoto et al., 2000). Pol η can bypass a BPDE lesion in an error-prone manner, frequently inserting an A residue (Zhang et al., 2000a). Finally, pol η is able to insert a nucleotide across from an abasic site, preferentially inserting an A residue (Masutani et al., 2000; Zhang et al., 2000a; Haracska et al., 2001).

In vivo studies using GFP-tagged pol η revealed that pol η is localized in the nucleus, and upon UV irradiation, pol η accumulates into replication foci (Kannouche et al., 2001; Kannouche et al., 2002). Localization of pol η to the nucleus requires the C-terminal 70 amino acids, and localization of pol η to replication foci requires the C-terminal 120 amino acids. The localization pattern for pol η was shown to be similar to that for pol ι, suggesting that these two homologs interact. Furthermore, a significantly reduced number of GFP-pol ι localized into UV-induced replication foci in XPV cells that lack pol η, suggesting that pol η plays an important role in recruiting or bringing pol ι to replication foci (Kannouche et al., 2002). Another important interaction was suggested by the presence of PCNA binding motifs in the C-terminus of pol η. Mutating the PCNA binding site in pol η abolished localization to foci, suggesting that an interaction with PCNA is critical to the accumulation of pol η into foci (Kannouche et al., 2004).

In vivo studies using XPV cell lines as a natural pol η knockout have demonstrated a UV-induced delay in S-phase progression compared to cells that contain functional pol η (Bullock et al., 2001; Cordeiro-Stone et al., 2002). Because XPV cells have a reduced ability to synthesize DNA after UV irradiation, the delay in S phase most likely provides these cells with extra time for repair or tolerance functions. This field of study has not received much attention until recently, when S phase delays have been observed in cell lines engineered to lack other TLS polymerases (Bi et al., 2005, McNally unpublished data). These data suggest a link between TLS and DNA damage-induced cell cycle checkpoints.

A link to human cancer for pol η was clearly established when mutations in pol η were found to be responsible for the XPV phenotype. All XPV cell lines tested to date have nonfunctional forms of pol η. The majority of the mutations are early termination codons, leading to severely truncated pol η protein in these cell lines (Masutani et al., 1999; Johnson et al., 1999b; Itoh et al. 2000; Broughton et al., 2002). This information indicates that pol η is not essential for life. However, the high frequency of skin cancer in XPV patients demonstrates the important protective role that pol η plays in human cells.

2.2 Polymerase ι

A second human homolog of the yeast Rad30 protein was identified shortly after pol η, called Rad30B (McDonald et al., 1999). This protein shares homology with human pol η, and it is believed that Rad30B evolved from gene duplication of pol η. Like pol η, Rad30B was shown to possess DNA polymerase activity and has since been called pol iota (ι) (Tissier et al., 2000). Pol ι has only been found in humans (McDonald et al., 1999), mouse (McDonald et al., 1999), and *Drosophila* (Ishikawa et al., 2001), and is ubiquitously expressed, with the highest level of expression in the testis. Like other Y family polymerases, the catalytic domain of human pol ι is contained in the N-terminal region of the protein encoded by the conserved motifs I-V (Figure 1).

In vitro experiments have demonstrated that the fidelity of pol ι is template dependent. For example, pol ι is extremely inaccurate when inserting nucleotides opposite a template T or C, but is relatively accurate when inserting nucleotides across from a template A. Pol ι performs the latter insertion with a misincorporation rate of 1×10^{-4} (Tissier et al., 2000; Frank et al., 2001). In fact, pol ι incorporates the incorrect nucleotide more frequently than pol η. *In vitro* experiments have shown that pol ι is able to incorporate a nucleotide opposite the 3' T of a TT 6-4 PP, but is unable to insert opposite the TT CPD lesion (Zhang et al., 2000b; Johnson et al., 2000a; Vaisman et al., 2000; Tissier et al., 2000; Zhang et al., 2001). Pol ι is capable of inserting nucleotides opposite an abasic site and an AAF lesion, but extension for complete bypass is inhibited (Zhang et al., 2000; Tissier et al., 2000; Vaisman et al., 2000). Pol ι plays a

minor role at the extension step of TLS by efficiently extending mismatched nucleotides. The efficiency of these extensions depends on the mismatch composition and the next nucleotide encountered on the template (Vaisman et al., 2001). These studies demonstrated that pol ι is more efficient at extending mismatched termini than pol η is.

In vivo experiments have been performed to gain insight into the localization of pol ι in human cells. These experiments used GFP-tagged pol ι, as described for pol η, and revealed that pol ι is localized in the nucleus, with a small percentage specifically localized at replication foci with pol η (Kannouche et al., 2001). After UV treatment, the number of foci containing colocalized pol ι and pol η increased. In XPV cells, localization of pol ι to replication foci was significantly decreased, suggesting a function for pol η in bringing pol ι to the replication foci, although the exact role remains unclear as a small amount of GFP-pol ι was present in replication foci in the absence of pol η (Kannouche et al., 2002).

A unique role for pol ι compared to other TLS polymerases is that pol ι possesses a 5'-deoxyribose phosphate lyase activity (Bebenek et al., 2001). In reconstituted reactions, pol ι used its lyase activity to repair guanine-uracil and adenine-uracil base pairs, implicating pol ι in specialized base excision repair. Further characterization of this function is lacking, but this evidence suggests a potential link between DNA repair and TLS.

A link to human cancer is hypothesized for pol ι based on the XPV phenotype. These cells lack pol η, which is highly accurate at bypassing UV-induced TT CPD's (Masutani et al., 1999; Johnson et al., 1999b). The high mutation frequency observed in this cell line after exposure to UV radiation is thought to be due to another polymerase bypassing the UV-induced damage in an error-prone manner. Because *in vitro* data suggests that pol ι preferentially inserts a T residue opposite the 3' T of a TT 6-4 PP, the increased T→A transversions characteristically seen in UV-irradiated XPV cells suggests a role for pol ι in this process. *In vivo* studies reducing pol ι using antisense in an XPV cell line have demonstrated that the mutation frequency is decreased in cells with decreased pol ι, suggesting that pol ι is responsible for the majority of mutations in the absence of pol η (Wang, unpublished data). Unfortunately, data regarding the UV-induced spectrum of mutations in the XPV cell line with reduced pol ι, was generally inconclusive.

Another link to human cancer was established for pol ι when Yang and colleagues demonstrated increased mRNA and protein expression of pol ι in human breast cancer cell lines compared to normal breast cell lines (Yang et al., 2004). Using an *ex-vivo* supF shuttle vector replication system, they determined that both the spontaneous and UV-induced mutation frequency was increased in the breast cancer cell lines compared to the normal breast cell lines. In order to link this phenomenon to pol ι, they observed the spectrum of UV-induced

mutations in the cancer cell lines compared to normal cell lines. They found an increase in transversions in the breast cancer cell lines, specifically a significant increase in C→A and T→A transversions. Because *in vitro* data previously demonstrated a preference for pol ι to insert a T across from the 3' T of a 6-4 PP, the increase in C→A and T→A transversions in breast cancer cell lines is consistent with the *in vitro* pol ι TLS signature when bypassing UV-induced DNA damage. While the previous studies provide useful information about the process of mutagenesis in the breast cancer cell lines studied, they do not provide a general mechanism for pol ι mutagenesis, because only three breast cancer cell lines were investigated. Studies manipulating endogenous pol ι and observing the mutation frequency and spectrum will provide further insight into the *in vivo* function of pol ι.

2.3 Polymerase κ

Homologs of polymerase κ have been found in *E.coli*, *C. elegans*, *S.pombe*, mouse, rat, chicken, *Arabidopsis*, and humans (Ohmori et al., 1995; Ogi et al., 1999; Johnson et al., 2000c; Ohashi et al., 2000a; Ohmori et al., 2001; Okada et al., 2002). Human pol κ is located on chromosome 5q.13 and is ubiquitously expressed, with the highest expression in testis, a common pattern for DNA repair genes. Like pol η and ι, the catalytic domain of pol κ is located in the N-terminal region of the protein (Figure 1). The C-terminus contains a putative nuclear localization signal (NLS) and a PCNA binding site, most likely required for *in vivo* pol κ function. In addition, two C_2H_2 zinc clusters are found upstream

of the NLS, the function of which is currently unknown. Most pol κ homologs share these protein motifs, suggesting that the function of pol κ has been conserved throughout evolution.

Human pol κ replicates undamaged DNA with very low fidelity (Zhang et al., 2000c; Zhang et al., 2000d; Ohashi et al., 2000a). *In vitro* data from several groups demonstrated that human pol κ is unable to fully bypass a TT CPD (Johnson et al., 2000c; Ohashi et al., 2000a; Ohashi et al, 2000b; Zhang et al., 2000d; Gerlach et al., 2001). Pol κ was, however, able to efficiently extend from a nucleotide placed opposite the 3' T of a TT CPD, but not the 3' T of a 6-4 PP (Washington et al., 2002). Pol κ was also able to extend from a nucleotide incorporated opposite an 0_6-methylguanine lesion and an 8-oxoguanine lesion, suggesting a major role for pol κ in TLS as an extender (Haracska et al., 2002a).

Pol κ is able to efficiently and accurately bypass lesions induced by BPDE *in vitro* by preferentially inserting a C across from the adducted guanine residue. The high efficiency with which pol κ bypasses a BPDE lesion *in vitro* suggests a major role for pol κ in bypass of this type of damage *in vivo*. This *in vitro* data, indicates that pol κ is involved in insertion during TLS as well as extension (Zhang et al., 2000d; Suzuki et al., 2002; Zhang et al., 2002a). There are currently no *in vivo* studies involving the role of human pol κ in the tolerance of BPDE-induced DNA damage. However, studies using mouse cells demonstrate that when cells lacking a functional pol κ were treated with BPDE, they had an

increased mutation frequency, reduced survival, and a change in the spectrum of mutations compared to wildtype cells, indicating a physiological role for pol κ in bypass of BPDE-induced lesions, at least in mouse cells (Ogi et al., 2002). Future studies with human cells will likely translate *in vitro* data into an *in vivo* function for pol κ in bypass of BPDE-induced lesions.

Pol κ contains a PCNA binding site, and direct binding of pol κ to PCNA was shown by the Prakash group (Haracska et al., 2002b). In addition to a physical interaction, PCNA stimulated the polymerase activity of pol κ. Pol κ also interacts with the C-terminus of Rev1, but the relevance of this interaction is still unknown, however, the fact that Rev1 binds to several other TLS polymerases suggests a complicated interplay of TLS proteins *in vivo*.

A role for pol κ in human cancer was elucidated when O-Wang and colleagues discovered that pol κ is overexpressed in several lung cancer tissues compared to normal lung tissue samples (O-Wang et al., 2001). Further investigation demonstrated that elevated expression of pol κ in lung cancer tissues strongly correlated with p53 inactivation, suggesting a mechanism for pol κ mutagenesis in lung cancer carcinogenesis (Wang et al., 2004). The *in vivo* functions of human pol κ in mutagenesis have not been characterized to date. However, the *in vitro* evidence that pol κ efficiently bypasses a BPDE adduct strongly suggests that pol κ is a likely candidate for the major polymerase involved in *in vivo* bypass of BPDE-induced DNA damage.

2.4 Rev 1

Homologs of Rev1 have been identified in *Saccharomyces cerevisae*, *Schizosaccharomyces pombe*, *Neurospora*, *Arabidopsis*, *C. elegans*, chicken, mouse, and humans (Lin et al.,1999a; Simpson and Sale, 2003; Masuda et al., 2003; Sakai et al., 2003; Takahashi et al., 2005). The human homolog of *S. cerevisiae* REV1 was identified by two independent groups (Lin et al., 1999a; Gibbs et al., 2000). hRev1 contains two N-terminal regions with 41% and 20% identity, and an additional region with 31% identity at the amino acid level to the yeast Rev1 protein (Gibbs et al., 2000). hRev1 was mapped to chromosome 2q.11 by sequence analysis and radiation hybrid analysis and is ubiquitously expressed as shown by Northern blot analysis (Wixler et al.,1999; Lin et al., 1999a; Masuda et al., 2001; Murakumo et al., 2001). hRev1 contains the conserved sequence motifs, I-V, characteristic of Y family polymerases (Figure 1) (Lin et al., 1999a).

Human Rev1 contains 1251 amino acids and has a predicted molecular weight of over 138kDa. Masuda and colleagues (Masuda et al., 2001) identified a splice variant of Rev1 that encodes a 1250 amino acid protein that lacks an alanine residue at position 479, which they called Rev1S. Expression of hRev1 and hRev1S was detected in mononuclear cells from 10 individuals at similar levels by quantitative RT-PCR, suggesting that both forms are expressed and at similar levels (Masuda et al., 2001). hRev1 is thought to be kept at very low cellular

levels because of an out-of-frame ATG in the 5' untranslated region (UTR), which creates a very early termination at position +2. The sequence upstream of this out-of-frame ATG is very close to the consensus Kozak sequence, suggesting that this ATG is an efficient translational start site. Thus, the translational efficiency of full length hRev1 is most likely reduced due to competition for the correct ATG start site, leading to low cellular protein levels. This theory is supported by the inability of investigators to visualize hRev1 protein using Western blot analysis (Maher and Wang, unpublished data). Recently, however, Hanoaka's group in Japan has developed an antibody that is sensitive enough to detect endogenous levels of hRev1 (communication from Dr. Hanoaka to Dr. VM Maher, 2005).

Human Rev1, like its yeast homolog, is believed to have at least two cellular functions. *In vitro* primer extension reactions demonstrated that purified hRev1 protein was able to transfer a C residue across from a template G, indicating that hRev1 has intrinsic deoxycytidyl transferase activity, like the yeast Rev1 protein (Lin et al., 1999a). hRev1 did not efficiently transfer a G, T, or A residue across from the template G, and tranferase activity across from a template T, A, or C was not observed, suggesting that this transferase activity is a highly specific, template dependent deoxycytidyl transferase. In addition, using a primer template containing an AP site, the investigators demonstrated that hRev1 specifically transferred a C residue across from an AP site (Lin et al, 1999a). Similar experiments were performed using the hRev1S protein and indicated that

hRev1S was also able to transfer a C residue opposite an AP site (Masuda et al., 2001). Taken together, this evidence indicates that hRev1 has an intrinsic template-dependent deoxycytidyl transferase activity across from a template G and an AP site, *in vitro*. Further investigation demonstrated that this deoxycytidyl transferase activity is closely associated with the conserved polymerase domains because transferase activity was abolished in a mutant containing a C-terminal deletion (Masuda et al., 2001). A highly conserved region in several Rev1 proteins was found in this C-terminal region, indicating a critical conserved domain for tranferase activity. Mutations in two conserved residues, found to be critical for polymerase activity in the yeast Rad30 protein, also abolished the transferase activity of hRev1S, strongly suggesting that the structure of the catalytic site for the dCMP transferase in hRev1S is similar to the catalytic site of Y-family polymerases.

hRev1 contains a BRCA-1 C-terminal (BRCT) domain in its N-terminal region (Lin et al., 1999a). The BRCT domain was originally identified in the breast cancer suppressor protein BRCA1, and is important for protein-protein interactions (Koonin et al.,1996; Zhang et al., 1998). Masuda and colleagues created deletion mutants of the Rev1S protein and demonstrated that the BRCT domain is dispensable for transferase activity, *in vitro*, because an N-terminal deletion of the region containing the BRCT domain exhibited a similar transferase activity to wildtype hRev1S (Masuda et al., 2001).

In contrast, the BRCT domain is hypothesized to be important for the second function of Rev1 during in TLS. Studies using mice cells with a targeted mutation in the BRCT domain of the Rev1 gene displayed reduced UVC induced mutations in the HPRT gene, suggesting that the BRCT domain of Rev1 has a role in UV-induced mutagenesis, at least in mouse cells (Jansen et al., 2005). Furthermore, mutations at T-T dimers were absent in these cells, implicating Rev1 in a mutagenic process at sites of UV-induced T-T dimers. These studies suggest a second role for Rev1 in cells involving induced mutagenesis, in addition to the deoxycytidyl transferase activity. In summary, the BRCT domain is not required for deoxycytidyl transferase activity *in vitro*, but is required for UV-induced mutagenesis *in vivo*, suggesting these two functions are distinct.

Mouse cells with the Rev1 mutation also demonstrated delayed progression through the S and G2 phases of the cell cycle, similar to what was seen in XPV cells lacking pol η, lung cancer cells with decreased pol κ, and human fibroblast cells with decreased hRev7 (Bullock et al., 2001; Cordiero-Stone et al., 2002; Bi et al., 2005; McNally unpublished data). While the previous studies indicate a clear role for the Rev1 BRCT domain in mutagenesis in mouse cells, whether the BRCT domain of human Rev1 is as critical remains to be determined.

The *in vitro* biochemical properties of hRev1 have been well characterized. In addition to the previously mentioned transferase assays, the ability of hRev1 to bypass different types of DNA lesions has been investigated. Using primer

template reactions, hRev1 was found to insert a dCMP residue opposite template 8-oxoguanines, BPDE-adducted guanines, and 1,N6-ethenoadenine lesions (Zhang et al., 2002). Complete bypass of the BPDE-adducted guanine and the ethenoadenine lesion was achieved by adding pol κ to the reaction. Insertion by hRev1 opposite the lesions was efficiently extended by pol κ, implicating these proteins in multi-polymerase TLS. hRev1 was additionally able to insert, with a very limited efficiency, opposite an AAF adducted guanine, but complete bypass was inhibited. Insertion by hRev1 opposite all lesions tested showed a strong preference for dCMP residue insertion. When tested using a template TT CPD or TT 6-4 PP, however, hRev1 was unable to insert a nucleotide opposite these types of lesions.

A possible regulatory role for hRev1 in TLS is suggested by its many interactions with other TLS polymerases. hRev1 was found to bind to hRev7 in a yeast-two-hybrid assay, and this interaction was confirmed using *in vitro* and *in vivo* binding assays (Murakumo et al., 2001). The important region of hRev1 for this interaction is located between amino acids 1130-1251, the extreme C-terminus. This interaction does not occur between the yeast proteins, suggesting additional roles for human Rev1 and Rev7 compared to the yeast proteins. This physical interaction has not yet lead to a functional interaction; Masuda and colleagues (Masuda et al., 2003) demonstrated that hRev7 did not influence the stability, substrate specificity, or kinetic parameters of the transferase activity of hRev1. There may be an as-yet undefined role for the hRev1-hRev7 interaction in TLS

and mutagenesis. In addition to interacting with hRev7, hRev1 has been found to interact with human pol η, pol κ, and weakly with pol ι in yeast-two-hybrid assays, but an interaction between hRev1 and hRev3 has not been identified (Murakumo et al., 2001; Masuda et al., 2003; Tissier et al., 2004). These interactions were confirmed in co-immunoprecipitation experiments using exogenously expressed proteins, and found to be dependent on the C-terminal 136 amino acids of hRev1.

Immunofluorescence experiments determined that hRev1 is localized in the nucleus and further localizes to replication foci during S-phase, independently of pol η. Because of its localization in replication foci during S-phase, and its ability to bind to several different TLS polymerases, it is hypothesized that hRev1 may regulate TLS activities by bringing several different polymerases to sites of damage. For example, hRev1 could potentially bring pol η to a UV-induced TT 6-4 PP to insert a nucleotide, and subsequently bring pol ζ (via its interaction with hRev7) to extend this nucleotide, resulting in full bypass of the lesion. Whether hRev1 plays such a role in human cells is unknown.

A specific *in vivo* role for hRev1 in tolerance of UV-induced DNA damage has been best shown by Gibbs and colleagues. Cells expressing high levels of antisense targeted against hRev1 mRNA exhibited a significantly reduced UV-induced mutation frequency, implicating hRev1 in the tolerance of UV-induced DNA damage in human cells (Gibbs et al., 2000). hRev1 has a role in UV-

induced mutagenesis and its deoxycytidyl transferase activity seems relevant for bypass of abasic sites *in vivo*, however, whether these two functions are distinct has not been clearly established.

2.5 Polymerase ζ

a. hRev3

Pol ζ is a human polymerase that has been implicated in TLS, but is not a member of the Y family of DNA polymerases. Homologs of Rev3 are found in yeast, mouse, *Aspergillus*, *Drosophila*, *Neurospora*, *Arabidopsis*, chicken, and humans (Lawrence and Christensen, 1979; Gibbs et al., 1998; Han et al., 1998; Van Sloun et al., 1999; Eeken et al., 2001; Sakai et al., 2002; Sakamoto et al., 2003; Okada et al., 2005). The human homolog of yeast Rev3 was identified by three independent groups that searched for sequences homologous to the yeast protein (Gibbs et al., 1998; Xiao et al., 1998; Lin et al.,1999b). hRev3 has 43% identity and 74% similarity to the yeast protein at the amino acid level. hRev3 is a large protein with a predicted molecular weight of over 350 kDa, and like yeast Rev3, human Rev3 was shown to contain six conserved sequence motifs characteristic of classical DNA polymerases in the C-terminus. Because of this homology, hRev3 is classified as a member of the B family of DNA polymerases, but has not been shown to possess polymerase activity to date. Initial northern analysis showed that hRev3 is variably expressed in different tissues.

Very little is currently known about human Rev3 because it has never been isolated from human cells or expressed as a cDNA. This inability to isolate hRev3 is thought to be due to very low cellular levels. Because hRev3 is known to be involved in mutagenesis, cells tightly regulate the activity of hRev3 by two mechanisms. First, there is a putative splicing variant that creates a large insertion leading to an early termination within the ORF. Second, an out of frame ATG in the 5' UTR that results in early termination of the hRev3 protein is also present. It appears that this alternate ATG is an efficient translation start site, which reduces the translational efficiency of the correct hRev3 protein. The hypothesized low level of hRev3 in cells is supported by the inability of several groups using multiple antibodies to detect hRev3 protein by Western blot analysis (Li et al., 2002).

In vivo experiments have shown that cells expressing high levels of antisense against hRev3 have a reduced UV- and BPDE-induced mutation frequency compared to cells without antisense (Gibbs et al.1998; Li et al., 2002). While reduced protein level of hRev3 could not be confirmed, these experiments strongly suggest a role for hRev3 in bypass of lesions induced by UV and BPDE. More recent studies using siRNA to reduce hRev3 protein levels demonstrated a role for hRev3 in bypass of UV-induced 6-4 PPs (Nakajima et al., 2004). These experiments were performed in an XPA cell line lacking NER in order to control the rate of repair using photolyases specific to CPDs or 6-4 PPS. The authors showed that cells with decreased hRev3 mRNA were more sensitive to the

cytotoxic effect of UV radiation than control cells containing hRev3. In addition, this increased sensitivity was prevented by photorepair of the 6-4 PP's but not the CPD's, suggesting that hRev3, and therefore pol ζ, is primarily involved in the tolerance of 6-4 PP's (Nakajima et al., 2004). These data correlate with *in vivo* experiments done in yeast that also show that Rev3 is important for tolerance of 6-4 PP's (Gibbs et al., 2005). As previously stated, *in vitro* data using human Rev3 is lacking, but based on data using yeast Rev3, it is hypothesized that the major contribution to TLS by hRev3 (pol ζ) is at the extension step of TLS. However, the relative contribution of hRev3 to TLS comparing insertion vs. extension has not been investigated *in vivo*.

