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THE ROLE OF HUMAN REV7, THE ACCESSORY SUBUNIT OF HUMAN DNA POLYMERASE ζ, IN CELL SURVIVAL AND DNA DAMAGE INDUCED MUTAGENESIS

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Major Professor's Signature

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THE ROLE OF HUMAN REV7, THE ACCESSORY SUBUNIT OF HUMAN DNA POLYMERASE ζ, IN CELL SURVIVAL AND DNA DAMAGE INDUCED MUTAGENESIS

By

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ABSTRACT

THE ROLE OF HUMAN REV7, THE ACCESSORY SUBUNIT OF HUMAN DNA POLYMERASE ζ, IN CELL SURVIVAL AND DNA DAMAGE INDUCED MUTAGENESIS

By

Jessica A. Neal

The presence of DNA damage can pose a considerable threat to cell survival because the high fidelity polymerases that are charged with the task of replicating DNA are often unable to synthesize past sites of damage. A considerable amount of the DNA damage incurred is removed by repair mechanisms. Nevertheless, repair processes are often overwhelmed by the abundance of DNA damage and consequently, some lesions persist during DNA replication. To overcome the presence of DNA lesions during replication, cells also employ damage tolerance mechanisms that promote cell survival by facilitating replication of damaged DNA. One important strategy for tolerating DNA damage is translesion synthesis (TLS). Translesion synthesis refers to the process of direct replicational bypass of DNA damage, which is conducted by a group of specialized DNA polymerases capable of inserting nucleotides opposite DNA lesions. Although TLS polymerases promote cell survival by avoiding replication arrest, many of these enzymes demonstrate reduced fidelity and as a result, also contribute to mutagenesis. The first of such TLS polymerases to be discovered was DNA polymerase zeta (Pol ζ). The function of Pol ζ has been well characterized in yeast and has been implicated in both spontaneous and damage-induced mutagenesis in that organism. However, the role of human Pol

ζ in such mutagenesis is much less well understood. To examine the role of hRev7, the accessory subunit of hPol ζ in the mutagenesis of human cells, we generated cell strains with significantly reduced levels of hRev7 and compared them to their parental strain and a vector control strain for cell survival, induction of mutations, and ability to progress through the cell cycle following exposure to UV radiation. Cells with reduced hRev7 progressed through S-phase more slowly and were more sensitive to the cytotoxic effects of UV radiation than the controls. In addition, cell strains with reduced hRev7 demonstrated a significantly reduced frequency of UV-induced mutations. These results strongly support the hypothesis that hRev7 is required for TLS past UV-induced DNA lesions. When these same cell strains were assayed for their response to the biological effects of benzo[a]pyrene diol epoxide (BPDE), cell strains with reduced hRev7 demonstrated a BPDE-induced delay in progression through S-phase and were also more sensitive to its cytotoxic effect. Surprisingly however, the frequency of mutations induced by BPDE in cell strains with reduced hRev7 did not differ from those of the control stains, indicating that hRev7 is not required for translesion synthesis past BPDE-induced DNA lesions. Taken together, these results demonstrate that the protective role hRev7 plays for cells exposed to BPDE is independent of the role of this protein in TLS. Combined with the results of the UV study, these data suggest that hRev7 has at least two distinct cellular functions: one as the accessory subunit of hPol ζ and an additional, as of yet uncharacterized role, in protecting cells from the cytotoxic effects of DNA damage.

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER II

LIST OF FIGURES

Images in this dissertation are presented in color.

CHAPTER I:

CHAPTER II:

CHAPTER III:

INTRODUCTION

Cellular DNA is continually damaged both by endogenous and environmental agents, causing a variety of DNA lesions. Many of these lesions are repaired by mechanisms that remove them directly, such as base excision repair or nucleotide excision repair. However, repair may be slow or the DNA damage may be extensive, resulting in DNA lesions that persist during replication. If the high fidelity, replicative polymerase complex encounters DNA damage that blocks elongation, potentially fatal stalling or arrest of DNA replication may occur.

To mitigate the threat of cell death due to replication arrest, cells have also evolved strategies for tolerating DNA damage. One strategy, called damage avoidance, refers to a mechanism in which a homologous piece of DNA, for example the newly replicated daughter strand of the sister duplex, is temporarily used as a template to replicate around a DNA lesion. Because damage avoidance does not require utilization of the lesion-containing portion of the DNA as a template, but instead, makes use of an undamaged, homologous DNA segment, it is typically considered to be an error-free mechanism of damage tolerance (for review see [1]).

Alternatively, cells may tolerate the presence of DNA damage by employing a process of direct replicational lesion bypass, called translesion synthesis (TLS). Translesion synthesis requires the use of one or more specialized DNA polymerases to replicate past the DNA lesion until the high fidelity replicative

polymerases are able to resume function. These specialized TLS polymerases possess active sites that are typically less restrictive then those of the classical replicative polymerases, which allows them to incorporate nucleotides opposite sites of DNA damage [2-5]. However, one consequence of the more open active sites of these polymerases is that they are also prone to erroneous nucleotide incorporation, and are therefore commonly associated with an increased frequency of mutations (reviewed in [6]).