Human Rev3 binds to human Rev7, and the important region of hRev3 for this interaction is residues 1847 to 1892 (Murakumo et al., 2000; Murakumo et al., 2001). This binding of hRev3 to hRev7, combined with a clear role for hRev3 in mutagenesis, strongly suggests that the human Rev3 protein has a similar function as the yeast Rev3 protein. Once hRev3 is purified and a specific and sensitive antibody to detect the endogenous protein is available, the role of hRev3 in the process of TLS will be better understood.

b. hRev7

Homologs of Rev7 have been found in *Saccharomyces* (Torpey et al., 1994), *Arabidopsis* (Takahashi et al., 2005), chicken (Okada et al., 2005), *Drosophila* (Takeuchi et al., 2004), *Neurospora* (Sakai et al., 2003), and humans (Murakumo

et al., 2000). Human Rev7 was initially isolated in a yeast-two-hybrid assay using part of the hRev3 protein as bait (Murakumo et al., 2000; for review see Murakumo, 2002). This protein has 23% identity and 53% similarity to the yeast Rev7 protein at the amino acid level. hRev7 encodes a small protein containing 211 amino acids with a molecular weight of 24 kDa, and is located on chromosome 1p36, a region of high loss of heterozygosity in human tumors (Murakumo et al., 2000). Northern analysis demonstrated ubiquitous expression of hRev7 with varied expression levels in tumor cell lines. High expression of hRev7 was detected in lymphoblastic leukemia and colorectal adenocarcinoma tumor cell lines. However, no transcript variations or protein mutations in hRev7 were detected (Murakumo et al., 2000; Ying and Wold, 2003). Like yeast Rev7, hRev7 contains a HORMA domain (Aravind and Koonin, 1998). The HORMA domain was named for yeast proteins Hop1, Rev7, and Mad2, all of which contain this moderate sequence conservation. Very little is known about this protein motif, but it is hypothesized to be involved in protein-protein interactions, although there is currently no evidence to support this. Alternatively, because Hop1, Rev7, and Mad2 all interact with DNA at some stage during the cell cycle, the HORMA domain is thought to involved in chromatin binding or recognition of chromatin (Aravind and Koonin, 1998).

As previously stated, hRev7 was initially isolated as a binding partner of hRev3 in a yeast-two-hybrid assay (Murakumo et al., 2000). This interaction was confirmed using an *in vitro* binding assay. Further interaction studies using

fragments of hRev3 showed that the minimal region on hRev3 required for interacting with hRev7 was located within amino acids 1847-1892 (Murakumo et al., 2001). The important region on hRev7 for interacting with hRev3 was shown to be located within amino acids 21-155. Because the total length of hRev7 is only 211 amino acids, this is a fairly large region of hRev7, and to date, this region has not been narrowed down. In addition to binding to hRev3, hRev7 was found in a yeast-2-hybrid assay to interact with hRev1. This interaction was confirmed using *in vivo* and *in vitro* binding assays (Murakumo et al., 2001; Masuda et al., 2003). The important region on hRev7 for interacting with hRev1 is the same region important for interacting with hRev3, amino acids 21-155 (Murakumo et al., 2001). This is an interesting finding because yeast Rev7 does not interact with yeast Rev1. The important region of hRev1 for binding to hRev7 is contained in the extreme C-terminus within amino acids 1130-1251. The fact that the critical 121 amino acid region in human Rev1 is not found in the yeast Rev1 protein explains the lack of interaction between yeast Rev7 and Rev1.

The investigators also observed in a yeast-two-hybrid assay that hRev7 can bind to itself, and that the important region for homodimerization is the same amino acid region important for binding to hRev3 and hRev1. Because hRev7 binds to hRev3 and hRev1, it is hypothesized that hRev3, hRev7, and hRev1 exist in a complex, although *in vitro* binding assays were not successful at isolating a complex of the three proteins together (Murakumo et al., 2001;). Recognition that the same binding region on hRev7 is used by all three proteins led to the

alternative hypothesis that these proteins compete for binding to hRev7; to date, no further evidence supports this theory. Because the amino acid region important for interaction involves such a large part of hRev7, this region will likely be narrowed down in the future, providing insight as to whether these proteins exist in a complex or whether they compete for binding to hRev7.

More recent studies by Masuda and colleagues (Masuda et al., 2003) were designed to test the interaction between hRev7 and hRev1. Specifically, because yeast Rev7 was found to enhance the polymerase activity of yeast Rev3, the investigators suggested that hRev7 modulates the activities of hRev1. They showed that hRev7 does not influence the stability, substrate specificity, or kinetic parameters of the transferase activity of hRev1. Whether hRev7 modulates the TLS activities of hRev1 or hRev3 in human cells has not yet been determined.

In addition to the hypothesized role for hRev7 in TLS, this protein may have additional functions in cells. hRev7 shares 23% identity and 54% similarity at the amino acid level with hMad2, a mitotic checkpoint protein (Murakumo et al., 2000). hMad2 functions to prevent the early onset of anaphase until all of the mitotic spindles are attached to the kinetochores, and all of the chromosomes are aligned at the metaphase plate (Li and Benezra, 1996; Cahill et al., 1999). The metaphase to anaphase transition is controlled by the anaphase promoting complex (APC) (Irniger and Nasmyth, 1997; Fang et al., 1998). APC is activated

by binding to cdc20 and cdh1, allowing progression through mitosis. cdc20 forms a complex with APC at the onset of anaphase, and cdh1 forms a complex with APC during late mitosis. In contrast, hMad2 regulates cell cycle progression by inhibiting APC activity by binding to the cdc20-APC complex (Fang et al.,1998; Hwang et al., 1998; Kim et al., 1998). A parallel mechanism regulating the cdh1-APC complex during the later stages of mitosis was lacking.

The high homology hRev7 shares with hMad2 suggests a potential cell cycle role for hRev7. Supporting this theory is evidence that hRev7 interacts with hMad2 in *in vitro* GST-binding assays (Murakumo et al., 2000). Additional studies by two independent groups demonstrated a functional role for hRev7 in the cell cycle. Chen and colleagues (Chen and Fang, 2001) used purified human Rev7 in *Xenopus* extracts and found that hRev7 blocked degradation of cyclin B in the presence of APC and cdh1 or cdc20, suggesting that hRev7 blocks activation of both the cdh1-APC and the cdc20-APC pathways. These investigators further showed that once APC was activated, hRev7 no longer had an inhibitory effect, strongly suggesting that the mechanism of inhibition by hRev7 is through binding to cdh1 and cdc20, or to these proteins complexed with APC. Immunoprecipitation experiments confirmed that hRev7 directly binds to both cdh1 and cdc20. Pfleger and colleagues performed similar experiments using the *Xenopus* homolog of hRev7, which is 95% identical to human Rev7 at the amino acid level (Pfleger et al., 2001). This group showed that xRev7 binds to the cdh1-APC complex and inhibits the activity of APC. In contrast to the

experiments described above, these investigators could not detect binding or inhibition by xRev7 to cdc20-APC, indicating a possible difference between human and *Xenopus* Rev7. Although these experiments indicate a role for hRev7 in cell cycle control, the specific role is not known. However, because of the results of my studies, hRev7 has now been implicated in not only cell cycle regulation, but also in TLS, leading to the hypothesis that these two functions are linked. If this hypothesis is correct, hRev7 may play a unique role in monitoring cell cycle progress, and upon a signal of stress, be stimulated to perform TLS. Alternatively, it is possible that after DNA damage has occurred and TLS is taking place, hRev7 is called upon to perform an inhibitory role during mitotic progression.

The two known roles for hRev7 may not be linked, however, as indicated by the fact that hRev7 interacts with several different proteins not involved in TLS or in cell cycle regulation. hRev7 interacts with PRCC, a rearranged protein in papillary renal cell carcinoma (Sidhar et al., 1996). The interaction between PRCC and hRev7 was initially demonstrated using a yeast-two-hybrid analysis (Weterman et al., 2001). In some renal cell carcinomas, a chromosomal translocation creates a fusion protein between PRCC and the transcription factor TFE3. Two fusion proteins are expressed in tumor cells: PRCC-TFE3 and TFE3-PRCC. Coimmunoprecipitation experiments confirmed a direct interaction between PRCC and hRev7. Colocalization experiments using transfected proteins suggested that hRev7 is translocated into the nucleus via PRCC.

However, these experiments used transiently transfected and overexpressed proteins, which may not recapitulate what actually occurs with the endogenous proteins. Yeast-two-hybrid analysis using the fusion proteins demonstrated that the PRCC-TFE3 protein had significantly reduced interaction with hRev7 compared to PRCC or the TFE3-PRCC fusion protein. In addition, colocalization experiments showed that hRev7 was not efficiently transferred to the nucleus in the presence of the PRCC-TFE3 fusion protein as it had been with PRCC. Finally, nocodazole treatment of renal carcinoma tumor cells demonstrated an impaired mitotic checkpoint in these cells. Taken together, these data suggest that hRev7 has a cellular function in a mitotic checkpoint because in renal carcinoma tumor cells where the PRCC-TFE3 fusion protein is expressed, hRev7 does not get translocated to the nucleus and is, therefore, not able to accurately perform the mitotic checkpoint. This ultimately leads to chromosomal instability and cancer in these cells.

hRev7 also interacts with the cytoplasmic domain of MDC9, also called ADAM9, in a yeast-2-hybrid assay (Nelson et al., 1999). MDC9 is a metalloproteinase disintegrin, a family of transmembrane glycoproteins involved in fertilization, sperm migration, myoblast fusion, and other developmental processes (Primakoff et al., 1987; Cho et al., 1998; Yagami-Hiromasa et al., 1995; Fambrough et al., 1996). More specifically, MDC9 is thought to be involved in shedding the heparin-binding epidermal growth factor-like growth factor (Izumi et al., 1998). A direct interaction was confirmed between hRev7 and MDC9 using *in vitro* GST-

binding assays. To date, a physiological function for this interaction has not been determined. Evidence of a parallel role for hMad2 binding to a different metalloproteinase disintegrin, TACE, suggests the possibility of a functional role between mitotic checkpoint proteins and metalloproteinase disintegrins, although there has been no further characterization of these interactions (Nelson et al., 1999).

hRev7 interacts with the adenovirus death protein (ADP), a type III integral membrane glycoprotein (Scaria et al., 1992; Ying and Wold, 2003). ADP mediates cell lysis and release of adenovirus particles (Tollefson et al., 1996). Previous studies suggest that ADP must reside in the nuclear membrane to perform its lytic function (Tollefson et al., 2003). Using the N-terminal 40 amino acids of ADP, Ying and colleagues (Yin and Wold, 2003) demonstrated binding between hRev7 and ADP using the yeast-two-hybrid system. *In vitro* GST pull down and *in vivo* coimmunoprecipitation experiments verified the interaction between hRev7 and ADP. In addition to physically interacting with ADP, a functional role for hRev7 was determined when the investigators overexpressed hRev7 in the human cell line A549. They measured release of lactate dehydrogenase as an indicator of cell viability and found that, when infected with adenovirus, cells overexpressing hRev7 were lysed much less efficiently than control cells. These experiments suggest that hRev7 acts as an antagonist to the cell lysis function of ADP. However, the specific role of hRev7 in this process remains to be determined.

Finally, hRev7 interacts with trichosanthin (TCS), a ribosome-inactivating protein (Chan et al., 2001). TCS is an active component of a Chinese medicinal herb used to induce mid-term abortions and treat ectopic pregnancies (Wang et al., 1986). TCS presumably alters the conformation of ribosomal RNAs, ultimately inhibiting protein synthesis. The interaction between hRev7 and TCS was initially observed using a yeast-two-hybrid assay and subsequently confirmed using *in vitro* binding assays (Chan et al., 2001). It is hypothesized that the interaction between hRev7 and TCS interferes with cell cycle regulation. However, the physiological function of this interaction is not known. Whether this interaction will prove to be relevant awaits further study.

It is intriguing that hRev7 has been found to interact with several different proteins that perform many different cellular functions. This property makes hRev7 unique among the group of proteins involved in TLS. The importance of these interactions is currently not established, but suggests that hRev7 is involved in several different cellular processes in human cells.

Until my dissertation studies, no *in vitro* or *in vivo* studies had indicated whether human Rev7 plays a role in mutagenesis. The high sequence homology to the yeast Rev7 protein and the binding evidence demonstrating that hRev7 binds to Rev3 and Rev1 strongly suggests a functional role for hRev7 in mutagenesis. My project was to determine if, in fact, hRev7 performed a similar function in

human cells as the yeast Rev7 protein does in yeast cells. The next chapter discusses the important data obtained to conclude that human Rev7 is involved in mutagenic TLS.

5. SOMATIC HYPERMUTATION

It is intriguing to consider why human cells have enzymes that are capable of making mutations. The selective advantage that mutations provide bacteria cells does not necessarily apply to higher eukaryotes. Nonetheless, human cells have several specialized polymerases that frequently make mutations via TLS. One explanation for the presence of these enzymes in higher eukaryotes is that mutations are advantageous for some cellular processes. For example, somatic hypermutation, i.e., the process of creating diversified antibody molecules by mutating variable regions, is such a process. This process, which generates a large number of point mutations in the variable region of immunoglobulin genes, is critical for humans to develop the antibodies needed to fight antigens. Interestingly, some of the TLS polymerases have been implicated in this process. Pol ζ was found to play a role in this process by measuring somatic hypermutation frequencies in human B cells expressing antisense against REV3 (Zan et al., 2001). These studies demonstrate a significantly reduced frequency of somatic hypermutation in cells with reduced REV3 mRNA levels. Rev1 and pol η were found to play a role in somatic hypermutation as well. DT-40 chicken cells, engineered to have a disrupted REV1 gene, exhibited greatly reduced hypermutation (Simpson and Sale, 2003). Pol η was implicated in the process of

somatic hypermutation specifically at AT base pairs (Rogozin et al., 2001; Zhen et al., 2001). Taken together, these data reveal that the specialized polymerases implicated in TLS, have additional important roles in cells in addition to TLS.

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Chapter II

hRev7, human homolog of *Saccharomyces cerevisiae* Rev7, is involved in translesion synthesis in human fibroblast cells

Experiments with clones that have significantly reduced hRev7

ABSTRACT

Translesion synthesis (TLS) refers to the mechanism by which specialized polymerases incorporate nucleotides opposite non-coding DNA damage and extend DNA replication until the regular DNA polymerases can resume their function. The first specialized polymerase discovered in eukaryotes was *S. cerevisiae* polymerase (pol) ζ, composed of catalytic subunit, Rev3, and non-catalytic subunit, Rev7. Human homologs of these two proteins have now been identified. Using human fibroblasts engineered to express antisense against hRev3, Gibbs and colleagues showed that the frequency of UV-induced mutations in such cells was nine times lower than control cells (Gibbs et al., 1998), strongly suggesting that hRev3 is required for UV-induced mutagenesis in human cells. To determine whether hRev7, the human homolog of yeast Rev7, also plays a critical role in mutagenic TLS, we transfected a human fibroblast cell strain with hRev7 siRNA, identified two cell strains with very significantly reduced levels of hRev7 protein, and compared them to their parental strain and a vector control, for their ability to carry out TLS. The derivative strains with reduced hRev7 were ~1.75 times more sensitive to the cytotoxic effects of UV radiation than the controls. The frequency of UV-induced mutations in their *HPRT* gene, however, was reduced ~4.4-fold compared to the frequency induced in control cells, strongly suggesting that hRev7 is involved in error-prone translesion synthesis of UV-induced DNA damage in human cells. In addition, the UV-induced mutation spectrum in the strains with reduced hRev7 was altered: the percentage of T→C transitions decreased ~5-fold; that of T→A transversions

increased ~3-fold. When the cell strains with reduced hRev7 were compared with control cell strains for their sensitivity to the cytotoxic and mutagenic effects of BPDE, they were ~2.2 times more sensitive to the cytotoxic effects of BPDE than control cells, but there was no significant difference among the four cell strains in their frequency of BPDE-induced mutations. These results indicate that hRev7 plays a critical role in UV-induced mutagenesis, but does not do so for BPDE-induced mutagenesis. These data also suggest that hRev7 interacts with hRev3 to constitute human pol ζ, and that pol ζ plays critical role in the TLS past UV-induced DNA damage, but not BPDE-induced DNA damage.

Introduction

DNA is constantly exposed to endogenous and exogenous damaging agents, many of which create lesions in the DNA that can block replication. If such DNA gets replicated past these blocking lesions, this may result in a mutation. Because mutations play a causal role in the development of cancer, it is important to understand the processes by which mutations develop in order to understand the process of carcinogenesis.

Human cells have many sophisticated DNA repair pathways to remove fork-blocking damage from DNA. In addition, they possess complex cell cycle checkpoints that can be activated to allow time for repair to occur before DNA replication begins. Despite such repair systems and cell cycle checkpoints, DNA damage may persist in the genome at the time of DNA replication. Under these circumstances, fork-blocking DNA lesions pose a challenge to the replication complex because most replicative polymerases are not efficient at replicating DNA containing damage. To overcome this obstacle, cells have evolved several damage tolerance pathways that allow them to cope with fork-blocking DNA damage during replication.

One damage tolerance pathway, called translesion synthesis (TLS), uses specialized DNA polymerases to incorporate nucleotides at sites of DNA damage. TLS is considered to be a two-step process. The first step involves insertion of nucleotides directly across from DNA damage. The second step

involves extension of the inserted nucleotides, which can involve extension of a mismatch. TLS can be error-free or error-prone depending on the type of DNA damage encountered, the specialized polymerases involved, and the sequence context surrounding the damage site (for review see Lawrence, 2004; Ohmori et al., 2004; Vaisman et al., 2004; Yang, 2005). However, because the process of TLS uses damaged DNA as the replication template, and most DNA damage is miscoding, it is often error-prone. Therefore, although TLS enhances cell survival, it comes with the risk of generating deleterious mutations.

Recent studies of newly discovered specialized DNA polymerases have provided insight into induced mutagenesis in human cells, however, several areas remain unknown. What is clear from previous studies is that many of these enzymes have the ability to insert an incorrect nucleotide across from DNA damage potentially leading to a mutation. Because of this ability to create mutations in DNA, these enzymes may play an important role in the development of cancer. In addition to creating mutations, many of these enzymes have evolved to accurately bypass DNA damage by inserting the correct nucleotide across from DNA damage. An example is polymerase η, which inserts two A residues across from a UV-induced thymine-thymine (TT) cyclobutane pyrimidine dimer (Johnson et al., 1999a; Johnson et al., 2000a; Masutani et al., 2000; McCullogh et al., 2004). The importance of these error-free enzymes is underscored by the clinical presentation of xeroderma pigmentosum variant (XPV) patients; cells from XPV patients lack a functional pol η (Johnson et al., 1999b; Masutani et al.,

1999). These patients have increased UV sensitivity and a predisposition to skin cancer. Thus, pol η deficiency results in the inability to tolerate UV-induced DNA damage and a remarkably high incidence of skin cancer. Many of the other specialized DNA polymerases are also being discovered to have a link to cancer. For example, polymerase ι has recently been shown to be up-regulated in some breast cancer cell lines and the spectrum of mutations in these cell lines is consistent with T→A and C→A mutations characteristic of pol ι *in vitro* (Yang et al., 2004). In addition, polymerase κ has been shown to be up-regulated in some lung cancer cell lines and this up-regulation of pol κ strongly correlated with inactivating mutations in p53 (O.Wang et al., 2001; Wang et al., 2004). Although a more recent study determined that pols κ, ι, and η were down-regulated in several cancer cell lines tested (Pan et al., 2005), the previous studies support the idea that TLS polymerases may be involved in mutagenesis, at least in some cancer cell lines, and are, therefore, an important area of investigation.

Much of what we know about one of the specialized polymerases, pol ζ, comes from yeast studies. The REV genes of *Saccharomyces cerevisiae*, encoding Rev1, Rev3, and Rev7 proteins, were identified by complementation of the phenotype of mutant strains that could not be reverted by treatment with DNA damage, such as ultraviolet radiation (UV_{254nm}) (Lemontt, 1971). The cells that were unable to yield damage-induced mutations were found to lack functional Rev proteins. This information indicated that these proteins play an important role in a mutagenic process involving UV-induced DNA damage (Lawrence et al.,

1984; Lawrence et al., 1985). A direct role for Rev3 in this process was confirmed when the sequence of the yeast REV3 gene was shown to contain polymerase domains in its C-terminal region. The structure of this polymerase domain placed it into the B family of DNA polymerases. The Rev3 protein was subsequently shown to have DNA polymerase activity (Nelson et al., 1996). Furthermore, the addition of yeast Rev7 protein was shown to enhance the polymerase activity of yeast Rev3 (Nelson et al., 1996). These proteins were also shown to interact in a yeast-two-hybrid assay. Together, Rev3 (the catalytic subunit) and Rev7 (the non-catalytic subunit) were designated *Saccharomyces cerevisae* polymerase ζ.

Pol ζ is currently thought to function in two aspects of TLS based on studies with yeast pol ζ. First, *in vitro* studies with yeast pol ζ demonstrate that pol ζ is able to insert nucleotides, albeit with low efficiency, across from a TT 6-4 photoproduct and an AAF-adducted guanine (Guo et al., 2001). Second, Pol ζ can function in extension of inserted nucleotides. Yeast pol ζ is very efficient at extending mispaired termini, suggesting that another polymerase may insert the nucleotide and pol ζ extends the mispair (Nelson et al., 1996; Johnson et al., 2000b). An example of this is an abasic site, where Rev1 can insert a nucleotide across from an abasic site and complete bypass is achieved when pol ζ extends from the inserted residue (Nelson et al., 1996; Haracska et al., 2001). These studies have demonstrated that pol ζ is involved at both the insertion and extension steps of TLS.

Human cells also contain polymerase ζ. While human Rev3 (hRev3) has been shown to be involved in the tolerance of UV-induced DNA damage *in vivo* (Gibbs et al., 1998; Li et al., 2002), *in vitro* studies with hRev3 have been limited because full length hRev3 has not to date been purified from cells or expressed as a cDNA. Therefore, it is unclear the exact role pol ζ plays in TLS in human cells.

Although it has not been possible to isolate hRev3 protein from human cells and demonstrate *in vitro* that it plays a role in DNA replication past fork-blocking lesions such as UV photoproducts or bulky adducts formed by benzo(a)pyrene diol epoxide (BPDE), Maher and Gibbs showed that human cells expressing high levels of antisense against hREV3 display significantly decreased frequencies of UV- and BPDE-induced mutations (Gibbs et al., 1998; Li et al., 2002). These data support the hypothesis that hRev3 is essential for a mutagenic process that deals with lesions induced by these agents in human cells, just as it does in yeast. However, it was not possible to detect and measure expression of the hRev3 protein in these studies because there was no antibody available to detect hRev3. Therefore, it was critically important to conduct similar studies of human pol ζ using hRev7, for which an antibody was available.

Human Rev7 (hRev7), the putative human homolog of the yeast Rev7 protein, was initially identified in a yeast-two-hybrid assay using parts of the hRev3

protein as bait. At the amino acid level, hRev7 shares 23% identity and 53% similarity with the yeast Rev7 protein (Murakumo et al., 2000). It also contains a HORMA domain, i.e., a moderate sequence conservation named for yeast proteins Hop1, Rev7 and Mad2 (Aravind and Koonin, 1998). The exact function of the HORMA domain is not known, but a common feature of these proteins is their association with chromatin, suggesting that the HORMA domain is involved in recognition of chromatin status (Aravind and Koonin, 1998).

The human Rev7 protein also shares 23% identity and 54% similarity at the amino acid level with hMad2, a mitotic checkpoint protein, suggesting that it also plays a role during mitosis (Murakumo et al., 2000). Although little is known about hRev7 playing a role during mitosis in human cells, it was found to inhibit activation of the anaphase promoting complex (APC) by binding to Cdh1 and Cdc20 in *Xenopus* extracts (Chen and Fang, 2001; Pfleger et al., 2001). If, indeed, hRev7 has a role in a mitotic checkpoint, this could indicate a potential link between cell cycle regulation and TLS.

Human Rev7, hypothesized to be the non-catalytic subunit of pol ζ, has not been tested for involvement in mutagenesis in human cells. Therefore, to test the hypothesis that hRev7 plays a role in mutagenic TLS in human cells, we used siRNA to decrease expression of this protein in human fibroblasts and compared cells with significantly reduced hRev7 protein with normal human cells for their response to the cytotoxic and mutagenic effects of UV radiation. The data from

these studies indicated that cells with reduced hRev7 protein were only slightly more sensitive than control cells to the cytotoxic effects of UV, but exhibited a ~4.4-fold decrease in the frequency of UV-induced mutations. In addition, there was a difference in the kinds of mutations induced by UV in the cells with reduced hRev7 compared to that seen in control cells. Cells with decreased hRev7 had 5-fold fewer T→C transitions and 3-fold more T→A transversions than those found in the control cells. Taken together, these data strongly suggest that hRev7 is essential for dealing with UV-induced lesions in human cells and is the functional homolog of the yeast Rev7 protein.

Materials and Methods

Cell culture and preparation of cell strains with reduced hRev7

MSU1.2.9N.58, a near diploid, infinite lifespan, human fibroblast cell strain, was grown in Eagle's minimum essential medium supplemented with L-aspartic acid (0.2 mM), L-serine (0.2 mM), sodium pyruvate (1 mM), 10% supplemented calf serum (HyClone), 100 units/ml penicillin, 100 µg/ml streptomycin, 1 µg/ml hydrocortisone and 1 µg/ml tetracycline. Oligonucleotides designed to target *hREV7* mRNA were annealed to a complementary oligonucleotide according to the manufacturer's protocol (Ambion). Annealed oligonucleotides were ligated into pSilencer3.1 (Ambion) using T4 DNA ligase (New England Biolabs). Purified siRNA vectors were transfected into MSU1.2.9N.58 cells using Lipofectamine (Invitrogen) according to the manufacturer's protocol, and stable clones were selected and maintained in medium supplemented with 1 µg/ml puromycin.

Growth curve analysis

Cells were plated at an initial density of 0.6×10^5 cells per 60-mm-diameter dish. Triplicate sample dishes for each cell strain were harvested using trypsin versene every 24 hr. The cells were then counted using a Coulter particle counter, and the average number of cells in each dish was calculated.

Preparation of nuclear protein extracts and Western blot analysis

Subconfluent monolayers of cells were washed with ice-cold phosphate buffered saline (PBS), scraped from 150-mm-diameter plates in 1 ml of lysis buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF), and incubated on ice for 15 min. Sixty-two μl of 10% NP-40 was added to each sample, which were then vortexed for 10 sec, and centrifuged at 4°C, 10,000 RPM, for 30 sec. The supernatant containing cytoplasmic proteins was removed and the nuclear pellet was washed once in 1 ml of buffer A containing 10% NP-40. Nuclear proteins were extracted by disruption of the nucleus in 40 μl of lysis buffer C (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF) and incubated on ice for 15 min with vortexing every 5 min. Nuclear extracts were centrifuged at 4°C, 16000 RPM, for 5 min. The supernatants containing the nuclear proteins were saved. Protein was quantified using the Bradford method (Pierce). Protein lysates were subjected to 14% SDS-polyacrylamide gel electrophoresis, transferred to a PDVF immobilon membrane (Millipore), and probed with a 1:600 dilution of a custom rabbit polyclonal antibody raised against the C-terminal 19 amino acids of the human Rev7 protein (Bethyl, Montgomery, Texas). The membrane was probed with a 1:7500 dilution of goat anti-rabbit secondary antibody (Sigma) and visualized using SuperSignal chemiluminescent detection reagent (Pierce). Equal protein loading was confirmed by probing with a 1:10,000 dilution of a

rabbit Ku80 antibody and a 1:10,000 dilution of anti-rabbit secondary antibody (Santa Cruz).

UV survival assay

The cytotoxic effect of $UV_{(254nm)}$ radiation was determined using a colony-forming assay. Briefly, cells in exponential growth were detached from the dishes with trypsin, plated at cloning densities (100-600 cells per 100-mm-diameter dish), and allowed 12 hr for attachment. The cells were rinsed twice with PBS, irradiated with the designated doses of UV, and fresh complete growth medium was added to the cells. The culture medium was renewed 24 hr after irradiation and again after seven days. After 14 days, the resulting clones were stained with crystal violet. The survival was determined by comparing the cloning efficiency of the irradiated cells with that of the sham-irradiated control cells. The survival value at each dose is expressed as a percent of the cloning efficiency of the control cells for each cell strain.

BPDE survival assay

The cytotoxic effect of BPDE was determined using the colony-forming ability of cells with reduced hRev7 and compared to parental cells. Parental cells and clones 2-2 and 2-6 were each plated into a 150-mm-diameter dish at a density of 1.5×10^6 cells and given approximately 12 hr to attach. Cells were rinsed twice with PBS, and Eagle's minimum essential medium was added to each dish of cells. BPDE was resuspended in dry DMSO, and the designated dose of BPDE

was added to the Eagle's medium in each 150-mm-diameter dish and the cells were incubated at 37°C for 1 hr. After BPDE treatment, the cells were washed twice with PBS, and complete medium was added to each dish. Cells to be used for the cytotoxicity assay were immediately harvested by treating with trypsin and counted using a Coulter particle counter. Each cell strain was plated into a series of 100-mm-diameter dishes at cloning densities (100-600 cells/dish). These cells were re-fed seven days post-treatment, and cultured for seven additional days to allow colony formation. The resulting colonies were stained with crystal violet, and survival was calculated using the average number of colonies from BPDE treated dishes as a percentage of the average number of colonies formed in non-treated controls for each cell strain.

Ionizing radiation survival assay

Single-cell suspensions for each cell strain were prepared at a density of 500 cells/ml in Eagle's minimum essential medium and gamma-irradiated at the designated doses. After irradiation, the cells were plated at a density of 500 cells/100-mm-diameter dish in triplicate for each dose. Cells were re-fed seven days later and stained with crystal violet on day 14 post-irradiation. Survival was calculated by taking the average number of colonies formed in IR-treated dishes as a percentage of the average number of colonies formed in non-treated controls for each cell strain. The resulting survival curve was generated from three independent experiments, except for the vector control, which was tested two independent times.

UV-induced *HPRT* mutation frequency assay

The mutagenic effect of UV radiation was determined from the frequency of 6-thioguanine-resistant, HPRT defective cells observed in each cell strain. Briefly, sufficient sets of cells, plated at densities of 0.5-1.5 x 10^6 cells per 150-mm-diameter dish, were used in order to have at least 1 x 10^6 surviving target cells per dose. These cells were allowed 12 hr for attachment. Cells were rinsed twice with PBS, irradiated at the designated doses, and immediately fed with fresh complete medium. The culture medium was renewed 24 hr after irradiation, and the cells were allowed to replicate for four days. They were then detached using trypsin, pooled, and plated at densities of 0.5-1.0 x 10^6 cells per 150-mm-diameter dish to allow for exponential growth for four additional days. This 8-day expression period is to allow for depletion of wildtype HRPT and expression of mutant HPRT. After eight days, cells were detached using trypsin, pooled, and plated at a density of 500 cells/cm^2 in medium containing 40 μM 6-thioguanine (TG) to select for cells with a mutant HPRT protein. At this time, a portion of cells from each population was also plated in non-selective medium at a density of 100 cells per 100-mm-diameter dish to assay the colony-forming ability of the cells at the time of selection. The culture medium was renewed after seven days. After 14 days, cells were stained with crystal violet and the frequency of 6-TG-resistant colonies was calculated using the cloning efficiency of the cells at the time of selection. The induced frequencies were calculated by

subtracting the background frequencies in the sham-irradiated control populations for each cell strain.

BPDE-induced HPRT mutation frequency assay

Parental cells and clones 2-2 and 2-6 were each plated into 150-mm-diameter dishes at a density of 1.5×10^6 cells and given approximately 12 hr to attach. Cells were rinsed twice with PBS, and Eagle's minimum essential medium was added to each dish of cells. BPDE was resuspended in dry DMSO and the designated dose of BPDE was added to the medium in each 150-mm-diameter dish and incubated at 37°C for 1 hr. After BPDE treatment, the cells were washed twice with PBS and complete medium was added to each dish. Cells to be used for the mutation frequency assay were cultured for four days after BPDE treatment. On day 4, cells were harvested by treating with trypsin and plated at a lower density to allow exponential growth for four additional days. On day 8, cells were harvested by treating with trypsin, and plated at a density of 500 cells/cm^2 in selective media containing 6-thioguanine. At the same time, each cell strain was plated in a series of 100-mm-diameter dishes at a density of 100 cells/dish in non-selective media to assay their cloning efficiency at the time of selection. Cells were allowed to grow for one week, and then re-fed, and cultured for an additional week. The resulting HPRT defective clones were counted and the frequency of BPDE-induced mutants was calculated using the cloning efficiencies at the time of selection. Induced mutation frequencies were

calculated by subtracting the background mutation frequencies for each cell strain.

HPRT mutation spectrum analysis

HPRT defective colonies were obtained essentially as described above for the UV-induced mutation frequency protocol, except that populations were kept independent to avoid sibling mutations. The HPRT mutant clones were isolated by treating with trypsin, and each independent mutant was subjected to reverse transcription and two rounds of PCR to amplify the HPRT coding region. PCR products were purified (Qiagen) and sequenced at the MSU macromolecular structure facility to determine the specific mutation in the HPRT coding region. Only base substitutions at adjacent pyrimidines were considered UV-induced.

Cell synchronization and flow cytometry analysis

Each cell strain was plated at a density of 0.2×10^6 cells per 100-mm-diameter dish and allowed 12 hr for attachment. Complete culture medium containing lovastatin (60 μM) was added to the dishes for 12 hr. The medium containing lovastatin was removed, the cells were washed twice with PBS, and complete medium containing aphidicolin (2 μg/ml) and mevalonic acid (6 mM) was added to the dishes for 12 hr to synchronize the cells at the G1/S border. The cells were released from synchrony by rinsing twice with PBS, and irradiated at the designated doses of UV immediately after release from synchrony. At the designated times post-irradiation, cells were detached using trypsin, fixed in 80%

ethanol, and stained with a propidium iodide solution (PBS, 1 mg/ml propidium iodide, 10% Triton X-100, 0.5 mM EDTA, 10 mg/ml RNase A) for cell cycle analysis by flow cytometry. Asynchronously growing cells were assayed in parallel experiments.

Results

Efficient reduction of the hRev7 protein using siRNA

The hRev7 nucleotide sequence was searched using the Oligoengine website for putative siRNA target sites. Approximately 60 sites were obtained in this search. These sites were used to search the BLAST database to find target sites with little or no homology to other known human genes. Six sites were chosen as target sites for siRNA (Figure 1). Vectors expressing each siRNA were created and tested for their effectiveness at targeting the hRev7 protein by transiently transfecting them into 293-HEK cells. Nuclear protein lysates were made 48 hr post-transfection and the lysates were analyzed by Western blot analysis (Figure 2). Results from this experiment demonstrate that three of the six vectors expressing siRNA were effective at targeting the hRev7 protein. siRNA vectors 2 (lane 4), 4 (lane 6), and 6 (lane 8) effectively reduced the hRev7 protein compared to the hRev7 protein level in the parental (lane 1) and vector control cells (lane 2).

To obtain stable clones with reduced hRev7, MSU1.2.9N.58 parental cells were transfected with the vectors expressing siRNA targeted against hRev7. Puromycin resistant clones were isolated, expanded, and nuclear protein lysates were analyzed for hRev7 protein expression by Western blot analysis. Figure 3 shows a representative Western blot analysis on lysates from cells transfected with an effective siRNA vector. Lanes 1 and 2 show the hRev7 protein migrating

Figure 1: Position of siRNA target sites in the hRev7 nucleotide sequence. The nucleotide sequence of hRev7 was searched using the Oligoengine website to find putative siRNA target sites. Approximately 60 putative sites were obtained in this search. These sites were analyzed using the BLAST database to find sites with little or no homology to other known human genes. Finally, six sites were chosen to target hRev7. The six siRNA target sites in the hRev7 nucleotide sequence can be seen in bold and underlined. The first two target sites overlap.

siRNA target sites in the hRev7 nucleotide sequence

atgaccacgctcacacgacaagacctcaactttggccaagtggtggccgatgtgctc
tgcgagttcctggaggtggctgtgcatctcatcctctacgtgcgcgaggtctaccccgt
gggcatcttccagaaacgcaa**gaagtacaacgtgccggtccag**atgtcctgcca
cccggagctgaatcagtatatccaggacacgctgcactgcgtcaagccactcctgga
gaagaatgatgtggagaaagtggtggtggtgattttggataaagagcaccgcccagt
ggagaaa**ttcgtctttgagatcaccc**agcctccactgctgtccatca**gctcagact**
cgctgttgtctcatgtggagcagctgctccgggccttcatcctgaa**gatcagcgtgt**
gcgatgccgtcctggaccacaacccccaggctgtaccttcacagtcctggtgcac
acgagagaagccgccactcgcaacatggagaagatccaggtcatcaaggatttcc
cctggatcctggcggatgagcaggatgtccacatgcatgaccccggctgataccac
taaaaaccatgacgtcggacattttaa**agatgcagctttacgtgga**agagcgcgct
cataaaggcagctga

Figure 1

Figure 2: Western blot analysis of 293-HEK cells transiently transfected with vectors expressing siRNA targeted against hRev7. 293-HEK cells were transiently transfected with six siRNA vectors designed to target the hRev7 mRNA. A vector expressing a scrambled siRNA insert was used as a control. Nuclear extracts were made from 293-HEK cells 48 hr post-transfection. Lane 1 shows the hRev7 protein in 293-HEK cells. Lane 2 shows a similar level of hRev7 protein in 293-HEK cells transfected with a scrambled siRNA vector control (VC). Lanes 3-8 show the hRev7 protein in 293-HEK cells transfected with one of six siRNA vectors targeting the hRev7 mRNA. Lane 9 shows a nuclear extract of 293-HEK cells transfected with a vector expressing the full length cDNA of hRev7 as a positive control (+).

Western Blot Analysis of hRev7 protein in 293-HEK cells transiently transfected with vectors expressing siRNA against hRev7

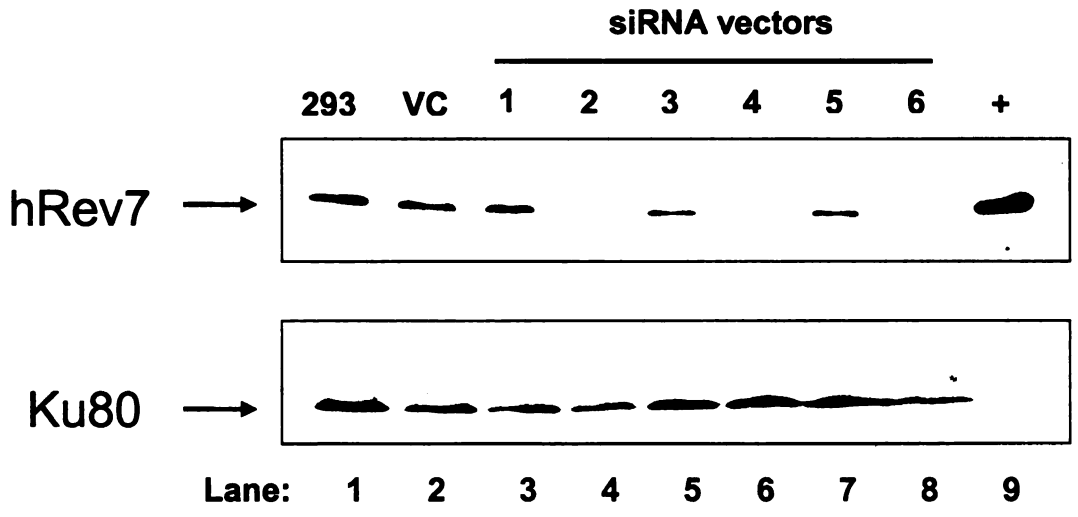

Figure 2

Figure 3: Western blot analysis of the hRev7 protein level in human fibroblast cells stably transfected with siRNA against hRev7. Nuclear protein lysates extracted from parental (P) and vector control cells (VC) demonstrate hRev7 protein at the expected size of 24 kDa. Stable clones (2-2 and 2-6) transfected with siRNA targeted against hRev7 exhibit significantly reduced hRev7 compared to the parent and vector control. Ku80 was used as the loading control.

Western blot analysis of stable clones with reduced hRev7

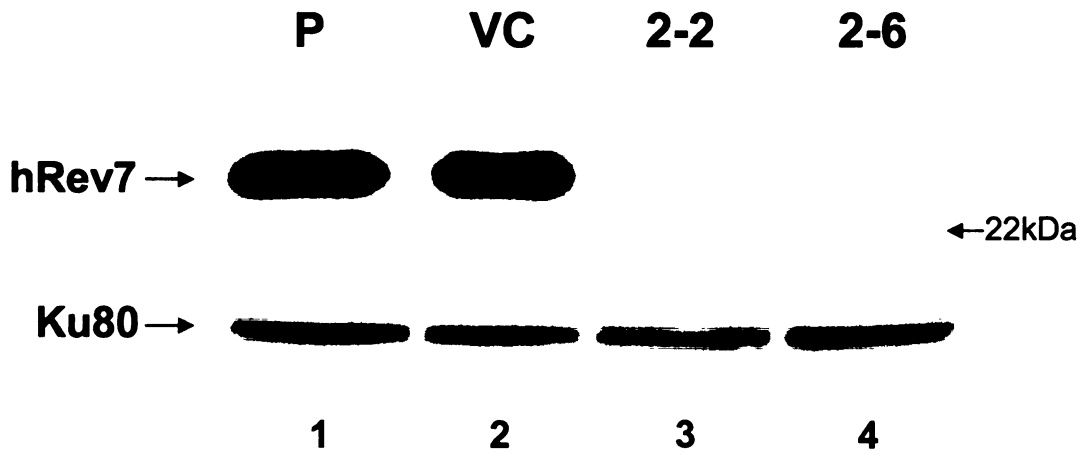

Figure 3

Figure 4: Growth curve analysis. Cells with decreased hRev7, clone 2-2 (closed squares) and clone 2-6 (closed triangles), were compared with the parental cells (open circles) for their growth rate under normal conditions. At each time point, the average number of cells from three 60-mm-diameter dishes for each cell strain is shown.

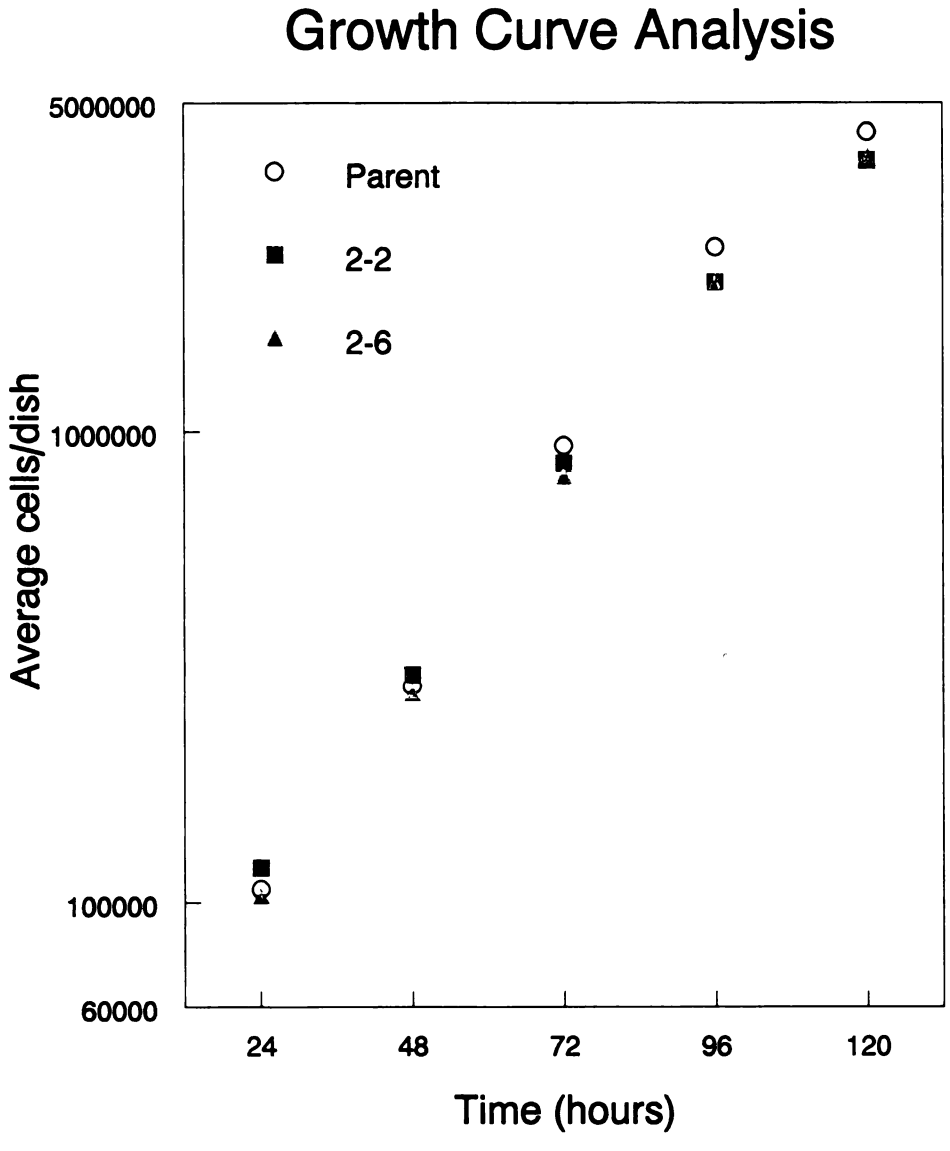
Growth Curve Analysis
5000000
1000000
100000
60000
Average cells/dish
Parent
2-2
2-6
24
48
72
96
120
Time (hours)

Figure 4

These results indicate that significantly reducing the hRev7 protein does not affect the growth rate of human fibroblast cells under normal conditions.