One such specialized TLS polymerase is DNA polymerase zeta (Pol ζ). Polymerase ζ is composed of two subunits, a catalytic subunit, called Rev3, as well as an accessory subunit, called Rev7 [7]. In 2002, our laboratory demonstrated that hRev3, the catalytic subunit of hPol ζ, is involved in mutagenesis induced by UV radiation as well as by the chemical carcinogen benzo[a]pyrene diol epoxide (BPDE) [8]. However, much less is known about the function of hRev7, the accessory subunit of hPol ζ, in such mutagenesis. Therefore, the focus of my research, which is presented in this dissertation, was to determine the role of hRev7 in human mutagenesis.

Chapter I consists of a review of the literature concerning two of the major biological responses to DNA damage, DNA repair and DNA damage tolerance, with a special focus on the process of translesion synthesis. **Chapter II** consists of a manuscript published in the April 2008 issue of DNA Repair [9], concerning the role of hRev7 in cell survival, induction of mutations, and cell cycle

progression of UV-irradiated human fibroblasts. In that manuscript I contributed data to Table 1 and I also contributed notably to the data interpretation and to the writing of the manuscript. **Chapter III** consists of a manuscript being prepared for submission to DNA Repair, detailing the role of hRev7 in cell survival, induction of mutations and cell cycle progression of BPDE-treated human fibroblasts. I carried out all of the experiments described, wrote the paper, and the interpretation of the data was essentially mine.

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UV(254nm)-irradiated human fibroblasts. *DNA Repair (Amst)* *7*, pp. 597-604.

CHAPTER I

Literature Review

Cellular Responses to DNA Damage

Cellular DNA is subjected to a variety of different types of agents that result in unfavorable chemical and structural modifications. Even under normal physiological conditions, DNA is subject to insult by water and reactive oxygen, resulting in the production of a variety of "spontaneous" DNA lesions (for review see reference [1]). For example, DNA hydrolysis can result in cleavage of the *N*-glycosidic bond, resulting in release of the DNA base and production of an apurinic or apyrimidinic site. Estimates indicate that in mammalian cells as many as 10,000 purines and 500 pyrimidines are lost due to hydrolysis each day [2-4]. Abasic sites can be cytotoxic if they block DNA replication, and because they are noninstructional lesions they have the potential to be highly mutagenic [5]. An additional consequence of DNA hydrolysis is removal of the exocyclic amino group of a DNA base i.e. deamination. Most frequently, cytosine is converted to uracil; however, adenine and guanine can also be deaminated resulting in conversion to hypoxanthine and xanthine, respectively. Loss of the amino group changes the base pairing properties of DNA and as result can be miscoding,

leading to mutation. Reactive oxygen species, generated as the byproducts of various metabolic processes, are another major source of spontaneous damage to DNA. Thus far, over 80 distinct types of oxidative DNA damage have been characterized, including oxidized bases, purine aldehyde adducts, DNA-protein cross-links, and DNA strand breaks [6, 7]. Even the process of DNA replication can result in a number of errors that, if left unrepaired, would cause a significant increase in mutagenesis.

In addition to endogenously generated DNA damaging agents, cellular DNA is also exposed to a variety of physical and chemical damaging agents that come from external sources (reviewed in [1]). Ultraviolet (UV) radiation from exposure to sunlight can cause adjacent pyrimidine residues to become fused, resulting in the formation of the cytotoxic and mutagenic, cyclobutane pyrimidine dimer or the pyrimidine-pyrimidone (6-4) photoproduct. Ionizing radiation from cosmic rays and from radioactive materials in the earth causes a broad spectrum of DNA lesions, including damaged bases and highly lethal DNA double-strand breaks. Finally, a host of chemical species can react with DNA to form a variety of potentially mutagenic lesions, including an assortment of alkylating agents, and cross-linking agents, as well as chemicals that are metabolized into electrophilic reactants, such as aromatic amines, and polycyclic aromatic hydrocarbons.

Given that DNA damage is so ubiquitous in nature and the fact that it poses such a significant threat to genomic stability, it is not surprising that cells have evolved

multiple strategies to respond to DNA damage. Many DNA lesions are repaired by mechanisms that either reverse or remove the damage directly. Repair of damaged DNA by excision mechanisms can result in release of the lesion either as a free base, referred to as base excision repair, or as a lesion-containing oligonucleotide, referred to as nucleotide excision repair. A third distinct form of excision repair, called mismatch repair, refers to the process of specifically removing mispaired bases from DNA (reviewed in [8]).

In addition to DNA repair, damage tolerance mechanisms exist, that allow the cell to mitigate the potentially lethal consequences of DNA damage, i.e. arrested DNA replication, without physical removal of the damage from DNA. One such tolerance strategy is called damage avoidance. In damage avoidance, replication arrest is prevented by using an undamaged, homologous portion of the DNA as a template to replicate around the DNA lesion. Alternatively, cells may employ a mechanistically distinct process of damage tolerance, called translesion synthesis. Translesion synthesis involves the temporary use of specialized DNA polymerases, capable of inserting nucleotides opposite DNA lesions, to replicate past sites of DNA damage. Because the specialized polymerases that perform translesion synthesis often exhibit relaxed fidelity, a property that confers the ability to replicate past DNA lesions, translesion synthesis is sometimes associated with an increase in mutagenesis (see [9] for review).

DNA repair and DNA damage tolerance represent two distinct, but equally important biological responses to DNA damage, which function to promote cell survival and maintain genomic stability in the face of constant DNA damage. Deficiencies in these processes have been linked to a number of different diseases, including several cancer predispositions. Consequently, DNA repair and damage tolerance have been and continue to be the focus of a significant amount of scientific investigation. The following is a review of the current literature concerning these two biological responses to DNA damage.