Decrease in hRev7 protein expression renders cells more sensitive to UV radiation

To determine the sensitivity of the different cell strains to the cytotoxic effect of UV radiation, survival of parental cells, vector control cells, and clones 2-2 and 2-6 was assayed using the colony forming ability of cells after UV irradiation. Figure 5 depicts a UV survival curve demonstrating the dose-dependent sensitivity of these cell strains to UV radiation. The parental cell strain (open circles) and vector control cells (open triangles) do not differ from each other with respect to their sensitivity to UV radiation. Clones 2-2 (closed squares) and 2-6 (closed triangles) do not differ from each other, but do have reduced survival compared to the parental and vector controls cells. These data indicate that with reduced hRev7, cells become more sensitive to the cytotoxic effects of UV radiation.

Decrease in hRev7 protein expression renders cells more sensitive to the chemical carcinogen BPDE

Because cells with reduced hRev7 were more sensitive to the cytotoxic effects of UV radiation, we wanted to test their response to other DNA damaging agents. hRev3 was previously shown to be important for tolerating BPDE-induced DNA and clone damage, therefore, we hypothesized that hRev7 is also involved.

Figure 5: UV-induced cytotoxicity. UV-induced cytotoxicity in parental cells (open circles), vector control cells (open triangles), and clones 2-2 (closed squares) and 2-6 (closed triangles) as determined by a colony-forming assay. Survival at each dose is represented by the average number of colonies in UV-treated dishes as a percentage of the average number of colonies in non-treated control dishes. Some points have been offset to make them visible. Lines represent least squares lines.

UV-induced cytotoxicity

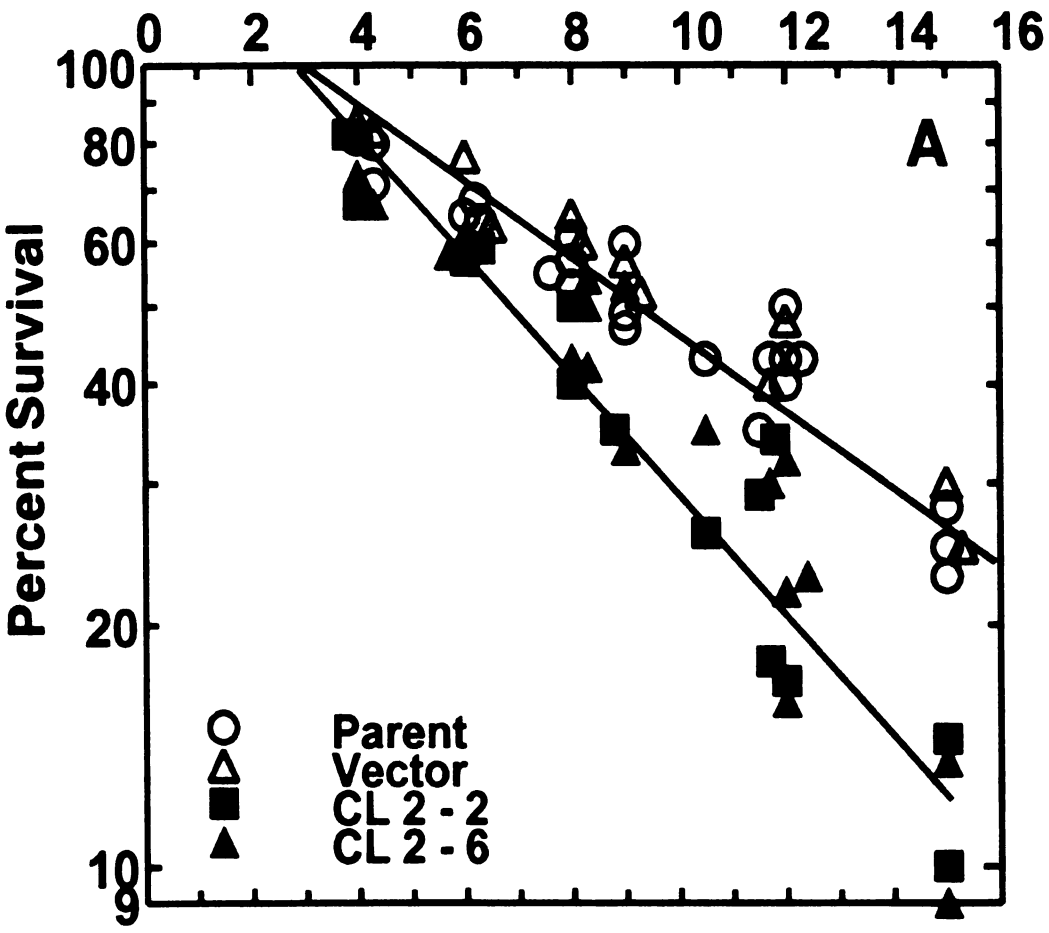

Figure 5

Clones 2-2 and 2-6 were subjected to BPDE treatment, and their sensitivity to BPDE was determined and compared with the parental cell strain (Figure 6). Results from these experiments demonstrate that the clones with reduced hRev7, 2-2 (closed squares) and 2-6 (closed triangles), were more sensitive to the cytotoxic effects of BPDE than parental cells (open circles). The sensitivity to BPDE was similar to the sensitivity seen in these cells after exposure to UV radiation.

Decrease in hRev7 protein expression does not affect the sensitivity of cells to ionizing radiation

Because cells with reduced hRev7 were shown to be sensitive to both UV and BPDE, we wanted to test a DNA damaging agent that induced a type of DNA damage that hRev7 was not expected to be involved in tolerating. Ionizing radiation (IR) was chosen because it induces double-strand breaks in DNA. hRev7 is not expected to be capable of repairing or tolerating double-strand breaks because both DNA strands are affected with breaks. The IR-induced cytotoxicity of the parent, vector control, and clones with reduced hRev7 is shown in Figure 7A. All cell strains examined demonstrated a dose-dependent sensitivity to IR. The clones with reduced hRev7, 2-2 (closed squares) and 2-6 (closed triangles) did not differ from parental (open circles) and vector control cells (open triangles) with respect to their sensitivity to IR. Because there was significant variation between experiments, the average percent survival of each

Figure 6: BPDE-induced cytotoxicity. Clones with reduced hRev7, 2-2 (closed squares) and 2-6 (closed triangles), were tested for their sensitivity to BPDE and compared with parental cells (open circles). Survival at each dose is represented by the average number of colonies in BPDE-treated dishes as a percentage of the average number of colonies in non-treated control dishes. Lines represent least squares lines.

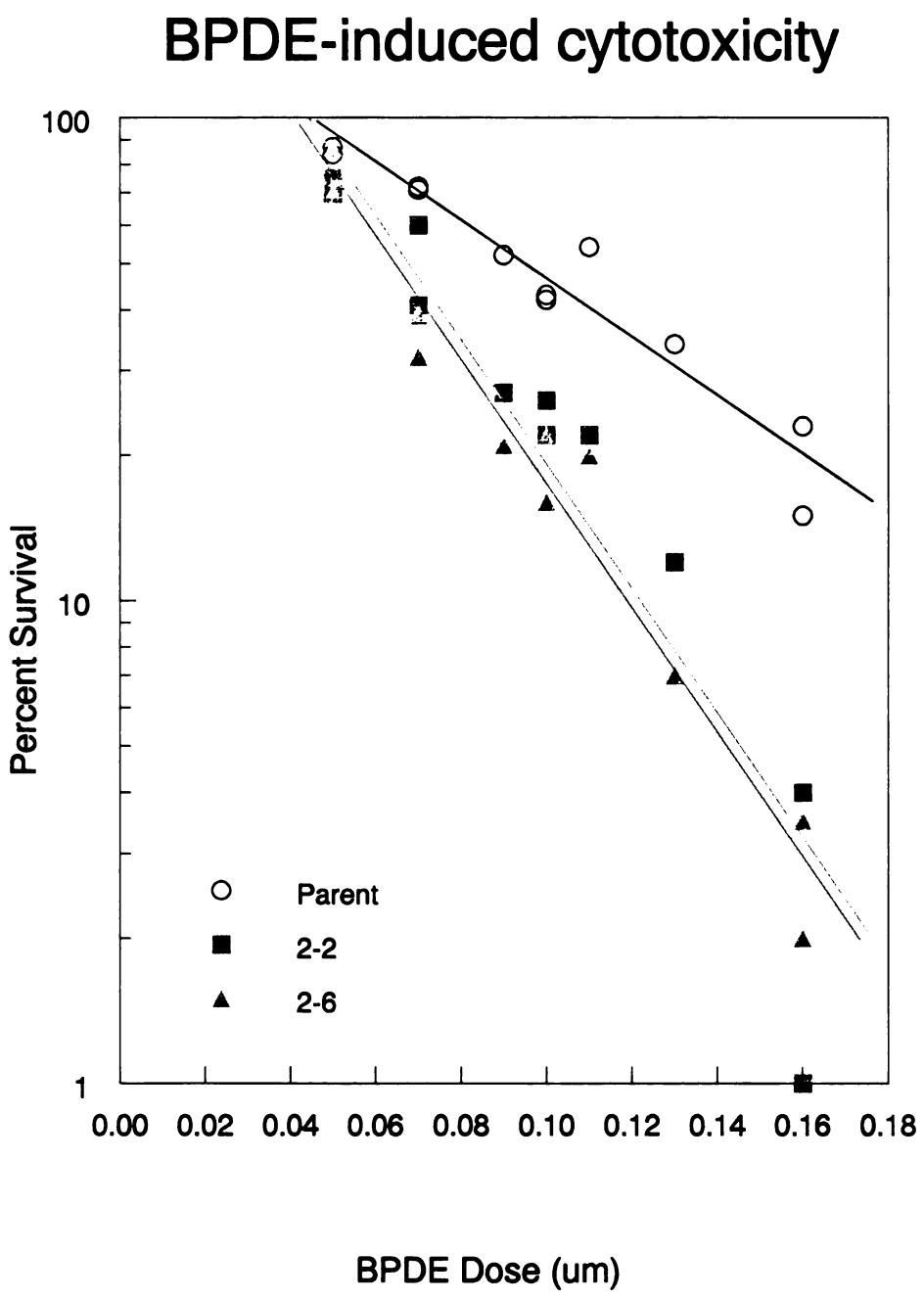
BPDE-induced cytotoxicity
100
10
1
Percent Survival
Parent
2-2
2-6
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
BPDE Dose (um)

Figure 6

Figure 7: Ionizing radiation-induced cytotoxicity. Cells with normal or reduced hRev7 were subjected to ionizing radiation to determine their sensitivity. Survival curves are shown from three independent experiments for parental cells and clones 2-2 and 2-6. For the vector control cells, results from two independent experiments are shown. **A.** Least squares lines are shown for parental cells (open circles), vector control cells (open triangles), and clone 2-2 (closed squares) and clone 2-6 (closed triangles). Survival at each dose is represented by the average number of colonies in ionizing radiation-treated dishes as a percentage of the average number of colonies in non-treated control dishes. **B.** A bar graph depicting the average percent survival at each dose is shown for parental cells (closed bars), vector control cells (open bars), and clones 2-2 and 2-6 (diagonal bars). Standard deviations are shown as light gray bars for each cell strain at each dose to indicate the variability between experiments.

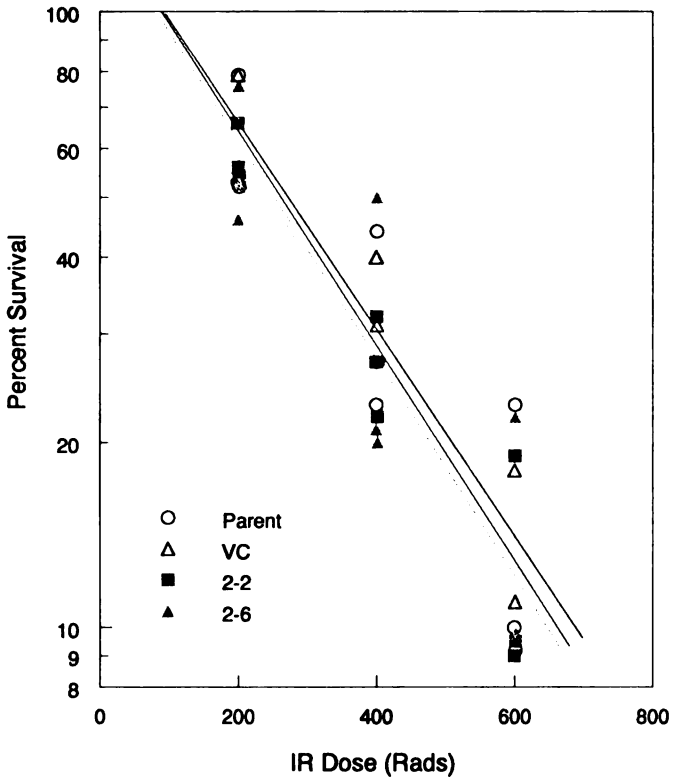

Figure 7A

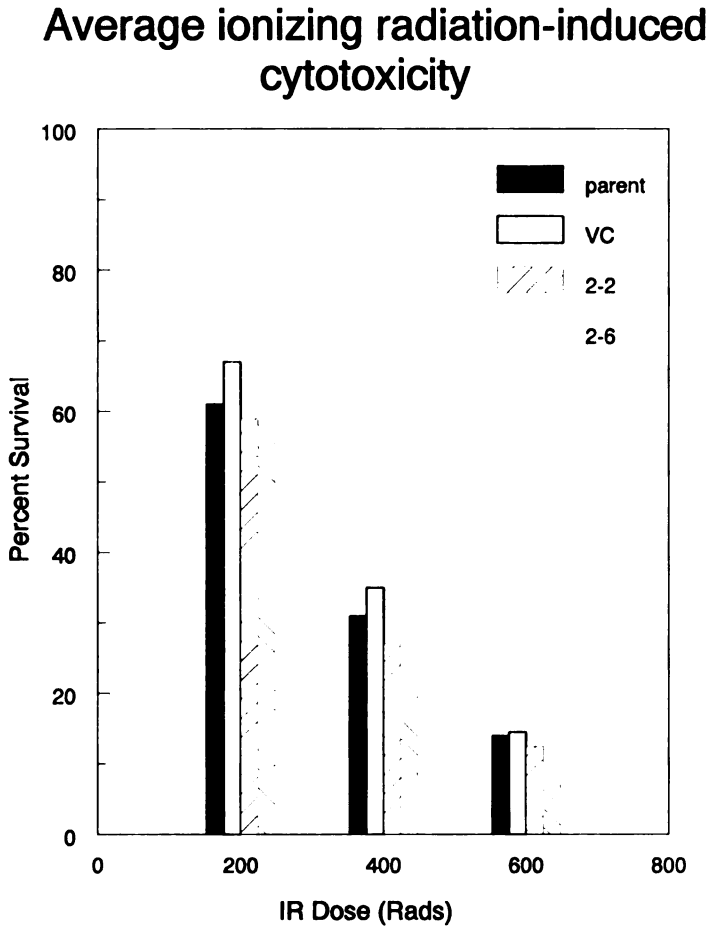

Figure 7B

cell strain at each dose is depicted in a bar graph (Figure 7B). Standard deviations are also included. Comparison of the average survival at each dose demonstrates that the cell strains did not differ from each other with respect to their sensitivity to IR. Taken together, these data indicate that cells with reduced hRev7 are not more sensitive than parental or vector control cells to the cytotoxic effects of ionizing radiation.

Decrease in hRev7 protein expression suppresses UV-induced mutations

The frequency of TG resistant mutants induced by UV radiation was measured in the parental cells, vector control cells, and clones 2-2 and 2-6. As shown in Figure 8, the frequency of UV-induced mutations was substantially the same in parental and vector control cells. Specifically, on average, the parental cells produced 109 mutants/10^6 cells and the vector control cells produced 106 mutants/10^6 cells at 9 J/m^2. Similarly, the parental cells produced 134 mutants/10^6 cells and the vector control cells produced 139 mutants/10^6 cells at 12 J/m^2. In contrast, clones 2-2 and 2-6 produced significantly fewer UV-induced mutants compared to parental and vector control cells. Specifically, TG-resistant mutants in clones 2-2 and 2-6 were reduced to an average of 33 and 30 mutants/10^6 cells at 12 J/m^2, respectively. This reduction in the frequency of UV-induced mutations in the HPRT gene is between 4-5-fold and indicates an important functional role for hRev7 in UV-induced mutagenesis in human cells.

Figure 8: UV-induced mutation frequency showing mutagenic TLS. The UV-induced HPRT mutation frequency assay showing mutagenic TLS in 9N.58 parental cells (open circles), vector control cells (open triangles), and cells with reduced hRev7, clone 2-2 (closed squares) and clone 2-6 (closed triangles). The frequency of thioguanine resistant cells containing a mutation in the HPRT protein induced by UV was calculated using the cloning efficiency of cells at the time of selection. Induced frequencies were calculated by subtracting background frequencies observed in non-treated populations.

UV-induced mutation frequency

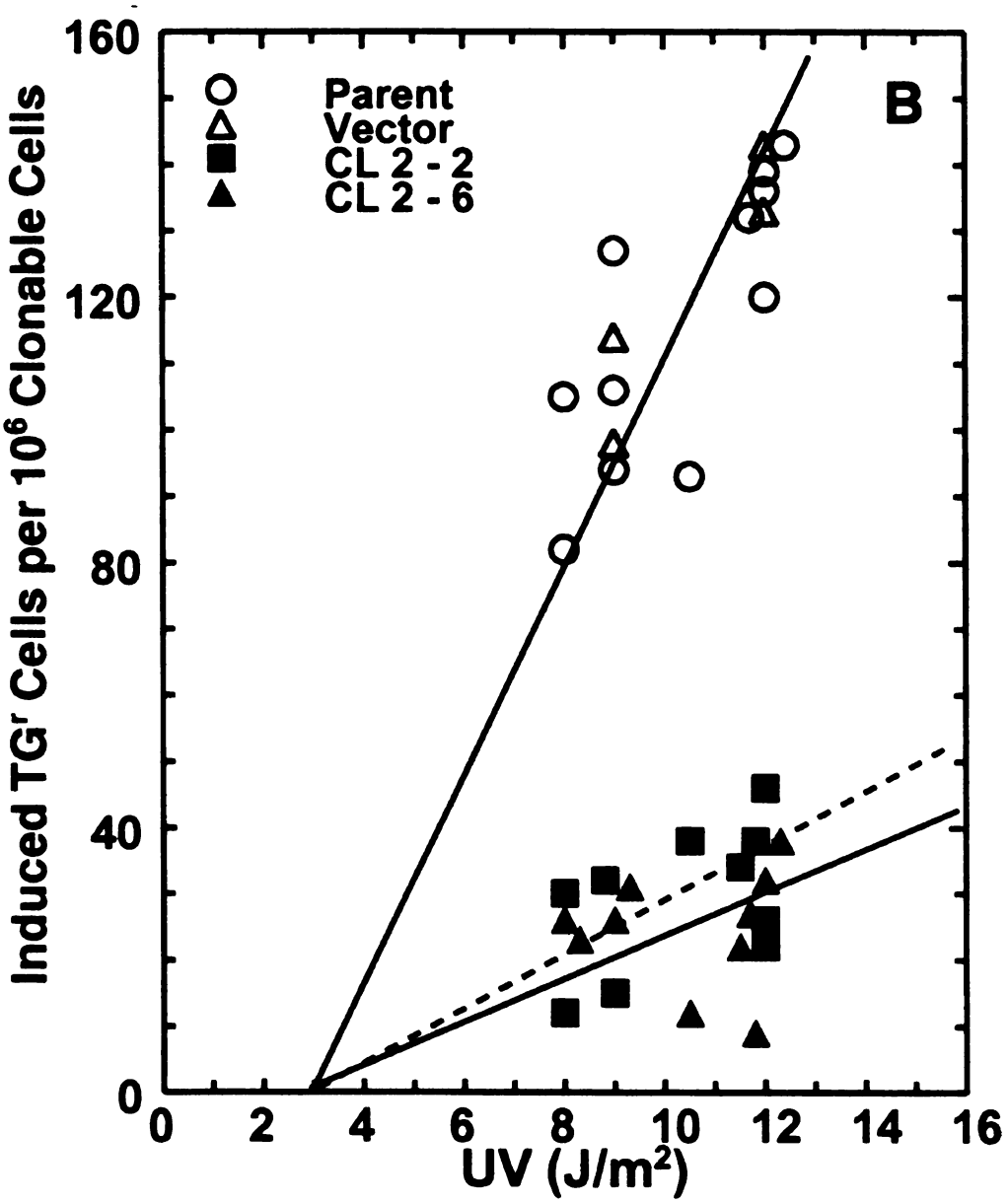

Figure 8

Decreased hRev7 protein expression changes the UV-induced spectrum of mutations

The kinds of UV-induced base substitutions in the HPRT coding region was compared for parental cells, vector control cells, and clones 2-2 and 2-6 as shown in Table 1. C→T mutations were the most frequent mutation in all cell strains as expected for UV-induced mutations. There were no significant changes in mutations found at sites of C. In contrast, there were two significant changes involving sites of T. T→C mutations in the control cells were 21% of the total mutations, whereas, in clones 2-2 and 2-6, T→C mutations decreased significantly to 4%. In addition, the frequency of T→A transversions increased significantly from 10% in the control cells to 30% in clones 2-2 and 2-6. These data indicate that the spectrum of base substitutions induced by UV-radiation is changed in cells with significantly reduced hRev7.

Cells with decreased hRev7 protein expression demonstrate a UV-induced S-phase delay

To assess potential effects of decreased hRev7 on cell cycle progression after UV irradiation, cell strains were synchronized at the G1/S border, released from synchrony, and UV irradiated with 12 J/m^2. Cell cycle progression was analyzed by flow cytometry as shown in Figure 9. The data at 0 hr show that cells from the parental, vector control, clone 2-2, and clone 2-6 synchronized equally (Figure 9A). All four cell strains released from the G1/S block in the absence of UV irradiation proceeded through the cell cycle at an equal rate (data not shown).

Table 1: UV-induced base substitutions in the HPRT protein in human cells that differ in expression of hRev7. UV-induced HPRT mutants were isolated and subjected to reverse transcription. The cDNA was subjected to two rounds of PCR to amplify the HPRT coding region. PCR products were sequenced to determine the exact mutation in the HPRT coding region. Only mutations at adjacent pyrimidines were considered UV-induced. The table shows the number of each type of mutation found in the control group (parent and vector control) and the cells with reduced hRev7 (clone 2-2 and clone 2-6) followed by the percentage of the total mutations that this number represents.

Base substitutions induced by UV in the *HPRT* gene of human cells that differ in expression of hRev7

Base substitutions	Parent and vector control	Clone 2-2 & Clone 2-6
C→T	34 (55 %)	24 (51%)
T→C	13 (21 %)	2 (4%)
T→A	6 (9.6%)	14 (30%)
C→A	5 (8 %)	5 (11%)
C→G	3 (4.8%)	1 (2%)
T→G	1 (1.6%)	1 (2%)
Total	62 (100 %)	47 (100%)

Table 1

Figure 9: Flow cytometry analysis of UV-treated cells. Parental cells (P), vector control cells (VC), clone 2-2 (2-2), and clone 2-6 (2-6) were subjected to different doses of UV radiation, stained with propidium iodide, and analyzed for their DNA content by flow cytometry. **A-D.** Cells were synchronized at the G1/S border, UV irradiated immediately after release from synchrony, and harvested at the designated timepoints post-irradiation: 0 hr (A), 10 hr (B), 16 hr (C), and 24 hr (D). **E-F.** Asynchronously growing populations were subjected to UV radiation and were harvested at 0 hr (E) and 10 hr (F) post-irradiation. These experiments were performed three independent times, and a representative result is shown.

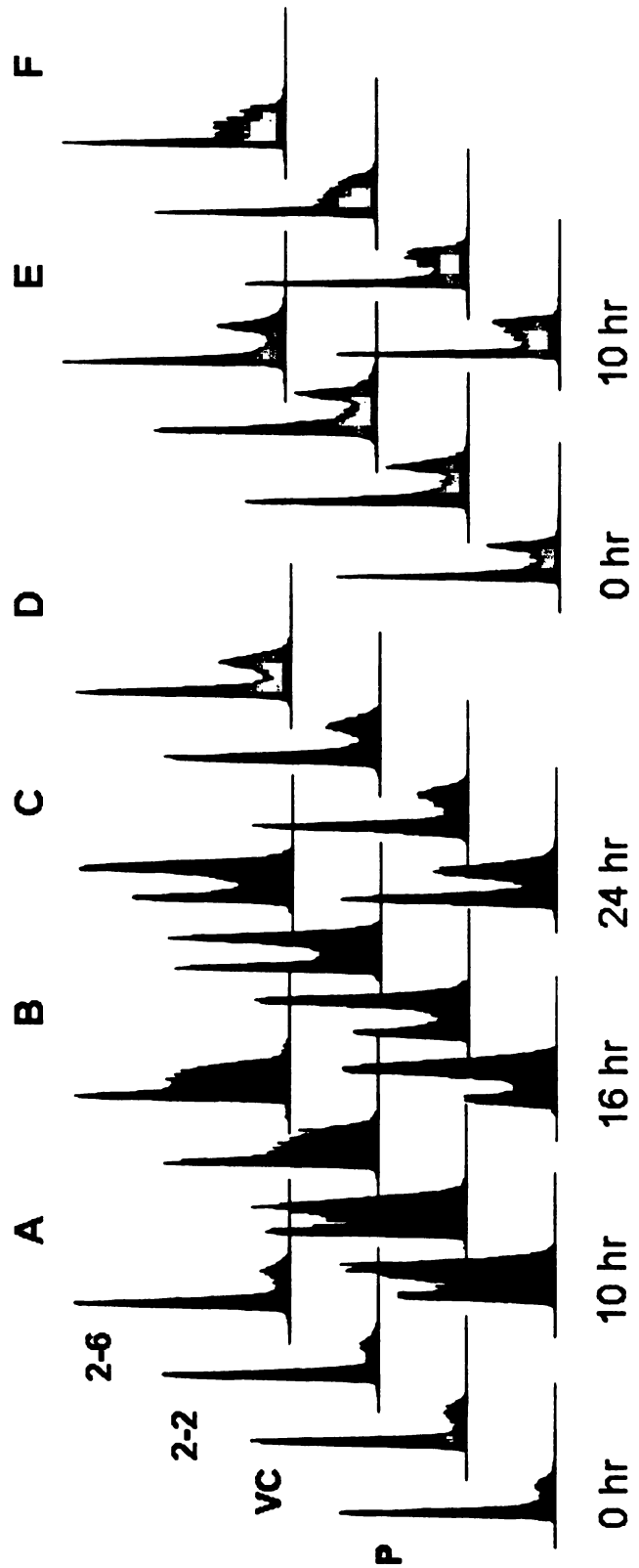

Figure 9

Ten hr after UV irradiation, parental and vector control cells had progressed from G1 into S and G2 (Figure 9B). In contrast to these two control cell strains, cells with decreased hRev7 showed delayed progression through S phase 10 hr after UV irradiation compared to the control cells (Figure 9B). By 16 hr after UV irradiation, all four cell strains had completed S phase, and the cell cycle progression in cells with decreased hRev7 was approaching that seen in the parental and vector control cells (Figure 9C). By 24 hr after UV irradiation, all four cell strains appeared to be cycling similarly (Figure 9D). The delay in cell cycle progression was also seen in asynchronously growing cells with decreased hRev7 10 hr after irradiation (Figure 9F).

Decrease in hRev7 protein expression does not affect the BPDE-induced mutation frequency

The BPDE-induced mutation frequency in cell strainss with reduced hRev7 was compared to that induced in parental cells. For such studies, the frequency of mutations induced in the HPRT gene can only be determined using doses that result in survival levels above 25% (Maher and McCormick, 1996). The results from these experiments showed that the BPDE-induced mutation frequency in clones 2-2 (closed squares) and 2-6 (closed triangles) did not differ greatly from that found in the parental cell strain (open circles). Because the doses of BPDE used are very low, it is difficult to be certain of the exact dose of BPDE in each experiment. Therefore, plotting the frequency of mutations against the percent survival is a more accurate way to determine the BPDE-induced mutation

frequency. The graph of mutation frequency against survival is shown in Figure 11. Unfortunately, even when the data is plotted versus survival, the results are not consistent between experiments. Overall, it appears that cells with reduced hRev7 did not differ greatly from parental cells with respect to their BPDE-induced mutation frequency.

Figure 10: BPDE-induced mutation frequency. The BPDE-induced mutation frequency was tested in clones 2-2 (closed squares) and 2-6 (closed triangles) and compared to the BPDE-induced mutation frequency in the parental cells (open circles). The frequency of thioguanine resistant cells containing a mutation in the HPRT protein induced by BPDE was calculated using the cloning efficiency of cells at the time of selection. Induced frequencies were calculated by subtracting background frequencies observed in non-treated populations

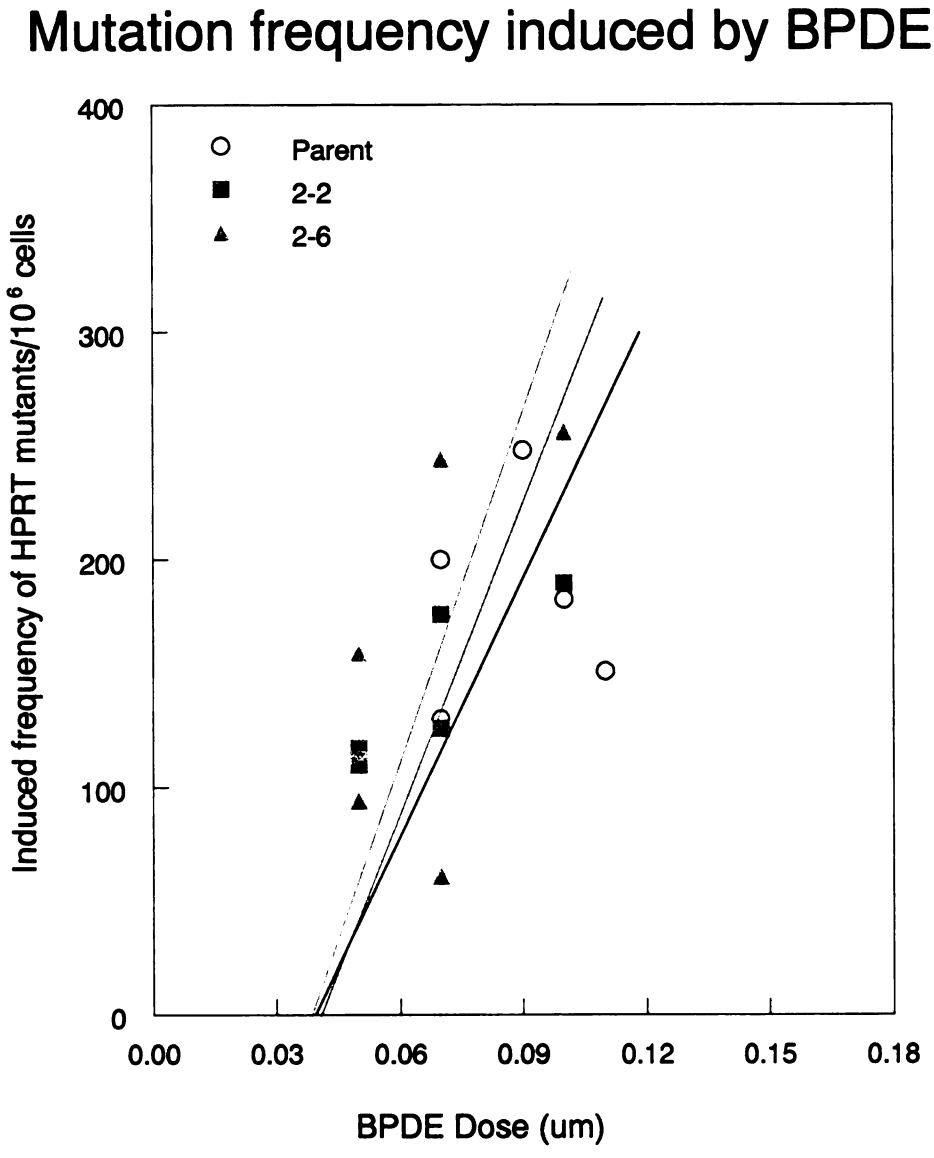
Mutation frequency induced by BPDE
Parent
2-2
2-6
Induced frequency of HPRT mutants/10 6 cells
400
300
200
100
0
0.00
0.03
0.06
0.09
0.12
0.15
0.18
BPDE Dose (um)

Figure 10

Figure 10: BPDE-induced mutation frequency plotted against percent survival. The frequency of BPDE-induced mutations in the HPRT gene is plotted as a function of percent survival to account for variations between experiments. Least squares lines are shown for parental cells (open circles), clone 2-2 (closed squares), and clone 2-6 (closed triangles). Clones with reduced hRev7 are shown as one dotted line.

BPDE-induced mutation frequency as a function of percent survival

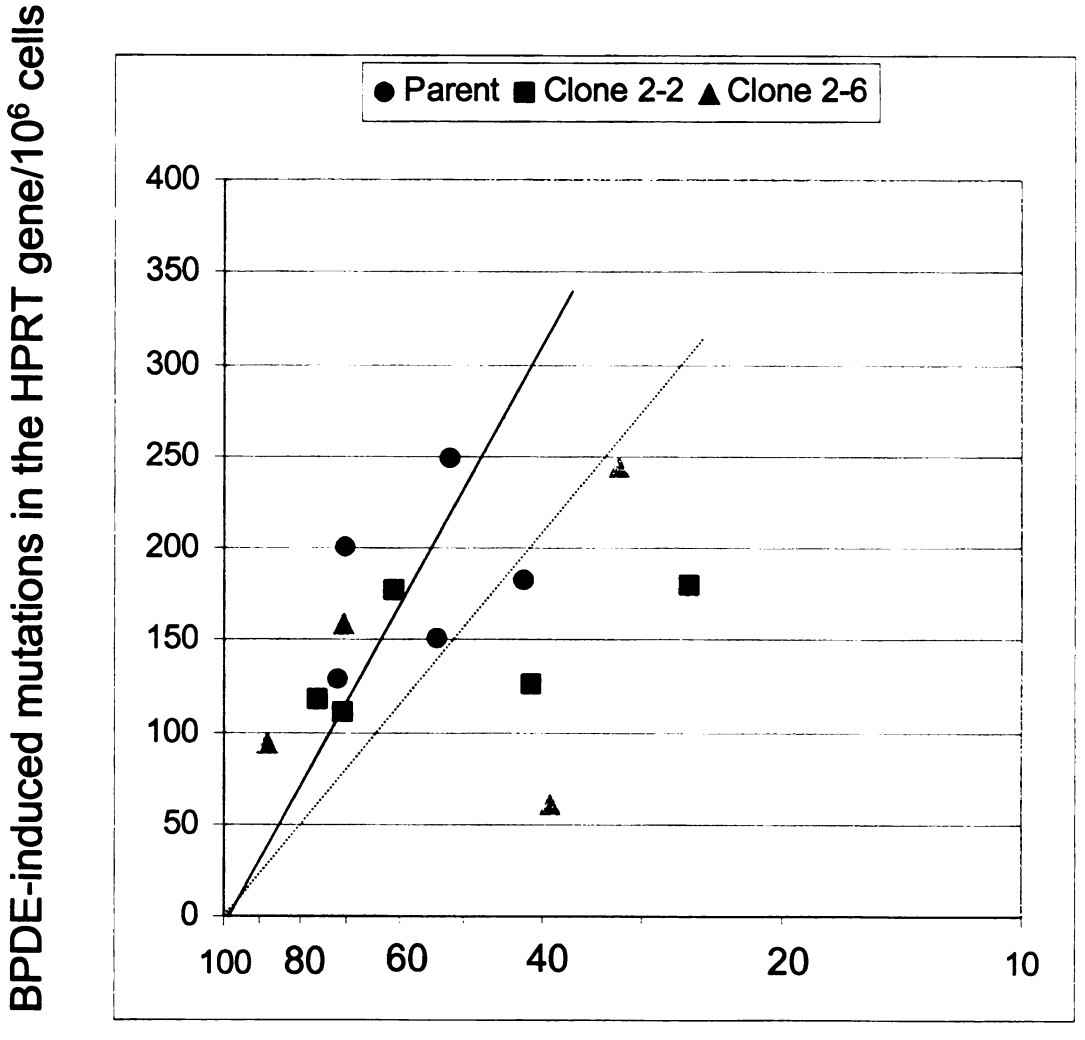

Figure 11

Discussion

A role for hRev7 as the non-catalytic subunit of pol ζ was suggested by its homology with the yeast Rev7 protein as well as its physical interaction with human Rev3 in a yeast-two-hybrid assay (Murakumo et al., 2000). The studies presented here establish a functional role for hRev7 in UV-induced mutagenesis. Cells with decreased hRev7 produced significantly fewer UV-induced mutations indicating that these cells have impaired TLS. This evidence supports the idea that hRev7 is involved in the tolerance of UV-induced DNA damage and, therefore, performs a similar function in human cells as its yeast counterpart. The decrease in UV-induced mutation frequency is similar to what was observed in cells expressing antisense against hRev3 (Gibbs et al., 1998; Li et al., 2002), suggesting that hRev7 and hRev3 function in a similar pathway, presumably as polymerase ζ. We hypothesize that in the cells with reduced hRev7, the pathway that allows the cells to continue DNA replication is error-free and is responsible for the decreased UV-induced mutation frequency. Although this decrease could be the result of an error-free specialized polymerase taking over for the missing hRev7, a more likely explanation is that the human cells with reduced hRev7 make use of an error-free homologous recombination pathway such as damage avoidance (Li et al., 2001).

The cells with significantly reduced hRev7 demonstrated reduced cell survival after exposure to UV irradiation, suggesting that hRev7 plays a protective role for

cells after UV irradiation. The increased UV sensitivity in these cells may result from the UV-induced delay in early S phase progression as shown by flow cytometry analysis. We hypothesize that after UV irradiation, cells with decreased hRev7 have difficulty bypassing UV-induced DNA damage because they can no longer efficiently perform TLS. This inability to bypass damage inhibits normal DNA synthesis leading to the delay in S-phase progression. Some cells with decreased hRev7 may not be able to resume DNA replication, and this would lead to replication fork breakdown, potentially leading to double-strand breaks and the induction of apoptosis. A similar effect was recently reported to occur in mouse cells with decreased pol κ (Bi et al., 2005). Mouse cells with reduced pol κ have a prolonged benzo [a]pyrene diol-epoxide (BPDE)-induced S-phase delay and are much more sensitive to the cytotoxic effects of BPDE than wild type cells.

Because a significant decrease in survival was not seen in human cells expressing antisense against hRev3 or hRev1 (Li et al., 2002; Gibbs et al., 2000), another factor may be contributing to the increased sensitivity of cells with reduced hRev7. Based on their mutational phenotype, it is hypothesized that hRev3, hRev7, and hRev1 function in the same pathway. In contrast to this hypothesis, we speculate that a decrease in the level of hRev7 protein is more cytotoxic to cells than a decrease in these other proteins because two TLS pathways are being inhibited simultaneously. First, reducing hRev7 reduces the interaction with hRev3, and therefore reduces polymerase ζ, leading to

decreased TLS. Second, reducing hRev7 reduces its interaction with hRev1. It is hypothesized that hRev1 has two functions in cells: a deoxycytidyl transferase function and an as-yet uncharacterized function in TLS. The fact that it has been shown that hRev7 does not affect the stability or deoxycytidyl transferase activity of hRev1 supports the idea that the interaction between hRev7 and hRev1 is most likely relevant to TLS. Therefore, a reduction of cellular hRev7 protein may lead to increased sensitivity to UV radiation because the hRev7-hRev3 and the hRev7-hRev1 TLS pathways are both being inhibited. The fact that a complex of hRev3-hRev7-hRev1 has not, to date, been isolated from human cells supports this idea. In addition, attempts to reconstitute the three protein complex *in vitro* have been unsuccessful (Murakumo et al., 2001). What is more, the important region on hRev7 for binding of hRev3 and hRev1 was found to be the same 21-155 amino acid region of hRev7, suggesting that hRev3 and hRev1 actually compete for binding to hRev7 (Murakumo et al., 2001).

Alternatively, the factor contributing to increased sensitivity may be unrelated to TLS. hRev7 was found to inhibit the anaphase promoting complex (APC) by binding to APC activators Cdh1 and Cdc20 in *Xenopus* extracts (Chen and Fang, 2001; Pfleger et al., 2001). These data suggest that hRev7 is involved in a mitotic checkpoint. While the exact function of hRev7 in mitotic cell cycle regulation is unknown, reducing a protein involved in mitosis could affect the sensitivity of cells to DNA damaging agents.

hRev7 is thought to be the noncatalytic subunit of pol ζ. Because of a lack of purified human Rev3, *in vitro* data about the bypass abilities of hRev3 is currently lacking. Previous *in vivo* studies demonstrated a role for hRev3 in tolerance of UV-induced DNA damage, however, whether this was due to an insertion or extension function is still unknown. The *in vivo* spectrum analysis of UV-induced mutations in the HPRT gene demonstrated that cells with decreased hRev7 had significantly decreased T→C transitions (21% to 4%) and increased T→A transversions (10% to 30%) compared to parental and vector control cells. These data indicate one of two possibilities. First, cells with reduced hRev7 no longer create T→C mutations because pol ζ was inserting a G across from a T, and in the absence of hRev7, pol ζ is no longer able to perform this insertion. In the absence of hRev7, another polymerase is inserting a T across from a T, leading to increased T→A mutations. It is interesting to hypothesize about what polymerase may be inserting in the absence of pol ζ. Because the T→A transversion is characteristic of pol ι *in vitro*, pol ι may be a candidate for inserting a T nucleotide across from UV-induced DNA damage, leading to increased T→A mutations in the absence of hRev7. An alternative explanation is that pol ζ efficiently extends a T-G mismatch and in cells with decreased hRev7, pol ζ is not available to perform this extension. In the absence of pol ζ, another polymerase must perform the extension role and this polymerase is not efficient at extending a T-G mismatch. Instead, this polymerase is more efficient at extending a T-T mismatch, leading to increased T→A mutations. This idea is supported by *in vitro* data demonstrating that yeast pol ζ is highly efficient at

extending a T-G mismatch with an extension efficiency of 54%. It is unclear what polymerase can extend in the absence of pol ζ as very little *in vivo* or *in vitro* data exists. Pol κ may be a likely candidate as pol κ was found to extend from a mismatch placed opposite UV-induced DNA damage. Nevertheless, these data demonstrate that in cells with reduced hRev7, the spectrum of UV-induced mutations is changed, strongly suggesting that hRev7 is involved in translesion bypass of UV-induced DNA damage in human cells.

Cells with reduced hRev7 were more sensitive to the cytotoxic effects of BPDE treatment compared to control cells. These data are consistent with the sensitivity observed in these cells after exposure to UV radiation, and demonstrate that hRev7 plays a protective role for cells after exposure to BPDE.

Because the catalytic subunit of pol ζ, hRev3, was found to be involved in error-prone bypass of BPDE-induced DNA damage, we hypothesized that hRev7 would also play a role in this process. However, cells with reduced hRev7 did not demonstrate a reduction in their BPDE-induced mutation frequency, suggesting that hRev7 is not involved in error-prone translesion synthesis of BPDE-induced DNA damage. It is currently unclear why the cells with reduced hRev7 are more sensitive to the cytotoxic effects of BPDE, but reduction of hRev7 protein does not affect the BPDE-induced mutation frequency. It is possible that hRev7 plays a protective role for cells after BPDE treatment, but that hRev7 is not involved in translesion bypass of BPDE-induced DNA damage.

Perhaps the BPDE-mutations created by pol ζ are reduced in these cells, but another error-prone specialized DNA polymerase is induced to perform translesion synthesis of BPDE-induced DNA damage, ultimately leading to a similar number of mutations as in the control cells. Alternatively, the different phenotypes observed in cells with reduced hRev7 after exposure to UV or BPDE may reflect different roles for hRev7 during TLS. For example, hRev7 may bind to hRev3 in order to tolerate UV-induced DNA damage, whereas, hRev7 may bind to hRev1 in order to tolerate BPDE-induced DNA damage. Whether the different phenotypes suggests distinct roles for hRev7 remains unknown.

Cells with reduced hRev7 demonstrated a similar sensitivity to ionizing radiation as control cells, indicating that hRev7 does not play a protective role for cells after exposure to ionizing radiation. These data suggest that hRev7 is not involved in the tolerance of ionizing radiation-induced DNA damage in human cells. Interestingly, chicken cells engineered to lack hRev7 were more sensitive to the cytotoxic effects of ionizing radiation, demonstrating differences in the function of the human Rev7 protein compared to that of chicken Rev7 (Okada et al., 2005).