I. DNA Repair

To prevent the harmful consequences of DNA damage, including induction of mutations and premature cell death, a variety of mechanisms have evolved that are responsible for repairing an enormous spectrum of DNA lesions. DNA repair specifically refers to those cellular responses that result in the restoration of damaged DNA to normal nucleotide sequence and DNA structure (see for example [1, 8]). DNA repair can be divided into several broad classes based on mechanism. Direct repair refers to processes in which the damage to the DNA is reversed by an enzyme in a single step reaction. Excision DNA repair mechanisms are defined as those in which DNA is repaired by removal, i.e. excision, of the damaged portions, followed by resynthesis using the complementary strand as a template. Finally, strand breaks can be repaired by mechanisms involving recombination or by end-joining mechanisms. Although each one of these DNA repair mechanisms are of fundamental importance to maintaining genomic integrity, the primary focus of this review is on the repair processes that operate on damaged or inappropriate nitrogenous bases in DNA. Specifically, the mechanisms of base excision repair, nucleotide excision repair and mismatch repair are covered in detail.

A. Base Excision Repair

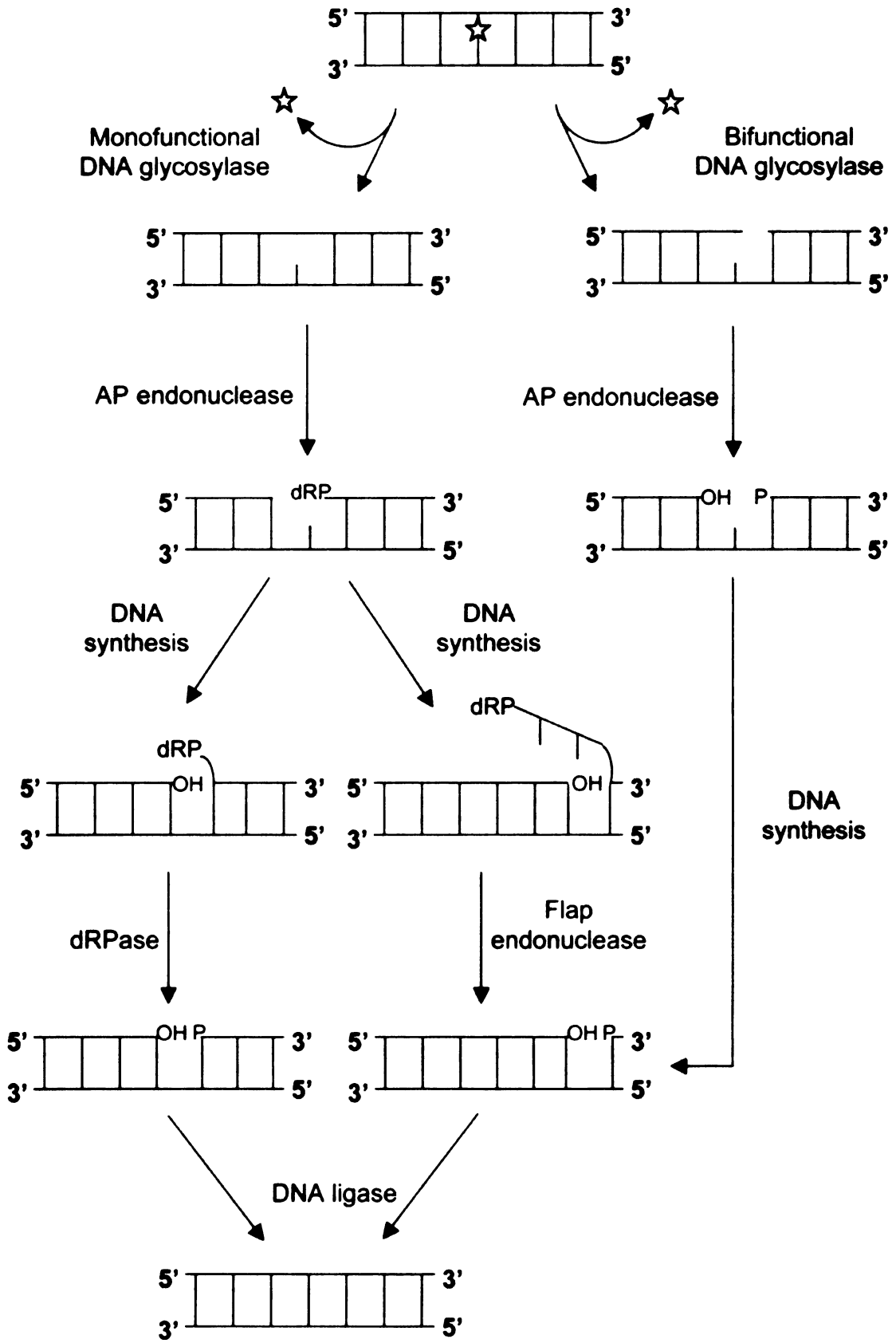
Base excision repair (BER) is considered to be the most frequently utilized pathway for removal of DNA lesions. The base excision repair pathway is involved in the repair of many different types of DNA lesions, including those generated by hydrolytic deamination of bases, oxidative damage, alkylating agents and ultraviolet light [10].