Several interesting areas still need to be addressed for hRev7. For example, the exact role of hRev7 in TLS remains to be determined. Does hRev7 enhance the polymerase activity, stability, or kinetic parameters of hRev3? Recently, Masuda and colleagues demonstrated that hRev7 did not affect the deoxycytidyl

transferase activity or stability of hRev1 *in vitro* (Masuda et al., 2003). Whether hRev7 affects the TLS function of hRev1 or hRev3 has not been established. Binding studies showing that hRev7 homodimerizes with itself as well as binding to hRev1 and hRev3 raise several questions about the function of hRev7 during TLS. Does hRev7 link hRev1 and hRev3 in human cells bringing them into proximity for functional cooperation, or do hRev3 and hRev1 compete for binding to hRev7, suggesting hRev7 has two distinct roles during TLS, one with hRev3 and one with hRev1? Another area of future research is the potential link between the cell cycle and TLS. While the exact role of hRev7 in both the cell cycle and TLS remains to be determined, it is curious that hRev7 has a functional role in both.

hRev7 has also been found to interact with several proteins not involved in TLS or cell cycle regulation. hRev7 has been found to interact with PRCC, a frequently rearranged protein in renal cell carcinoma (Weterman et al., 2001). hRev7 has also been found to interact with MDC9, a metalloproteinase disintegrin, Adenovirus death protein (ADP), and Trichosanthin, a ribosome-inactivating protein (Chan et al, 2001; Nelson et all, 1999; Ying and Wold, 2003). The importance of these interactions with hRev7 remains to be determined. While there are many questions remaining about hRev7, it is now clear that hRev7 has an important role in UV-induced mutagenesis in human cells, presumably as a subunit of human polymerase ζ.

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Chapter III

Experiments with a clone that overexpresses hRev7

Introduction:

Polymerase ζ from *Saccharomyces cerevisiae* has been well characterized both *in vitro* and *in vivo*. Yeast pol ζ is composed of Rev3, the catalytic subunit, and Rev7, a noncatalytic subunit. Rev3 contains DNA polymerase motifs characteristic of B family DNA polymerases and *in vitro* primer extension experiments using purified Rev3 first revealed the inherent polymerase activity of Rev3 (Nelson et al., 1996). Furthermore, the polymerase activity of Rev3 was significantly stimulated by the addition of Rev7 to the reaction, suggesting that Rev7 affects the stability or processivity of Rev3. The exact role of Rev7 as a subunit of polymerase ζ has not been further characterized in yeast.

hRev7 was discovered by its ability to bind to hRev3 in a yeast-two-hybrid assay, and sequence analysis demonstrated that hRev7 shares a high amount of homology with the yeast Rev7 protein (Murakumo et al., 2000). These characteristics suggest a role for hRev7 as a subunit of polymerase ζ in human cells. However, there have been no reported studies examining the role of hRev7 in mutagenesis other than this dissertation. An important characteristic to define hRev7 as the human homolog of the yeast Rev7 protein is whether hRev7

stimulates the polymerase activity of hRev3, like yeast Rev7 stimulates yeast Rev3. Because human Rev3 has not been isolated from cells or expressed as a cDNA, *in vitro* experiments have not been able to provide the answer to this question. By overexpressing hRev7 in a normal cell strain, it may be possible determine if hRev7 can increase the polymerase activity of hRev3. It is my hypothesis that overexpressing hRev7 will stimulate the polymerase activity of hRev3, and the induced mutation frequency will increase in cells with high levels of hRev7 compared to control cells with normal levels of hRev7 protein.

Materials and Methods

Preparation of the hRev7 expression vector

The Carcinogenesis Laboratory received pcDNA3.1-hRev7 from Dr. Yoshiki Murakumo, which contains the full length hRev7 cDNA expressed from the CMV promoter. The selectable marker on this vector is neomycin, which cannot be used for selection in the 9N.58 cell strain. The following primers were designed to amplify the hRev7 cDNA from pcDNA3.1-hRev7: 5'-CGGAATTCGCCGCCAT GACCACGCTCACA-3' and 5'-CGCGCTCGAGCCCTCAGCTGCCTTTATGAGC-3'. PCR was performed using Pfu polymerase (Stratagene) to ensure high fidelity. The PCR product was purified (Qiagen) and ligated into the *Eco*RI and *Xho*I sites in the multiple cloning region of the pcDNA6A-Blasticidin vector (Invitrogen) using DNA ligase (New England Biolabs). The products of the ligation reactions were transformed into *E.coli* competent cells (Invitrogen), and the bacterial transformants were plated on selective LB agar plates containing

100 µg/ml ampicillin (Roche). Plates containing bacteria were incubated at 37°C overnight, and bacterial colonies were isolated, inoculated into LB broth containing ampicillin, and shaken overnight at 37°C. Plasmid DNA was extracted from the bacterial cultures following the manufacturer's protocol (Qiagen), and the hRev7 cDNA was sequenced at the Michigan State University macromolecular facility to confirm that it did not contain any mutations.

Stable Transfection

MSU1.2.9N.58 cells were plated at a density of 1 x 10^5 cells per 100-mm-diameter dish and allowed to attach for approximately 18 hr. The cells were rinsed twice with PBS, and the pcDNA6A-hRev7 DNA/Lipofectamine solution was added to each dish of cells for approximately 7 hr. Eagle's minimum essential medium containing 20% supplemented calf serum was added to each dish 7 hr after transfection, and cells were re-fed 24 hr post-transfection with Eagle's medium containing 10% supplemented calf serum (HyClone), 100 units/ml penicillin, 100 µg/ml streptomycin, 1 µg/ml hydrocortisone, and 1 µg/ml tetracycline. Cells were re-fed 48 hr post-transfection with complete medium containing 17 µg/ml blasticidin to select for transfected cells. Transfected cells were grown for 14 additional days in blasticidin selection until they formed colonies, which were isolated and expanded in medium containing blasticidin.

Western blot analysis

Subconfluent monolayers of cells were washed with ice-cold PBS, scraped from 150-mm-diameter plates in 1 ml of lysis buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF), and incubated on ice for 15 min. Sixty-two μl of 10% NP-40 was added to each tube, and the lysates were vortexed for 10 sec, and centrifuged at 4°C, for 30 sec at 10,000 RPM. The supernatent was removed, and the nuclear pellet was washed one time in 1 ml of buffer A containing 10% NP-40. Nuclear proteins were extracted with disruption in 40 μl of lysis buffer C (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF) and incubated on ice for 15 min, with vortexing every 5 min. Nuclear extracts were centrifuged at 4°C, 16000 RPM, for 5 min. The supernatant contained nuclear proteins and was saved. Protein was quantitated using the Bradford method (Pierce). Nuclear proteins (50 μg) for each sample were subjected to 14% SDS-polyacrylamide gel electrophoresis, transferred to a PDVF immobilon membrane (Millipore), and probed with a 1:600 dilution of a custom rabbit polyclonal hRev7 antibody raised against the C-terminal 19 amino acids of the human Rev7 protein (Bethyl). The membrane was then probed with a 1:7500 dilution of an anti-rabbit secondary antibody (Santa Cruz) and visualized using SuperSignal chemiluminescent detection reagent (Pierce). Equal protein loading was confirmed by probing with a 1:10,000 dilution of a rabbit Ku86 antibody and a 1:10,000 dilution of an anti-rabbit secondary antibody (Santa Cruz).

UV survival assay

The cytotoxic effect of $UV_{(254nm)}$ radiation was determined using a colony-forming assay. Parental cells and cells with overexpressed hRev7 (Clone A) were each plated into a series of dishes at cloning densities (100-600 cells per 100-mm-diameter dish) and given 12 hr to attach. Cells were rinsed twice with PBS, UV-irradiated using the designated doses, and immediately fed with fresh complete medium. Cells were re-fed 24 hr after irradiation, and cultured for 14 days. The resulting colonies were stained with crystal violet and percent survival was calculated using the average number of colonies formed in UV-treated dishes as a percentage of the average number of colonies formed in non-treated control dishes for each cell strain.

HPRT mutation frequency assay

The mutagenic effect of UV radiation was determined from the frequency of HPRT-defective, 6-thioguanine resistant mutants from parental cells and their derivative cells that overexpress hRev7 (Clone A). Parental cells and Clone A were plated at densities of 0.5-1.5 x 10^6 cells per 150-mm-diameter dish and allowed to attach for 12 hr. Cells were rinsed twice with PBS, irradiated at the designated doses, and immediately fed with fresh complete medium. Cells were re-fed 24 hr after irradiation and allowed to replicate for four days. On day 4, cells were detached with trypsin, pooled, and replated at a lower density. Cells were cultured for four additional days, at which time they were detached with trypsin, pooled, and selected at a density of 500 cells/cm^2 for resistance to 6-

thioguanine (40 μM). At this time a portion of cells from each population was plated in non-selective medium at a density of 100 cells per 100-mm-diameter dish to assay the colony-forming ability of the cells at the time of selection. After 14 days of selection, cells were stained with crystal violet, and the frequency of HPRT-defective, 6-thioguanine resistant colonies was calculated using the cloning efficiencies at the time of replating for each cell strain. Induced mutation frequencies were determined by subtracting the background mutation frequencies for each cell strain.

Results

Nuclear protein extracts from several clones transfected with pcDNA6A-hRev7 were tested to determine the hRev7 protein level in each clone compared to that in the parental cells. A representative Western blot analysis is shown in Figure 1A. The hRev7 protein migrates at the expected size of 24 kDa. Lanes 2-6 contain nuclear proteins extracted from independent blasticidin-resistant, stably-transfected clones. Compared to the parental cell strain (P, lane1), Clone A (A, lane 2) expresses significantly higher levels of hRev7 protein. Other blasticidin-resistant clones (lanes 3-6) did not exhibit significant overexpression of hRev7. A second Western analysis, shown in Figure 1B, confirms that Clone A (A, lane 2) has significantly overexpressed hRev7 compared to parental cells (P, lane 1).

Because hRev7 is hypothesized to be involved in cell cycle regulation, growth curves were performed on cells with overexpressed hRev7 protein expression

and compared to parental cells to determine if overexpressing hRev7 has an effect on the normal growth rate. Results from the growth curve analysis demonstrated that cells with overexpressed hRev7 (inverted triangles) grew at approximately the same rate as parental cells (open circles) (Figure 2). These results indicated that overexpressing hRev7 does not affect the normal growth rate compared to cells with endogenous levels of hRev7 protein.

To determine if overexpression of hRev7 affects the sensitivity of human cells to the cytotoxic effect of UV radiation, the colony forming ability of Clone A was compared to that of the parental cell strain. Results from these experiments (Figure 3) demonstrated that cells from Clone A (inverted triangles) did not differ from the parental cell strain (open circles) in their survival after exposure to UV radiation.

To determine if overexpression of hRev7 stimulates the polymerase activity of hRev3, the frequency of HPRT-defective, 6-TG resistant mutants induced by UV radiation was measured in Clone A and compared to the parental cell strain. As shown in Figure 4, the frequency of UV-induced mutations in Clone A (inverted triangles) was essentially the same as that for parental cells (open circles). These data strongly suggest that overexpressing hRev7 does not affect the UV-induced mutation frequency in human fibroblast cells.

In addition to testing the UV-induced sensitivity of cells overexpressing hRev7, Clone A was also tested for its sensitivity to ionizing radiation. A survival curve showing the dose-dependent sensitivity of Clone A (inverted triangles) and parental cell strain 9N.58 (open circles) to the cytotoxic effects of ionizing radiation is shown in Figure 5. The results obtained from these experiments demonstrated that the survival of the cell strains did not differ from each other. Overexpression of hRev7, therefore, did not affect ionizing radiation-induced cell killing.

Figure 1: Western blot analyses of a clone that overexpresses hRev7. Nuclear protein extracts from parental cells and stable clones transfected with a vector expressing hRev7 were subjected to Western blot analysis. **A.** Lane 1, the hRev7 protein in parental cells (P). Lane 2, the hRev7 protein in stably-transfected Clone A (A). Lanes 3-6, the hRev7 protein in other stably-transfected clones that do not exhibit significant overexpression of hRev7. **B.** Western blot analysis to confirm that Clone A (A, lane 2) stably overexpresses hRev7 compared to parental cells (P, lane 1).

Western blot analyses of a stable clone that overexpresses hRev7

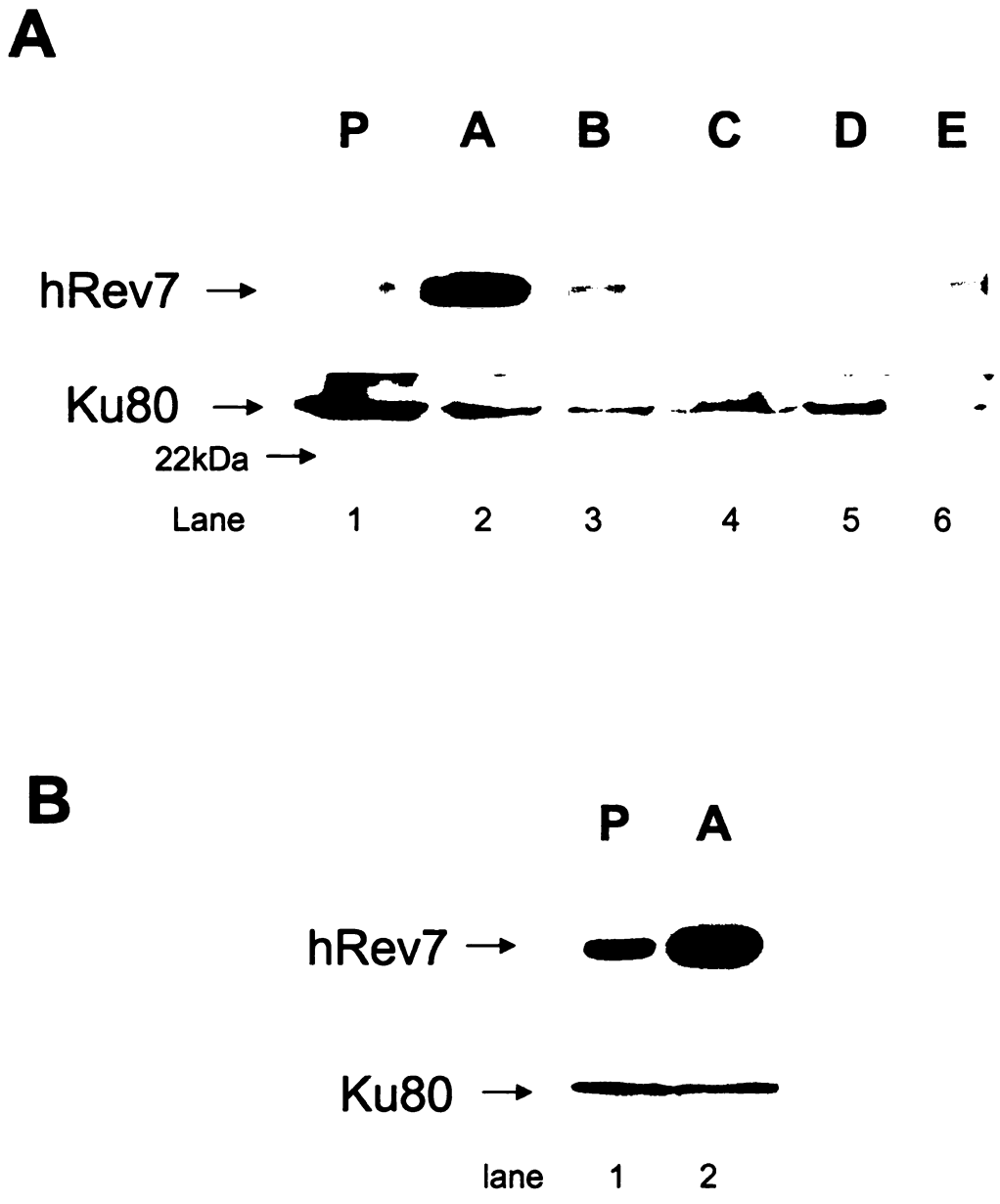

Figure 1

Figure 2: Growth curve analysis. Cells with overexpressed hRev7, Clone A, (inverted triangles) were compared with the parental cells (open circles) for their growth rate under normal conditions. At each time point, the average number of cells from three 60-mm-diameter dishes for each cell strain is shown.

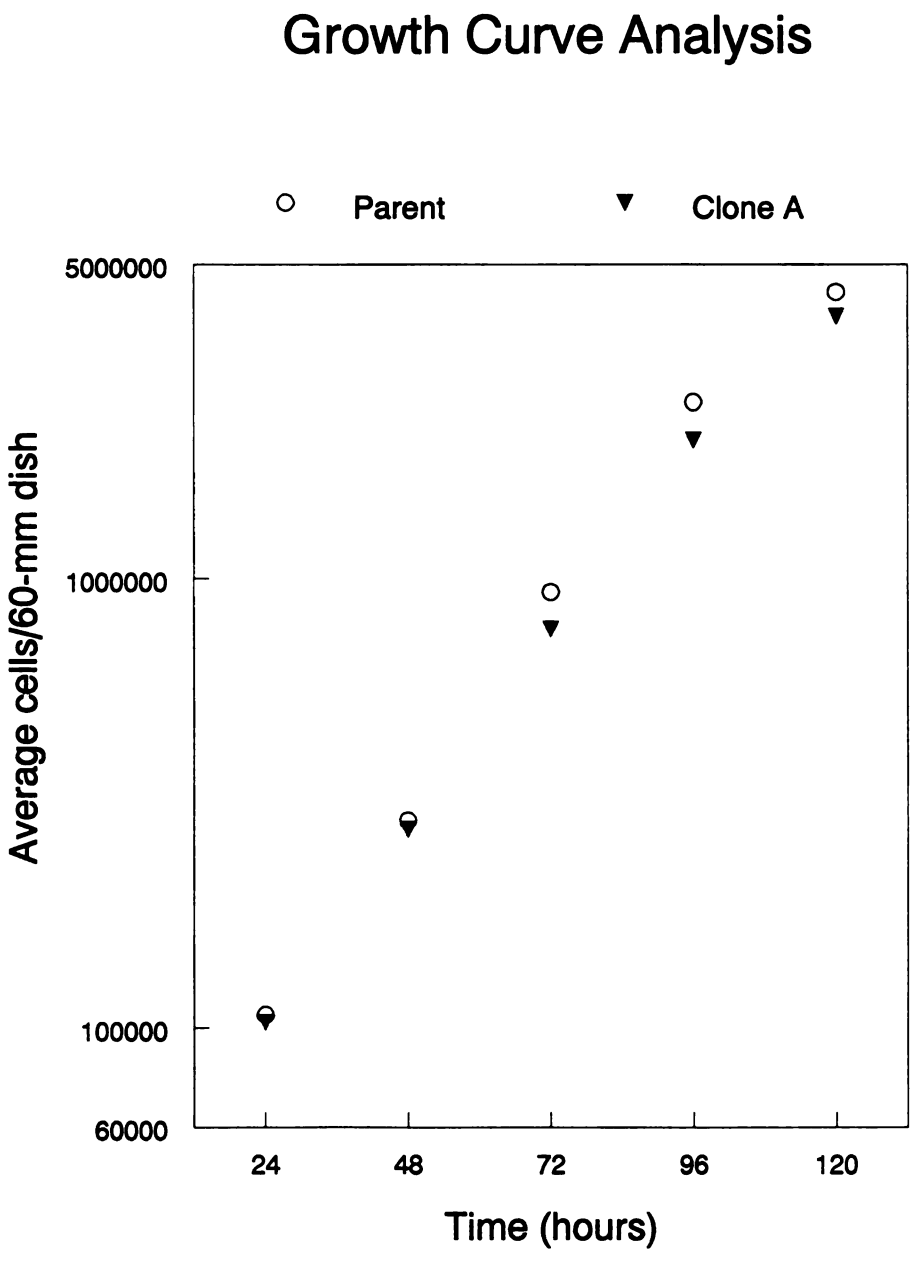
Growth Curve Analysis
Parent
Clone A
5000000
1000000
100000
60000
Average cells/60-mm dish
24
48
72
96
120
Time (hours)

Figure 2

Figure 3: UV-induced cytotoxicity in 9N.58 and Clone A. Parental cells (open circles) and cells from Clone A (inverted triangles) were subjected to UV radiation and analyzed for their sensitivity using a colony-forming assay. Survival at each dose is represented by the average number of colonies in UV-treated dishes as a percentage of the average number of colonies in non-treated control dishes. Lines represent least squares lines.

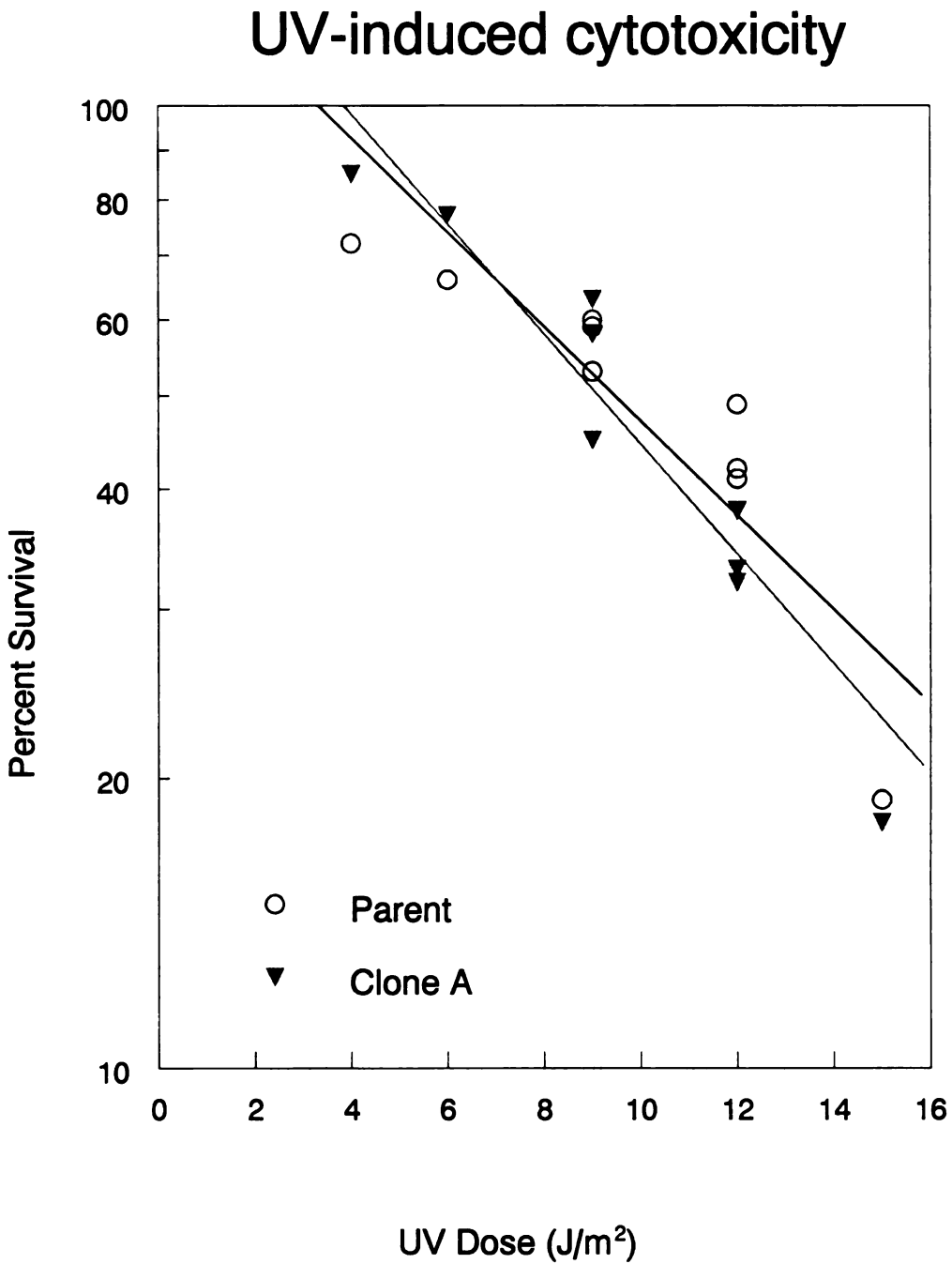
UV-induced cytotoxicity
Percent Survival
100
80
60
40
20
10
0
2
4
6
8
10
12
14
16
Parent
Clone A
UV Dose (J/m²)

Figure 3

Figure 4: Comparison of the UV-induced mutation frequency in parental cell strain 9N.58 and Clone A. The UV-induced HPRT mutation frequency indicating TLS in parental cells (open circles) was compared to that induced in Clone A (inverted triangles). The frequency of thioguanine resistant cells containing a mutation in the HPRT protein induced by UV was calculated using the cloning efficiency of cells at the time of selection. Induced frequencies were calculated by subtracting background frequencies observed in non-treated populations.