1. Base Excision Repair Pathways

The mechanism of base excision repair in prokaryotes and eukaryotes is well understood (for review see [1, 10, 11]). Base excision repair is initiated by enzymes called DNA glycosylases, which recognize DNA lesions and cleave the *N*-glycosidic bond linking the damaged bases to the DNA backbone (Figure 1). This cleavage event results in excision of the damaged base and thus, this repair process, where lesions are released as free bases, is referred to as "base excision repair".

DNA glycosylases fall into two general classes depending on their mechanism of catalysis. The first class of glycosylases, called monofunctional glycosylases, eliminate the damaged nucleoside, leaving behind an abasic site. The second class of glycosylases are called bifunctional glycosylases because, in addition to hydrolysis of *N*-glycosidic bonds, these enzymes support an apurinic/apyrimidinic

Figure 1 - The base excision repair pathway. Base excision repair is initiated by DNA glycosylases, which recognize DNA lesions and cleave the *N*-glycosidic bond linking the damaged bases to the DNA backbone. Cleavage by monofunctional glycosylases leaves an abasic (AP) site. Bifunctional glycosylases possess an additional AP lyase activity that results in cleavage of the DNA backbone on the 3' side of the newly generated abasic site. Immediately following removal of the damaged base by a DNA glycosylase, an AP endonuclease nicks the DNA backbone immediately 5' of the AP site. When AP endonuclease acts on the product of a monofunctional DNA glycosylase, cleavage results in a free 3'-OH and 5'-deoxyribosephosphate (dRP) group. At this point, BER continues through one of two distinct pathways termed short-patch repair (right) and long-patch repair (left). In short patch repair, the remaining one-nucleotide gap is filled by a repair DNA polymerase and the 5'-dRP group is removed by an enzyme possessing deoxyribosephosphate lyase (dRPase) activity. Finally, the DNA ends are sealed by a DNA ligase. In long-patch repair, a more extensive patch of DNA is synthesized, displacing the strand containing the 5'dRP group. This 5' flap is cleaved by a flap endonuclease and the resulting DNA ends are sealed by DNA ligase. When an AP endonuclease cleaves the product of a bifunctional DNA glycosylase, the blocked 3' end is removed and repair polymerization and ligation can occur directly. This figure was adapted from reference [10].


5'
3'
3'
5'
Monofunctional
DNA glycosylase
Bifunctional
DNA glycosylase
AP endonuclease
AP endonuclease
dRP
OH
P
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dRP
dRP
OH
OH
DNA
synthesis
dRPase
Flap
endonuclease
OH P
OH P
DNA ligase

(AP) lyase activity that results in cleavage of the DNA backbone on the 3' side of the newly generated abasic site. The lyase activity of bifunctional glycosylases generates a 3' end that lacks a hydroxyl and must be removed before DNA synthesis can occur.

After removal of the damaged base by a DNA glycosylase, processing of the abasic site is initiated by an AP endonuclease. These enzymes generate a nick in the DNA backbone immediately 5' of the abasic site. When AP endonuclease acts on the product of a monofunctional DNA glycosylase, cleavage of the DNA backbone 5' to the abasic site results in a free 3'-OH and 5'-deoxyribosephosphate (dRP) group. At this point, BER continues through one of two distinct pathways, termed short-patch repair and long-patch repair. In short patch repair, the one-nucleotide gap that remains after excision is filled by a repair DNA polymerase, and the 5'-dRP group is removed by an enzyme possessing deoxyribosephosphate lyase (dRPase) activity. Finally, the DNA ends are sealed by a DNA ligase. In long-patch repair, a more extensive length of DNA (2-20 nucleotides) is synthesized, displacing the strand containing the 5'dRP group. This 5' flap is then cleaved by a flap endonuclease, and the resulting DNA ends are sealed by DNA ligase. When an AP endonuclease cleaves the product of a bifunctional DNA glycosylase, the blocked 3' end is removed and repair polymerization and ligation can occur directly.

a. DNA Glycosylases

The base excision repair pathway is utilized to repair many different types of DNA lesions, including those generated by hydrolytic deamination of bases, oxidative damage, alkylating agents and ultraviolet light. To distinguish between such a large variety of DNA lesions, each species possesses many different DNA glycosylases that recognize one, or at most a few, specific types of DNA lesions based on their specific structural folds and motifs. DNA glycosylases have been divided into five classes based on their structure and substrate specificities (reviewed in [1, 10, 11]). The uracil-DNA glycosylase family consists of monofunctional DNA glycosylases, including the uracil N-DNA glycosylases (UNGs), the mismatch specific uracil-DNA glycosylases (MUGs), the thymine-DNA glycosylases (TDGs), and the single-strand-selective mono-functional uracil-DNA glycosylase (SMUGs). Uracil-DNA glycosylases recognize uracil or thymine mismatches in DNA. The Fpg/Nei superfamily, defined by the *E. coli* enzymes Fpg (also known as MutM) and endonuclease VIII (also known as Nei), specializes in recognizing oxidative DNA damage. This superfamily was thought to be unique to bacteria until 2002, when three homologous Nei-like proteins (NEIL1-NEIL3) were identified in humans [12-14]. Members of the helix-hairpin-helix (HhH) superfamily are characterized by a core helix-hairpin-helix motif found in many DNA-dependent enzymes [15]. The HhH superfamily is the most functionally diverse superfamily of DNA glycosylases. It includes both monofunctional and bifunctional glycosylases, that recognize a variety of different types of DNA lesions [10]. In fact, the HhH superfamily is so diverse that glycosylases from this superfamily cover nearly the entire spectrum of lesions

repaired by BER [11]. Finally, there are two single member superfamilies that have no known structural relatives: the alkyladenine glycosylase (AAG) superfamily, which is defined by the DNA glycosylase AAG, a monofunctional glycosylase that recognizes alkylation damage [16], and the endonuclease V superfamily, which is defined by endonuclease V from the T4 virus, a small bifunctional glycosylase that recognizes UV-induced thymine-thymine dimers [17].