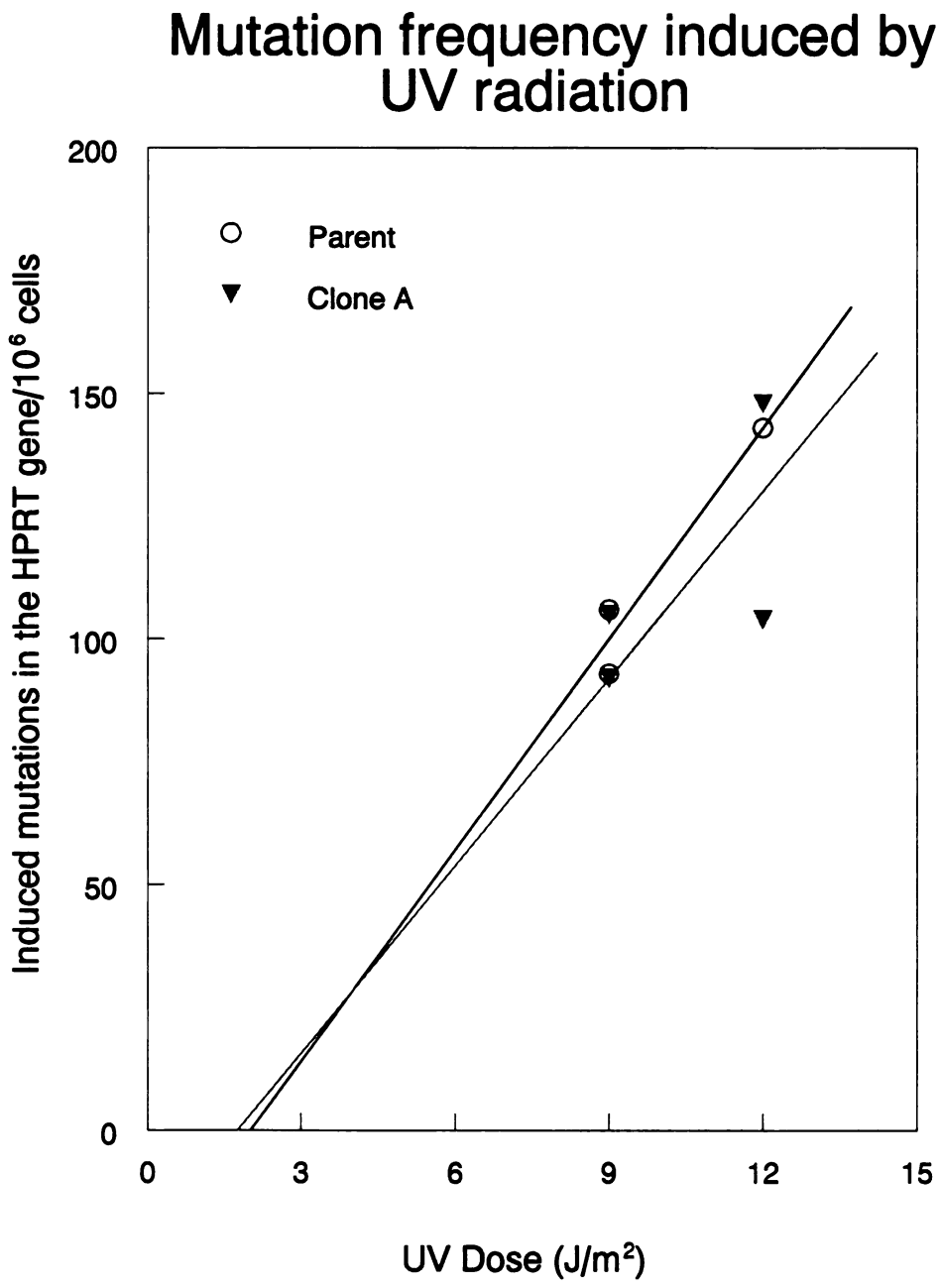
Mutation frequency induced by UV radiation
Induced mutations in the HPRT gene/10^6 cells
200
150
100
50
0
Parent
Clone A
0
3
6
9
12
15
UV Dose (J/m^2)

Figure 4

Figure 5: Comparison of the ionizing radiation-induced cytotoxicity in 9N.58 and Clone A. Cells with normal or overexpressed hRev7 were subjected to ionizing radiation to determine their sensitivity. Survival curves are shown from two independent experiments for the parental cells and Clone A. Least squares lines are shown for parental cells (open circles) and Clone A (inverted triangles). Survival at each dose is represented by the average number of colonies in ionizing radiation-treated dishes as a percentage of the average number of colonies in non-treated control dishes.

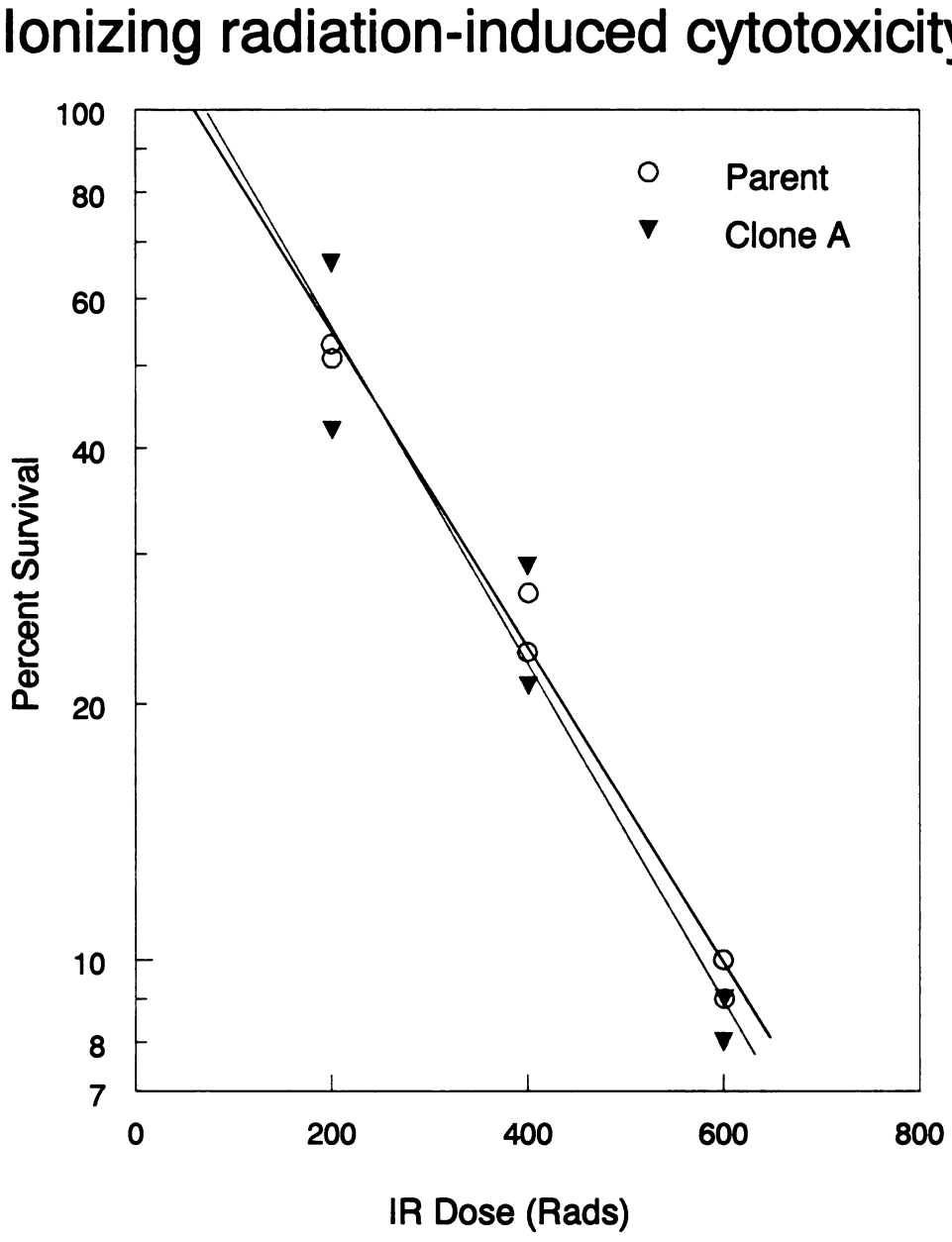
Ionizing radiation-induced cytotoxicity
Parent
Clone A
Percent Survival
100
80
60
40
20
10
8
7
0
200
400
600
800
IR Dose (Rads)

Figure 5

Discussion:

Cells that overexpress hRev7 displayed a sensitivity to the cytotoxic effect of UV similar to that seen in the parental cell strain. In addition, cells overexpressing hRev7 displayed a UV-induced mutation frequency similar to parental cells. These data suggest that overexpressing hRev7, at least at the level observed in Clone A, does not affect the polymerase activity of hRev3 in human cells.

Several reasons could account for the fact that there was not a significant difference in the survival or mutation frequency after exposure to UV radiation. One reason is that there are not enough data contributing to all of the experiments involving Clone A. If these experiments were to be repeated, it would allow one to gather enough data to draw a statistically significant conclusion. Another reason is that a clone expressing hRev7 at a level even higher than seen in Clone A would be required to stimulate the polymerase activity of hRev3. Western blot analysis demonstrated that the hRev7 protein level in Clone A was significantly overexpressed compared to the parental cell strain. However, it is not known how much overexpression of hRev7 is needed to see an effect.

It is possible that the relatively high frequency of UV-induced mutations characteristic of the parental cell strain, 9N.58, prevents the effects of hRev7 overexpression from being detected. The factors contributing to this high frequency of induced mutations are not known, but it may be very difficult to

further increase the mutation frequency in this cell strain, which was the hypothesis of this study. Perhaps performing similar experiments with overexpressed hRev7 in a cell strain with a lower induced mutation frequency is a better strategy for investigation of this hypothesis.

A more likely explanation for the lack of a phenotype in Clone A is that hRev3 is the limiting factor for polymerase ζ activity, not hRev7. The very low cellular levels of hRev3 support this idea. If hRev3 is the limiting factor for pol ζ activity, overexpressing hRev7 may not have an effect on pol ζ polymerase activity, because the level of hRev3 has remained the same. Overexpressing both hRev3 and hRev7 simultaneously may stimulate pol ζ activity, but this experiment must wait until a human Rev3 is available as a cDNA.

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Nelson JR, Lawrence CW, Hinkle DC. (1996) Thymine-thymine dimer bypass by yeast DNA polymerase zeta. Science, 272(5268): 1646-1649.

Pfleger CM, Salic A, Lee E, Kirschner MW. (2001) Inhibition of Cdh1-APC by the MAD2-related protein MAD2L2: a novel mechanism for regulating Cdh1. Genes Development, 15(14): 1759-1764.

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Chapter IV

Proposed future experiments involving hRev7

There are several exciting areas of future research for hRev7. Because hRev3 is the catalytic subunit of pol ζ, hRev7 has not been an actively pursued area of investigation. There are very few publications involving any aspect of human Rev7, and no publications involving the role of hRev7 in mutagenesis. Because of this, the potential areas of investigation for hRev7 are unlimited. In addition, because we have an excellent antibody against hRev7 and the 24kDa protein is easily expressed as a cDNA from a vector, *in vitro* and *in vivo* experiments using hRev7 as a subunit of pol ζ are much more plausible than using hRev3. Appendix B consists of proposed future experiments involving hRev7 that are important to gain a better understanding of this protein during TLS, as well as generate a bigger picture of hRev7 function in human cells.

Is hRev7 involved in the tolerance of BPDE-induced DNA damage in human cells?

Hypothesis: hRev7 is involved in the tolerance of BPDE-induced DNA damage in human cells like hRev3 is.

Aim: To use cells with decreased and overexpressed hRev7 to determine the survival, mutation frequency, mutation spectrum, and cell cycle progression after BPDE treatment.

Studies in the Carcinogenesis Laboratory determined that hRev3 and hRev1 were important for the tolerance of BPDE-induced DNA damage (Li et al., 2002a;

Wang unpublished data). Because hRev7 interacts with both hRev3 and hRev1, it would be interesting to determine if hRev7 is also involved in tolerating BPDE-induced DNA damage like hRev3 and hRev1. Previous studies using cell strains with reduced hRev7 (clones 2-2 and 2-6) that were treated with BPDE are discussed in Appendix A. Initial experiments testing the sensitivity of clones 2-2 and 2-6 demonstrated that these cells were more sensitive to BPDE than parental cells. Results from these experiments are similar to the sensitivity seen in these cells after UV-irradiation, suggesting that the sensitivity to BPDE is real. Taking this information into consideration, clones 2-2 and 2-6 could be tested for their BPDE-induced mutation frequency. Previous experiments demonstrated that the clones with reduced hRev7 had a similar BPDE-induced mutation frequency as parental cells, however, because of the very low survival of these cells at the time of BPDE treatment, the mutation frequency in these experiments may be skewed, and may not accurately reflect the BPDE-induced mutation frequency.

These experiments should be repeated using doses of BPDE that result in a similar survival in the parental cells and clones 2-2 and 2-6. Once the accurate BPDE-induced mutation frequency in clones 2-2 and 2-6 is obtained, the spectrum of mutations could be determined to see if cells with reduced hRev7 exhibit a change in the types of mutations induced by BPDE compared to the parental cells. In addition, a significant delay in S-phase progression was

observed in clones 2-2 and 2-6 after UV-irradiation. It could be determined if treatment with BPDE also induces such a delay in cells with reduced hRev7.

Finally, the cell strain that overexpresses hRev7 could also be tested to determine if overexpressing hRev7 has an effect on tolerating BPDE-induced DNA damage, however, the caveats that surround the overexpression experiments are still valid. Perhaps a new cell strain that overexpresses hRev7 at a level even higher than seen in clone A would be required for these experiments.

Previous studies (discussed in Appendix A) demonstrate a clear increase in BPDE-induced sensitivity in cells with reduced hRev7, providing evidence that hRev7 is involved in some aspect of tolerance after BPDE treatment. Future studies should focus on determining the accurate BPDE-induced mutation frequency in cells with reduced and overexpressed hRev7 to determine of hRev7 is involved in bypass of BPDE-induced DNA damage in human cells.

What other types of DNA damage is hRev7 involved in tolerating via TLS?

Hypothesis: hRev7 is involved in tolerating many different kinds of DNA damage in human cells.

Aim: To use cells with reduced or overexpressed hRev7 to determine the survival, mutation frequency, mutation spectrum, and cell cycle progression after treatment with different DNA damaging agents.

The focus of this dissertation research was to determine if hRev7 was involved in a mutagenic process after DNA damage. UV radiation was used as the damaging agent because yeast Rev7 was found to play a role in tolerating UV-induced DNA damage (Torpey et al., 1994). In addition, hRev3, the catalytic subunit of polymerase zeta was found to be involved in tolerating UV-induced DNA damage (Li et al., 2002a). Based on this information, I hypothesize that hRev7 is also involved in bypassing other types of DNA damage. In the process of my research, cell strains with reduced hRev7 were established using siRNA and a stable cell strain overexpressing hRev7 was also established. Using these cell strains, the role of hRev7 in the tolerance of different types of DNA damage could be easily tested. Several different carcinogens could be used to determine the survival, mutation frequency, and mutation spectrum of cells with reduced or overexpressed hRev7. Because cells with reduced hRev7 demonstrated a UV-induced S-phase delay, other DNA damaging agents could be tested to see if they also induce an S-phase delay. The following agents could be used for these experiments: ionizing radiation, N-ethyl-N-nitrosourea (ENU), N-2-acetyl-2-aminofluorene (AAF), cisplatin, O^6-methylguanine, 8-oxoguanine, and alkylating agents. While these experiments would be costly and time consuming, they would provide a plethora of information and are the most accurate way to determine the *in vivo* role of hRev7 in response to different DNA damaging agents.

Does tranfecting a vector expressing a full length hRev7 cDNA into clones 2-2 and 2-6 with reduced hRev7 complement the UV-induced sensitivity and decreased mutation frequency observed in these clones?

Hypothesis: Expressing hRev7 in clones with reduced hRev7 will complement the sensitivity and mutation frequency seen in these cells after UV treatment.

Aim: To use clones 2-2 and 2-6 with more normal levels of hRev7 protein to test their survival, mutation frequency, mutation spectrum, and cell cycle progression after UV treatment.

A confirmatory experiment that is fairly straightforward to perform would be to express hRev7 in cells that have reduced hRev7. An expression vector that has been transiently transfected in 293-HEK cells and expresses full length hRev7, migrates at the expected size of 24kDa, exactly where endogenous hRev7 migrates. This vector has blasticidin resistance and can be used to select for stable transfectants expressing hRev7 in the clones derived from 9N.58. This vector could be stably transfected into late-passage 2-2 and 2-6 cells with reduced hRev7. Blasticidin resistant clones could be isolated, expanded, and tested to determine if their hRev7 protein level has increased to normal levels.

A potential caveat to this experiment is that these two cell strains still express the siRNA targeting hRev7. Presumably, ectopically expressed hRev7 would also be targeted by the siRNA, leading to no stable clones with increased hRev7 protein expression. There are two ways to potentially overcome this caveat. First, we could stop selecting for the siRNA vector and only select for the hRev7 expression vector. Second, there is some information suggesting that the promoter driving siRNA expression becomes silenced in human cells. It could be

possible to stop selecting for the siRNA vector and continually passage the clones with reduced hRev7 until the promoter becomes fully silenced. Over time, these cells may have an increase in hRev7 protein as the siRNA vector becomes silenced.

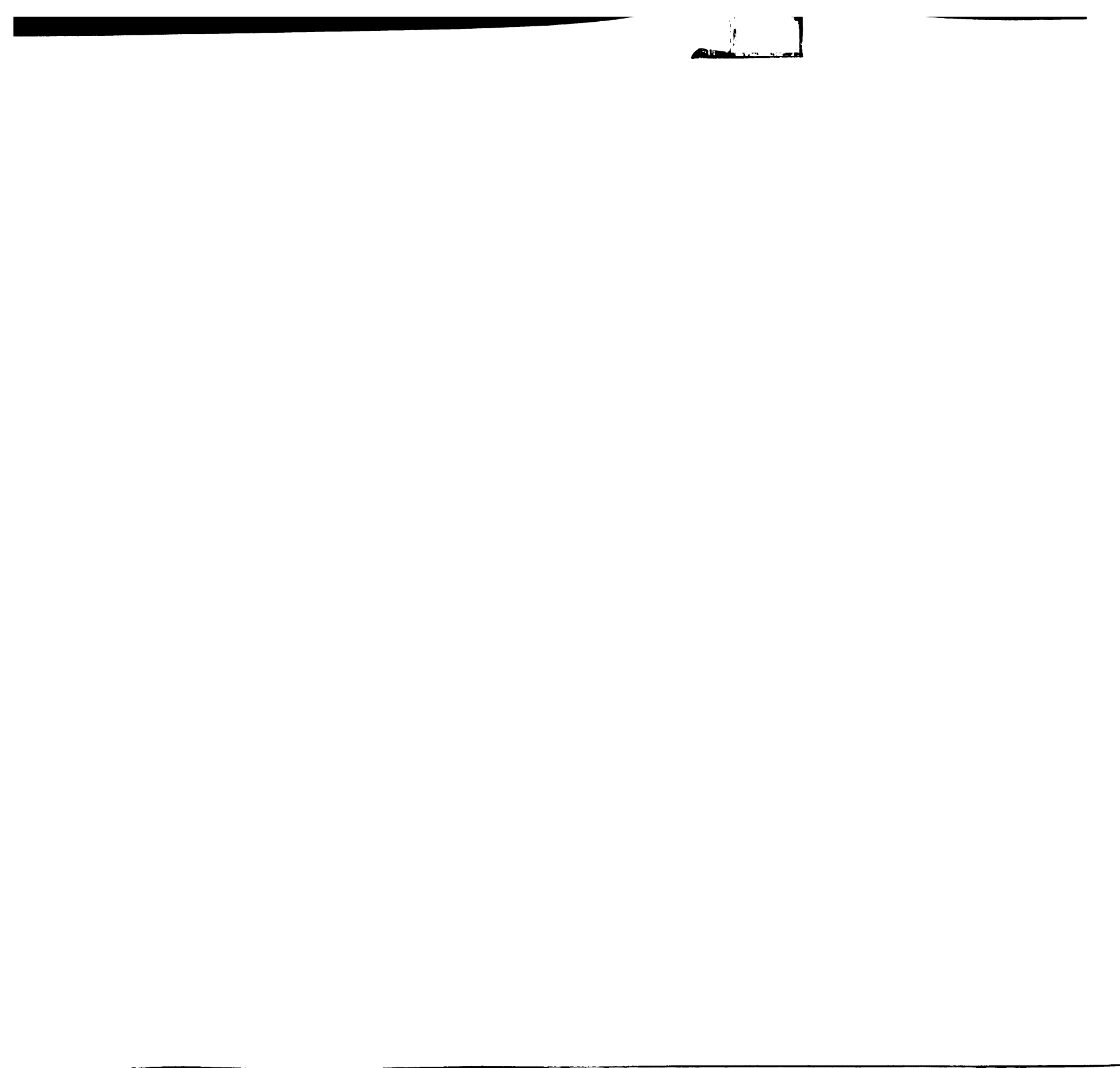
If clones with a more normal level of hRev7 protein were isolated, several experiments could be performed. First, a UV-induced survival curve could be performed to determine if increasing the hRev7 protein back to normal levels complements the increased sensitivity observed when hRev7 was reduced. If so, the mutation frequency could be tested to determine if increasing the hRev7 protein to normal levels complements the UV-induced mutation frequency. If this occurred, it would allow us to definitively conclude that the reduction in hRev7 protein was responsible for the increased sensitivity and decreased frequency of mutations after UV-irradiation. The cells expressing hRev7 could be used to isolate HPRT mutants to determine if the UV-induced spectrum was restored to that seen in parental and vector control cells. Finally, flow cytometry could be performed to determine if expressing hRev7 complements the UV-induced delay in cell cycle progression seen in cells with decreased hRev7. All of these experiments would confirm that the changes observed in cells with reduced hRev7, were specifically due to the lack of hRev7 protein.