b. Apurinic/Apyrimidinic (AP) Endonucleases

In contrast to the multitude of DNA glycosylases that exist, each organism possesses only a few distinct AP endonucleases. AP endonucleases have thus far been divided into four structural classes. Classes I and II are defined by the bacterial AP endonucleases, Xth (also known as exonuclease III) and Nfo (also known as endonuclease IV) (reviewed in [10]). Xth is the predominant AP endonuclease in *E. coli*. In addition to AP endonuclease activity, Xth also possesses 3'→5' exonuclease activity for which it was originally characterized [18]. The major human AP endonuclease, APE1, and its recently discovered paralog, APE2, are homologous to Xth [19, 20]. Bacteria contain a second AP endonuclease, Nfo, although it is thought to account for only 10% of the total bacterial AP endonuclease activity [21, 22]. The major *S. cerevisiae* AP endonuclease, APN1, is homologous to Nfo [23]. Two groups have also reported the existence of a third class of AP endonucleases, defined by the UV damage endonuclease, UVDE, which is found in some fungi and bacteria [24, 25]. More

recently, a fourth class of AP endonucleases has been defined based on the discovery of a third human AP endonuclease, called PALF [26].

c. Post-incision Events in Base Excision Repair

When an AP endonuclease acts on the product of a monofunctional DNA glycosylase, cleavage of the DNA backbone 5' to the abasic site results in a one nucleotide gap and a dRP group that must be removed. If the base excision repair process proceeds through the short-patch repair pathway, the one-nucleotide gap is filled by a repair DNA polymerase, and the dRP group is removed by an enzyme possessing dRPase activity. In bacteria, the major DNA repair polymerase, Pol I is used for gap filling in short-patch BER [27]. Bacterial Pol I also possesses a 5'→3' activity and can degrade the blocked dRP terminus. However, several other enzymes from *E. coli*, including exonuclease I, RecJ, and Fpg have also been proposed as *E. coli* dRPases [28-30]. In mammalian short-patch BER, DNA polymerase β can fill in the one-nucleotide gap and can subsequently remove the 5' dRP group from a blocked terminus [31]. The dRPase activity of Pol β is apparently required for repair of at least some types of DNA lesions because the hypersensitivity of cells lacking Pol β to methyl methane sulfonate can be complemented by overexpression of the dRPase domain of Pol β, but not by overexpression of the polymerase domain [32]. Once the dRP group is removed, the newly generated DNA ends are sealed by DNA ligase I in *E. coli*, or the DNA ligase 3α-XRCC1 complex in humans.

If processing of the dRP residue is not efficient, for example, because the dRP residue becomes reduced, long-patch BER can occur. In long-patch BER, a more extensive patch of DNA is synthesized displacing the strand containing the 5'dRP group. This displaced strand must then be cleaved by a flap endonuclease. In *E. coli*, if the 5' dRP residue cannot be removed, Pol I displaces the dRP-containing strand and then cleaves the strand using its 5'→3'exonuclease activity [33]. In human cells, if Pol β is unable to remove the 5' dRP group, a polymerase switch may occur and Pol δ or Pol ε together with accessory factors RFC, PCNA and RPA add several nucleotides generating a flap, which is then removed by the flap endonuclease, FEN1 [34]. In humans, ligation of the DNA ends generated in long-patch BER is achieved by DNA ligase I rather than DNA ligase 3α (as in short-patch BER), probably as a consequence of its interaction with PCNA.

Finally, if an AP endonuclease acts on the product of a bifunctional DNA glycosylase, it removes the blocked 3' end, leaving a free 3'-OH which is suitable for repair polymerization and ligation by Pol I and DNA ligase I in *E. coli*, and by Pol β and Ligase 3α in humans.

B. Nucleotide Excision Repair

The idea that DNA can be repaired by the physical removal of a lesion was first suggested in the 1960s, when two independent groups reported that in bacteria,

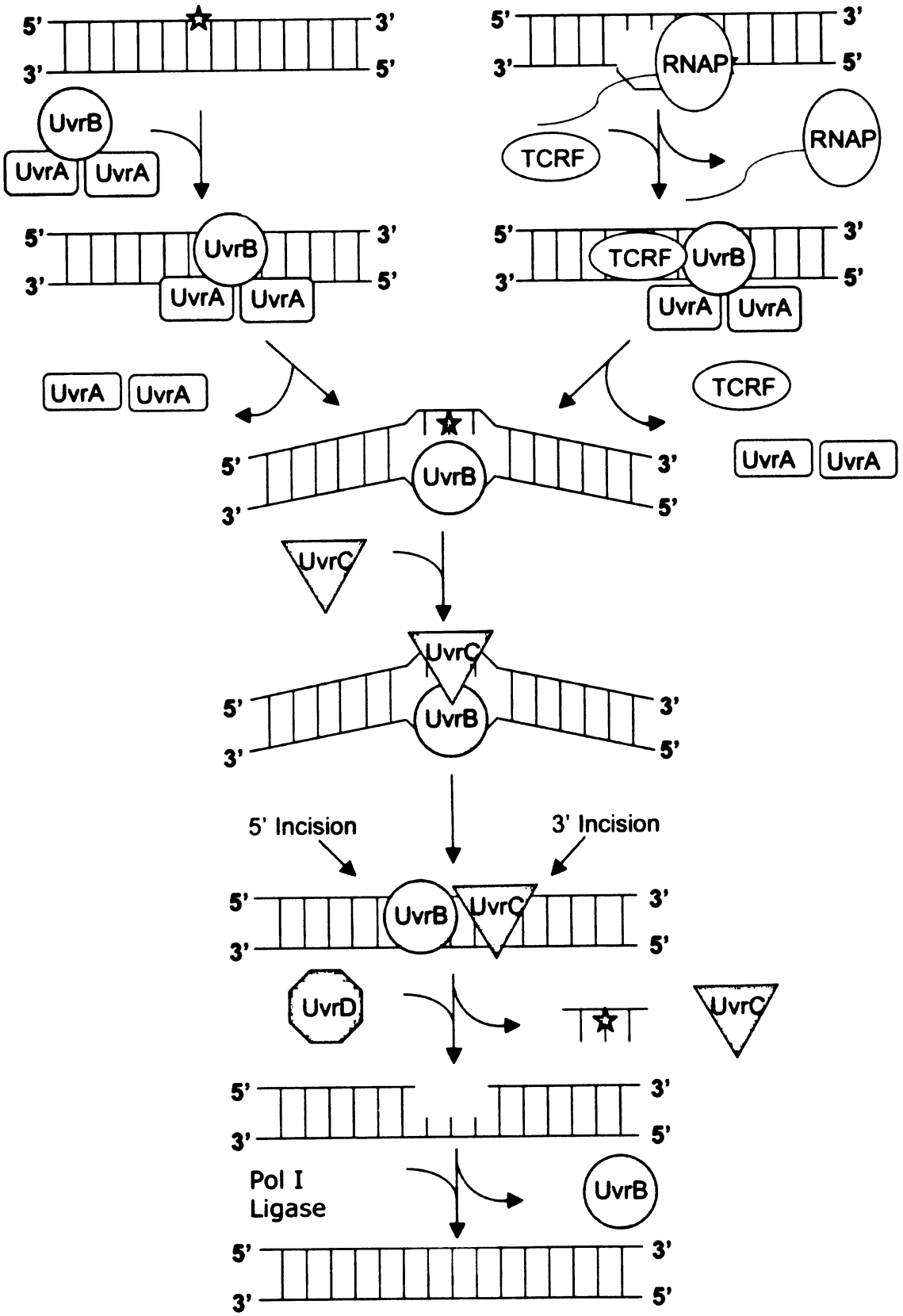
UV-induced cyclobutane thymine-thymine dimers (T-T dimers) were eliminated from DNA by excision [35, 36]. It is now understood that excision of damaged sequences from DNA is one step in a complex pathway that has come to be known as nucleotide excision repair (NER). The nucleotide excision repair process works through a "cut and patch" mechanism whereby a stretch of DNA containing damage is excised and then restored by DNA repair synthesis (reviewed in [37]). Nucleotide excision repair is the primary repair process for removal of a variety of bulky DNA adducts, such as those formed by exposure to UV radiation i.e. T-T dimers and 6-4 photoproducts, as well as those formed by exposure to chemicals such as benzo[a]pyrene or acetylaminofluorene. In addition, single base lesions can be removed through the NER pathway, and therefore, NER can function as a back-up system for lesions escaping base excision repair. Because NER is required for repair of such a diverse set of genotoxic DNA lesions, defects in NER can be detrimental to an organism's survival. Nucleotide excision repair consists of two mechanistically similar pathways termed global genomic NER (GG-NER) and transcription-coupled NER (TC-NER). The global genomic NER pathway removes DNA lesions both from transcriptionally active and transcriptionally silent portions of the genome, discriminating the frequency of lesion removal only based on chromatin status. In contrast, transcription-coupled NER is highly specific for removal of DNA lesions generated in genes that are being actively transcribed (reviewed in [38]).

1. Global Genomic Nucleotide Excision Repair in Prokaryotes

The nucleotide excision repair process consists of four basic steps: (1) recognition of damaged DNA, (2) excision of the DNA damage by dual incisions, (3) DNA synthesis to repair the resulting gap, and (4) ligation. Prokaryotic NER is primarily controlled by the Uvr proteins, which were originally identified based on their ability to complement strains of *E. coli* defective in the ability to repair DNA lesions induced by UV radiation [39].