What is the effect of altering multiple TLS polymerases simultaneously in human cells?

Hypothesis: Decreasing hRev7 in an XPV cell strain will decrease the high UV-induced mutation frequency observed in XPV strains.

Aim: To transfect an XPV cell line with the siRNA against hRev7 and test the UV-induced mutation frequency and spectrum.

Another future direction for the study of specialized DNA polymerases is to knock out combinations of polymerases to see how that specific combination may change the balance of polymerase activity. This is an easily achievable project in yeast, mouse, or chicken cells where knock-out experiments are fairly easy to accomplish. For most cases in human cells we have to rely on reducing protein levels instead of complete knock-outs. XPV cells, however, naturally lack a functional pol η due to early stop codons leading to severely truncated forms of the protein. Currently, there are no reports of a human cell strain that lacks pol η and pol ζ. The siRNA against hRev7 could be transfected into an XPV cell strain in an attempt to create a cell line lacking both pol η and hRev7 (pol ζ). There are some advantages to using hRev7 in these experiments. First, experiments using normal cells with decreased hRev7 demonstrated an important role for hRev7 in UV-induced mutagenesis, strongly suggesting that hRev7 is involved in mutagenesis as a subunit of human pol ζ. Second, because there is an effective siRNA against hRev7 and a specific and sensitive antibody to detect the hRev7 protein, using hRev7 (instead of hRev3) to determine the effect of reducing pol ζ in XPV cells ensures confirmation of a quantitative reduction in the amount of hRev7 protein. A clear disadvantage is that cells with reduced hRev7 exhibited a



UV-induced sensitivity raising the concern that cells with reduced hRev7 and no pol η may be too sensitive to the cytotoxic effects of UV radiation to perform mutagenesis experiments. This could be easily tested using a UV survival curve.

If the survival curve demonstrated that cells with reduced hRev7 and no pol η were not extremely sensitive to UV radiation, the UV-induced mutation frequency could be tested. My hypothesis is that with a reduction of hRev7 (pol ζ), the extremely high UV-induced mutation frequency observed in XPV cells will be greatly decreased demonstrating an important role for hRev7 (pol ζ) in UV-induced mutagenesis in the absence of pol η. I speculate that in the absence of pol η, a more error-prone TLS polymerase inserts nucleotides across from DNA damage, and another polymerase extends these insertions leading to a high frequency mutations. It is my hypothesis that pol ζ is the major polymerase involved in these extensions, and in the absence of hRev7, the frequency of mutations would ultimately decrease because there is not another polymerase that is as efficient as pol ζ at extending. HPRT mutants from these experiments could be isolated to determine the spectrum of UV-induced mutations in cells with decreased hRev7 and no pol η, to gain insight into what polymerases might be making mutations in the absence of these two polymerases known to be important for tolerating UV-induced DNA damage.

Finally, because several other polymerases are being actively investigated in the Carcinogenesis Laboratory, it would be possible to use cells with reduced hRev7

and attempt to reduce other TLS proteins such as pol κ, pol ι, and Rev1 in combination with hRev7 to determine what effect this might have on survival, mutation frequency, mutation spectrum, and cell cycle progression after treatment with different DNA damaging agents.

Do cells with reduced hRev7 exhibit greater UV-induced apoptosis?

Hypothesis: Cells with reduced hRev7 are more sensitive to UV radiation because they exhibit greater UV-induced apoptosis.

Aim: To use cells with decreased hRev7 to test their UV-induced apoptosis using annexin-V.

One of the most recent areas of my research involved studies to determine why cells with reduced hRev7 were more sensitive to the cytotoxic effects of UV radiation than parental or vector control cells. This is an interesting finding because cells expressing antisense against hRev3 or hRev1 were not more sensitive to UV radiation than their parental cell strains (Li et al., 2002a; Wang unpublished data). The hRev3 and hRev1 protein levels were not able to be determined in these studies, but these cells express high levels of antisense against hRev3 or hRev1. In addition, these cells demonstrated significantly reduced UV- and BPDE-induced mutation frequencies suggesting that the hRev3 and hRev1 proteins were decreased.

Why are cells with hRev7 more sensitive to UV radiation? There are several possible reasons for this finding. First, hRev7 has been found to interact with several other proteins, suggesting hRev7 may have alternative cellular roles in

addition to TLS (Nelson et al., 1999; Chen and Fang, 2001; Pfleger et al., 2001; Weterman et al., 2001; Chan et al., 2001; Yin and Wold, 2003). Reducing hRev7 could be detrimental to cells because of another function of hRev7, unrelated to TLS. It should be noted that cells with reduced hRev7 grow at the same rate and have the same morphology as parental and vector control cells, therefore, lacking hRev7 is not immediately detrimental under normal growth conditions. My hypothesis is that cells with reduced hRev7 have trouble bypassing DNA damage in UV-treated cells because TLS is impaired. This difficulty replicating DNA containing damage leads to a delay in S-phase progression in an attempt to give the cells more time for repair or tolerance. Eventually, this impairment in replicating damaged DNA results in the breakdown of replication forks, leading to double-strand breaks. The presence of double-strand breaks induces apoptosis, leading to the UV-induced sensitivity seen in these cells.

Preliminary studies using Annexin-V to detect apoptotic cells after UV irradiation provided some information that the cells with reduced hRev7 had a greater number of Annexin-V positive cells than parental or vector control cells, indicating more UV-induced apoptosis (Figure 1). Conditions need to be optimized for these experiments because I was unable to adequately confirm these results. Ohmori's group in Japan has recently reported a similar effect in mouse cells lacking pol κ (Bi et al., 2004). Cells that lack pol κ are significantly more sensitive to BPDE and have a BPDE-induced S-phase delay compared to wild-type cells. In addition, XPV cells reportedly have a similar UV-induced S-

Figure 1: Apoptosis positive cells 10 hours after UV treatment of asynchronously growing cells. Annexin V-FITC was used to detect early apoptotic events in parental cell strain 9N.58 and clones 2-2 and 2-6 with reduced hRev7. Ten hours after UV irradiation, cells were harvested and stained with annexin V. Samples were subjected to analysis by flow cytometry to detect cells positive for annexin V. Annexin V positive cells for the parental cell strain were normalized to 1. Clones 2-2 and 2-6 are shown as fold increase above the parental cell strain. Results from one experiment are shown.

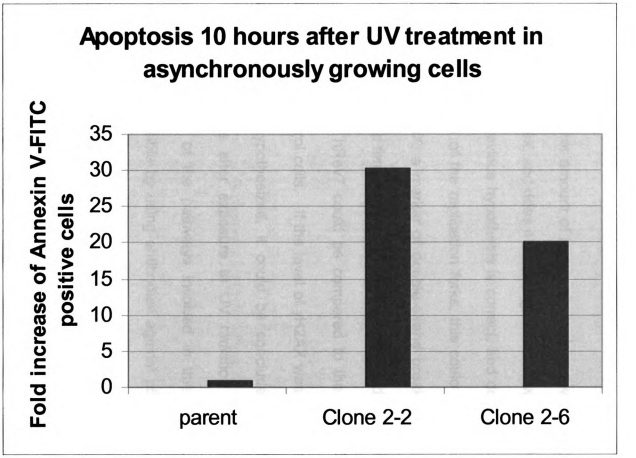

Apoptosis 10 hours after UV treatment in asynchronously growing cells
Fold increase of Annexin V-FITC positive cells
35
30
25
20
15
10
5
0
parent
Clone 2-2
Clone 2-6

Figure 1

phase delay, suggesting that cells with impaired TLS activate S-phase checkpoints leading to the delay in cell cycle progression seen in these cells (Bullock et al., 2001; Cordeiro-Stone et al., 2002).

Finally, if there is a greater amount of apoptosis in clones with reduced hRev7, what pathways are at work, and does it occur regardless of what DNA damaging agent is used? If my previous hypothesis is correct, and double strand-breaks occur due to breakdown of the replication forks, this could be tested using an antibody to detect γH2AX, a marker of double-strand breaks. Protein lysates could be made at specific timepoints after UV-treatment, and the level of γH2AX in cells with decreased hRev7 could be compared to the level of γH2AX in parental and vector control cells. If the level of γH2AX was higher in cells with decreased hRev7 as hypothesized, it could be concluded that these cells accumulate more DSB's after exposure to UV radiation than control cells. Further characterization of the pathways involved in this induced apoptotic response would be possible by using antibodies against p53, ATM, ATR, and other proteins involved in the response to DNA damage to gain insight into the pathways involved in the apoptotic response in cells with decreased hRev7.

Does reducing hRev7 increase the rate of DNA damage-induced homologous recombination in human cells?

Hypothesis: Cells with reduced hRev7 (and therefore, reduced TLS) have an increased rate of homologous recombination after treatment with DNA damaging agents.

Aim: To use cells with decreased hRev7 to determine the induced rate of recombination in human cells.

Previous studies in the Carcinogenesis Lab have shown that cells expressing antisense against hRev3 have significantly reduced UV- and BPDE-induced mutations (Gibbs et al., 1998; Li et al., 2002a). The cells which produced a low frequency of mutations had increased homologous recombination events, suggesting that when TLS is impaired, homologous recombination increases (Li, unpublished data). In addition, cells with reduced hMMS2, a protein required for recombination, had drastically decreased homologous recombination and an increased mutation frequency, suggesting that the tolerance of DNA damage by TLS and recombination is carefully balanced in human cells (Li et al., 2002b).

It would be interesting to determine if reducing hRev7 has an effect on homologous recombination as tested by our system (Figure 2). Our system uses an intrachromosomal substrate containing two copies of the hygromycin (hyg) gene, oriented in the same direction. Each hyg copy is nonfunctional because of a mutation from a HindIII linker insertion. The gpt gene is used as a selectable marker. As shown in Figure 2, when homologous recombination using a homologous copy of the gene as an undamaged template occurs, a functional copy of the hyg gene is produced after one round of replication. By adding hygromycin to the culture medium, recombination events can be selected for and calculated.

Figure 2: Homologous recombination substrate. A. The intrachromosomal substrate contains two nonfunctional copies of the hygromycin (hyg) gene, each containing a mutation by a HindIII linker insertion. The gpt gene is a selectable marker. The triangle represents UV-induced DNA damage. **B.** The process of homologous recombination using the newly replicated daughter strand of the sister duplex (blue) as an undamaged template. **C.** The result of this process is that both products contain a hyg gene with the HindIII linker. **D.** After one round of replication, the hyg gene contains the HindIII linker so these homologous recombination events will not be detected by selection with hygromycin. **E.** The process of homologous recombination using the homologous copy of the gene as an undamaged template (red hyg gene). **F.** The result of this process is that the hyg1 gene (red) remains intact. In addition, The newly replicated strand of the hyg2 gene copied the sequence from the corresponding section of the hyg1 gene leading to a hyg gene with no HindIII linker. **G.** After one round of replication, a wildtype copy of the hyg gene has been generated and this homologous recombination product can be selected using hygromycin.

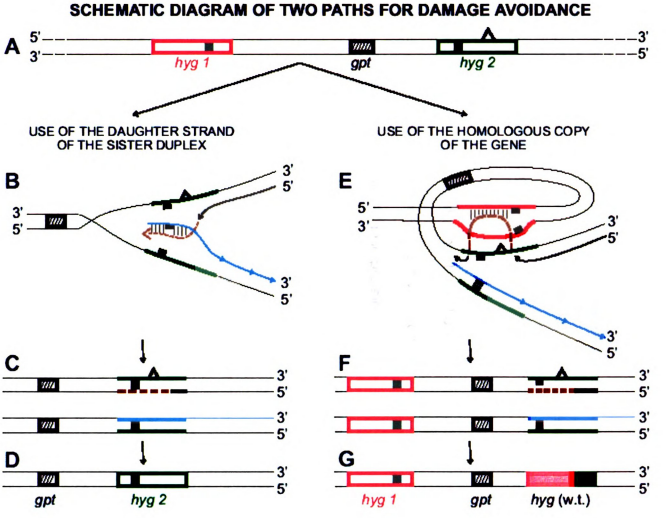

SCHEMATIC DIAGRAM OF TWO PATHS FOR DAMAGE AVOIDANCE
A
5'
3'
hyg 1
gpt
hyg 2
3'
5'
USE OF THE DAUGHTER STRAND OF THE SISTER DUPLEX
USE OF THE HOMOLOGOUS COPY OF THE GENE
B
C
D
E
F
G
gpt
hyg 2
hyg 1
gpt
hyg (w.t.)

Figure 2

Experiments testing the effect of reduced hRev7 on BPDE-induced recombination have been performed, however, results were difficult to interpret. Cells with reduced hRev7 did not produce any hyg-resistant recombinant colonies when treated with BPDE. This could be because of several reasons. First, the drug selection we use on the parental cell line is too high of a concentration for the clones with reduced hRev7. This can be easily tested using different doses of hygromycin. Second, data from our laboratory suggests that the two main proteins required for recombination, MMS2 and UBC13, are expressed at very different levels from the 9N.58 cell strain when it was clonally expanded. It is unclear why there is such variation in the levels of these proteins, but suggests that one or both of these proteins is expressed at very low levels in the clones with reduced hRev7, leading to no hyg-resistant recombination events. Preliminary Western blot analysis showed that both hMMS2 and hUBC13 are expressed in the clones with reduced hRev7. Finally, it could be that hRev7 is itself involved in the process of homologous recombination. Because there were no hyg-resistant recombinants in cells with reduced hRev7, it suggests that reducing hRev7 also reduces the frequency of recombination events, at least those detected by our substrate. It would be interesting to find a protein involved in both pathways of damage tolerance. In support of this idea, another TLS protein, Rev3, was found to be important for repair of double-strand breaks by homologous recombination in DT-40 chicken cells (Sonoda et al., 2003). Because of the many interactions that hRev7 has with other cellular proteins, a role for hRev7 in homologous recombination cannot be ruled out at

this time. Future experiments should focus on optimizing the concentration and timing of hygromycin selection. Then, experiments to test the role of hRev7 in recombination could be performed after treatment with DNA damaging agents.

Does reducing hRev7 in a cell strain with significantly reduced homologous recombination have an effect?

Hypothesis: Cells with reduced TLS and reduced recombination will be very sensitive to DNA damaging agents.

Aim: To, first, establish a cell line with impaired TLS (by reducing hRev7 protein expression) and impaired homologous recombination (by reducing hMMS2 protein expression) and, second, to test this cell line for its UV-induced cytotoxicity and mutation frequency.

The Carcinogenesis Laboratory currently has cell strains with reduced hRev7 as well as cell strains with reduced hMMS2. Cell strains with reduced hRev7 demonstrate a significantly reduced UV-induced mutation frequency and increased sensitivity to UV, strongly suggesting that cells with decreased hRev7 have impaired TLS. Cells with decreased hMMS2 have almost no detectable homologous recombination and an increased mutation frequency, suggesting that when the recombination pathway is impaired, the process of TLS is used more frequently, leading to increased mutations (Li et al., 2002b). It would be interesting to determine what effect the combination of reduced hMMS2 (decreased recombination) and hRev7 (decreased TLS) would have on mutation frequency and homologous recombination. It is difficult to predict the phenotype of these cells because both known pathways of damage tolerance would be impaired.

Antisense against hMMS2 could be transfected into clones with reduced hRev7 or the effective hRev7 siRNA could be transfected into cells with decreased hMMS2. The UV-induced phenotype of cells with both decreased hMMS2 and decreased hRev7 could be observed. One concern is that these cells may be very sensitive to the cytotoxic effects of DNA damaging agents because they have presumably lost both tolerance pathways, however, this has yet to be determined. Results from these experiments may prove to be very interesting if these cells are not too sensitive.

What regions of hRev7 are important for binding to other proteins and pol ζ activity?

Hypothesis: hRev3 and hRev1 compete for binding to hRev7

Aim: To mutate conserved residues in hRev7 and test the ability of the hRev7 mutants to bind to hRev3, hRev1 and hRev7.

hRev7 can homodimerize with itself, as well as bind to hRev3 and hRev1. The important region for interaction with these proteins is amino acids 21-155 of hRev7 (Murakumo et al., 2001). There has been no further characterization of this hRev7 interaction region to date. One hypothesis is that because the phenotypes for hRev3 and hRev1 deficient cells were very similar, that hRev3 and hRev1 function in the same pathway. Because hRev7 binds to both hRev3 and hRev1, it is thought that these three proteins function as a complex during TLS. While all three proteins bind at the same 21-155 amino acid region, this is a fairly large region of the 211 amino acid hRev7. Because the region important for binding is quite large, it could be that hRev7 binds to hRev3, hRev1, and

hRev7 at different regions of hRev7 within the 21-155 amino acids. However, a complex of hRev3, hRev7, and hRev1 has never been isolated from human cells. In addition, a complex of the three proteins has never been reconstituted *in vitro*, despite several attempts. Based on this evidence, an alternative hypothesis is proposed that because all of the interactions between hRev7, hRev3, and hRev1 occur at the same region of hRev7, these proteins compete for binding to hRev7. hRev7 may bind to each protein depending on what cellular function of hRev7 is needed. Which scenario occurs in human cells, however, is currently unknown.

Mutational analysis of different amino acids in this region could provide information about the important regions for binding to each protein. The nucleotide sequence could be examined and any important or conserved residues could be mutated and tested in *in vitro* binding assays using hRev7, hRev3 and hRev1 as binding partners. An additional area to target would be the hRev7 HORMA domain and any conserved residues contained within this motif. One of the hypothesized functions of the HORMA domain is protein-protein interactions, so mutating critical or conserved residues in this domain may affect binding to other proteins. If the *in vitro* binding assays provided useful information about binding to hRev7, these hRev7 mutants could be used in *in vitro* primer extension reactions to test their effect on TLS. Results from these experiments could provide information about whether hRev7 binds to hRev1 and hRev3 at the same time forming a complex, or whether these proteins compete

for binding to hRev7. The mutational analysis of hRev7 and its binding partners may provide insight into the different roles of hRev7 during TLS.

What is the cellular localization of hRev7 in human fibroblast cells?

Hypothesis: hRev7 is localized in the nucleus and after UV irradiation, hRev7 further localized into replication foci.

Aim: To use immunofluorescence to detect the localization of hRev7 in cells under normal conditions and after UV treatment.

One of the most interesting and unexplored areas of research for TLS is the localization of hRev7 before and after treatment with DNA damaging agents. Currently, hRev7 is thought to have at least two functions in cells. One function is that hRev7 is involved in tranlesion replication of DNA damage. A second function is that hRev7 is involved in mitosis, although the exact role remains unknown. Immunofluorescence experiments to determine the localization pattern of hRev7 might be useful in helping to understand the process of events that occurs after cells have been stressed with DNA damaging agents. Is hRev7 normally in the nucleus or the cytoplasm? Is there a redistribution of hRev7 after treatment with a DNA damaging agent? Immunofluorescence experiments using our hRev7 antibody may take some time to optimize, but they could provide interesting information about hRev7. Investigation into the following questions would be interesting.

1. Is there a difference in the localization of hRev7 in parental cells compared to cells with decreased hRev7 or do these cells just contain less hRev7, but in the same general distribution compared to parental cells?

2. Is there a difference in the localization of hRev7 in cells that overexpress hRev7 compared to parental cells?

3. Upon UV irradiation, does the distribution of hRev7 in cells change? Does hRev7 co-localize to replication foci in the nucleus of cells like that seen for polymerases η and ι? If so, is there a difference in cells with reduced or overexpressed hRev7?

4. If hRev7 localizes to replication foci, is this localization dependent on pol η, as the localization of pol ι is? Using an XPV cell line lacking pol η, this hypothesis could be easily tested.

In general, these experiments could provide useful information about the movement of hRev7 in cells after treatment with DNA damaging agents.

Does overexpression of hRev7 have an effect on the tolerance of UV-induced DNA damage.

Hypothesis: Overexpression of hRev7 will stimulate the polymerase activity of hRev3 leading to an increased mutation frequency after treatment with DNA damaging agents.

Aim: To use a cell strain that overexpresses hRev7 to test the UV-induced mutation frequency

Primer extension reactions demonstrated that yeast Rev3 had minimal polymerase activity, but the addition of hRev7 to the reaction stimulated the polymerase activity of Rev3 over 20-fold (Nelson et al., 1996). Confirmation for hRev7 as a subunit of polymerase ζ can be shown if hRev7 stimulates the polymerase activity of hRev3 in human cells. The lack of a purified hRev3 prevents this experiment being performed *in vitro*. A possible way to test this is

to overexpress hRev7 in a cell strain and determine the induced mutation frequency. The hypothesis is that overexpressed hRev7 will stimulate hRev3, and the mutation frequency will increase. Initial experiments are discussed in Appendix A, but showed no difference in mutation frequency for cells that overexpress hRev7 compared to parental cells. It is possible that overexpressing hRev7 does not stimulate the polymerase activity of hRev3, however, these experiments have several caveats. First, the 9N.58 cell strain was chosen for our studies because it has an incredibly high induced mutation frequency. Using a different cell strain with a lower induced frequency may increase our chances of seeing the frequency increase. Second, hRev3 may be the limiting factor for pol zeta activity, so overexpressing hRev7 and not changing hRev3 may not have an effect. Third, the level of overexpression of hRev7 in the cell line tested may not be high enough. Finding clones with a much higher level of hRev7 expression and using a cell strain with a lower induced mutation frequency would be beneficial to these studies.

Is hRev7 required for the mitotic checkpoint in response to spindle disruption in human cells?

Hypothesis: hRev7 is involved in a mitotic checkpoint in human cells

Aim: To treat cells with decreased hRev7 with an inhibitor of spindle formation to test for a mitotic checkpoint defect.

Studies using human and *Xenopus* Rev7 in *Xenopus* extracts demonstrated that hRev7 inhibits the anaphase promoting complex by binding to cdc20 and cdh1 (Pfleger et al., 2001; Chen and Fang, 2001). In addition, a paper characterizing

the interaction between hRev7 and papillary renal cell carcinoma protein (PRCC), provides evidence that when the interaction between PRCC and hRev7 is impaired as seen in renal cell carcinoma cells, these cells have a defective mitotic checkpoint (Weterman et al., 2001). There are only a few papers suggesting a role for hRev7 in the regulation of mitosis and the specific role for hRev7 in mitosis remains largely undefined. An experiment to test if hRev7 is involved in a mitotic checkpoint in human cells would be to treat cells with reduced hRev7 with nocodazole and compare their response to parental cells. Nocodazole disrupts spindle microtubules, which results in accumulation of cells in mitosis if an intact mitotic checkpoint is present. If hRev7 is involved in mitotic arrest, cells with decreased hRev7 will have fewer cells arrested in mitosis compared to control cells, indicating that these cells have an impaired mitotic checkpoint. Quantitation of mitotic cells can be achieved by counting mitotic nuclei as well as by FACS analysis to determine DNA content of the cells. In addition, nocodazole treatment before and after UV treatment might provide useful information about the mitotic role of hRev7 and how it may be linked to the TLS role of hRev7.

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