In prokaryotes, the damage recognition factor is generated in solution, when two subunits of the UvrA protein dimerize in an ATP-dependent manner and then bind to the DNA helicase, UvrB (Figure 2). Once this "damage recognition complex" is formed, UvrA, which is highly specific for binding damaged DNA, binds at or near the site of a DNA lesion. When the damage recognition complex has identified a DNA lesion, UvrB, using its helicase activity, unwinds and bends the DNA at the site of damage. Following this conformational change, UvrA dissociates resulting in formation of the UvrB-DNA pre-incision complex. The pre-incision complex is then able to be recognized and bound by the endonuclease, UvrC. The UvrC endonuclease generates two incisions in the DNA; a 3' incision, 4-5 nucleotides from the site of the lesion, followed by a 5' incision that occurs exactly 8 nucleotides from the lesion. This results in excision of the DNA oligonucleotide containing the lesion, but it remains bound to the post-incision complex until the UvrD helicase displaces both the UvrC protein and the lesion-containing oligonucleotide. The UvrB protein remains bound to the single

Figure 2 - The two pathways of prokaryotic nucleotide excision repair (NER). In prokaryotes, global genomic NER (left) is initiated when the damage recognition complex, consisting of two subunits of the UvrA protein and one subunit of the UvrB DNA helicase, recognizes and binds to DNA at the site of damage. Transcription-coupled NER (right) is initiated when a lesion on the transcribed strand of DNA blocks the progression of RNA polymerase. The stalled RNA polymerase is identified and bound by the transcription-repair coupling factor (TRCF) at the site of DNA damage. Upon binding the damaged DNA, TRCF displaces RNA polymerase along with the unfinished transcript. The $UvrA_2B$ complex then recognizes TRCF bound at the site of DNA damage. Although the mechanisms for lesion recognition differ between the two classes of NER, once the DNA lesion has been identified, the two pathways converge and proceed through the same series of mechanisms. First, the area surrounding the lesion is unwound and bent by the UvrB helicase. The UvrB-DNA complex is then bound by the endonuclease UvrC. The UvrC endonuclease generates an incision on either side of the DNA damage. After the DNA oligonucleotide containing the lesion has been excised, it remains attached until the UvrD helicase displaces both the UvrC protein and the lesion-containing oligonucleotide. The UvrB protein remains bound to the single stranded DNA to prohibit degradation, until it is displaced by DNA polymerase I. Finally, the resultant gap is filled by DNA polymerase I, and the newly synthesized DNA end is sealed by DNA ligase. This figure was adapted from reference [38].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TCRF
UvrB
UvrA
UvrA
UvrC
UvrC
UvrB
5' Incision
3' Incision
UvrB
UvrC
UvrD
UvrC
Pol I
Ligase
UvrB

2. Transcription-Coupled Nucleotide Excision Repair in Prokaryotes

In *E. coli*, DNA lesions that occur in actively transcribed portions of the genome are preferentially repaired [41]. Such transcription-coupled repair occurs via an NER pathway that overlaps the global genomic NER pathway described previously. In transcription-coupled NER, however, recognition of the DNA lesion is initiated by a stalled RNA polymerase complex (Figure 2). If RNA polymerase becomes stalled by the occurrence of a DNA lesion, its very presence inhibits global genome NER by interfering with the ability of the damage recognition complex, $UvrA_2B$, to bind to the site of DNA damage. In this case, the transcription-repair coupling factor, TRCF binds to the site of DNA damage and displaces RNA polymerase along with the unfinished transcript. The $UvrA_2B$ complex recognizes TRCF bound at the site of DNA damage. Although the initial recognition step differs in TC-NER, requiring a stalled RNA polymerase and TRCF, excision of the DNA lesion, repair synthesis, and ligation proceed via the same process as in GG-NER [38].

3. Global Genomic Nucleotide Excision Repair in Eukaryotes

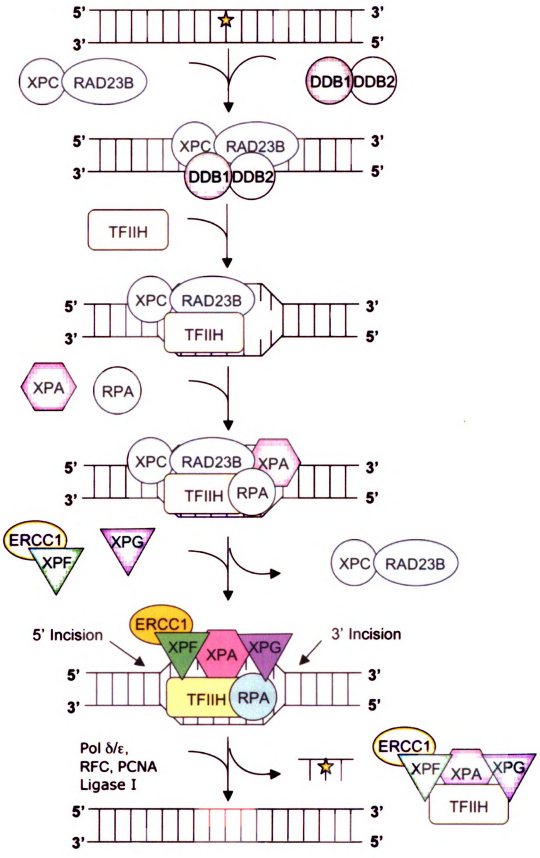
Nucleotide excision repair in eukaryotes proceeds through a similar set of reaction mechanisms as those used by prokaryotic NER, beginning with

recognition of DNA damage, followed by excision of the DNA lesion via dual incisions, and finally gap-filling DNA synthesis. However, eukaryotic NER requires the function of a larger set of proteins. Surprisingly, although the mechanistic properties of the eukaryotic NER proteins are highly similar to the prokaryotic Uvr proteins, there is no evolutionary relationship between the proteins required for NER in prokaryotes and eukaryotes [42].

Most researchers agree that, in human NER, the formation of the pre-incision complex requires at least four factors, replication protein A (RPA), XPA, the XPC-RAD23B complex and the transcription factor TFIIH. However, it remains unclear which of these proteins is the true damage recognition factor. All of these factors show a preference for binding damaged DNA, but the order of assembly of these factors (if a specific order of assembly is necessary) remains unproven [43]. Adding to the already confusing assembly scheme in human NER is the fact that the damaged DNA binding protein (DDB) has been shown to play a role in recognition of cyclobutane pyrimidine-pyrimidine dimers (CPDs), the most frequent lesion induced by UV radiation [44].

Currently, the most commonly accepted scheme for assembly of the pre-incision complex (Figure 3) is as follows. First, the site of DNA damage is recognized by the XPC-RAD23B complex in collaboration with the DDB complex. After the DNA lesion has been recognized, XPC-RAD23B and/or the DDB complex recruit the

Figure 3 - The global genomic nucleotide excision repair pathway in eukaryotes. The site of a DNA lesion is recognized and bound by the XPC-RAD23B complex in collaboration with the DDB complex. After the DNA lesion has been identified, the transcription factor TFIIH is also recruited to the site of the DNA lesion. TFIIH unwinds DNA at the lesion site using the helicase activity of its subunits XPB and XPD. The single-stranded DNA generated by the combined action of XPC-RAD23B and TFIIH facilitates recruitment of the single-stranded binding protein, RPA, along with XPA, to verify the presence of DNA damage. The XPG nuclease makes the first incision 3' of the lesion and then the XPF-ERCC1 complex makes an incision 5' of the lesion. The damaged DNA is released as a 25-30 residue oligonucleotide. The resulting DNA gap is filled by DNA polymerase δ/ε along with the accessory factors, PCNA and RFC. In the final step of NER, the newly synthesized DNA end is sealed by DNA ligase I. This figure was adapted from reference [45].


5'
3'
3'
5'
XPC
RAD23B
DDB1
DDB2
TFIIH
XPA
RPA
ERCC1
XPF
XPG
5' Incision
3' Incision
Pol δ/ε,
RFC, PCNA
Ligase I

transcription factor TFIIH to the DNA lesion. TFIIH unwinds DNA at the site of the lesion using the helicase activity of subunits XPB and XPD, creating short stretches of single-stranded DNA surrounding the lesion. The single-stranded DNA generated by the combined action of XPC-RAD23B and TFIIH facilitates recruitment of the single-stranded binding protein, RPA, along with XPA, to verify the presence of DNA damage. The opening of the pre-incision complex results in 25-30 residues of single-stranded DNA, which is the substrate for cleavage by the nucleases XPG and XPF-ERCC1. The XPG nuclease makes the first incision 3' of the lesion, and then the XPF-ERCC1 complex makes an incision 5' of the lesion. The damaged DNA is then released as a 25-30 residue oligonucleotide. The resulting DNA gap is filled by DNA polymerase δ/ε along with the accessory factors, proliferating cell nuclear antigen (PCNA) and replication factor C (RFC). In the final step of NER, the newly synthesized DNA end is sealed by DNA ligase I (for review see [43, 45]).

4. Transcription-Coupled Nucleotide Excision Repair in Eukaryotes

The mechanism of transcription-coupled NER in mammalian cells is much less well understood then that of prokaryotes. However, many proteins thought to play a role in TC-NER have been identified. The two most well known factors that participate in human TC-NER are the CSA and CSB proteins. These two proteins were identified based on their ability to complement impaired TC-NER in cells derived from patients suffering from Cockayne syndrome (see below) [46]. The CSB protein interacts with the transcription machinery dynamically, and this

interaction is stabilized in the presence of DNA damage [47]. CSB serves as the transcription-repair coupling factor by recruiting the pre-incision NER factors and the CSA protein to the site of DNA damage [48, 49]. The CSA protein has been shown to be part of an E3-ubiquitin ligase complex also consisting of DDB1, Culin 4A and Roc1/Rbx1. In response to UV radiation, the CSA complex was shown to associate with the COP9 signalasome (CSN), resulting in inactivation of the ubiquitin ligase activity of the CSA complex. These data suggests that although CSA is absolutely required for TC-NER, the ubiquitin ligase function of the CSA complex is dispensable [50].

The XBA2 and the HMGN1 proteins also play a role in mammalian TC-NER. Both factors have been shown to interact with stalled RNA polymerase II (RNAPII) complexes in a UV and CS-dependent manner [48]. The XBA2 protein has also been shown to interact with the XPA protein, suggesting that XBA2 functions as a scaffold factor in TC-NER [51]. HMGN1 is a nucleosome binding protein that is thought to activate the pre-incision complex by facilitating chromatin remodeling and activating histone hyperacetylation by the histone acetyltransferase, p300 [48]. Changes in chromatin structure are known to occur during NER and remodeling of the DNA/RNAPII interface may be required for repair factors to access the DNA lesion and/or to facilitate replication restart [52, 53]. Finally, unlike prokaryotic TC-NER, in mammalian TC-NER, RNAPII likely remains bound at the site of the DNA lesion [48, 54-57]. This would be expected to be a barrier to access and repair the DNA lesion. This barrier might be

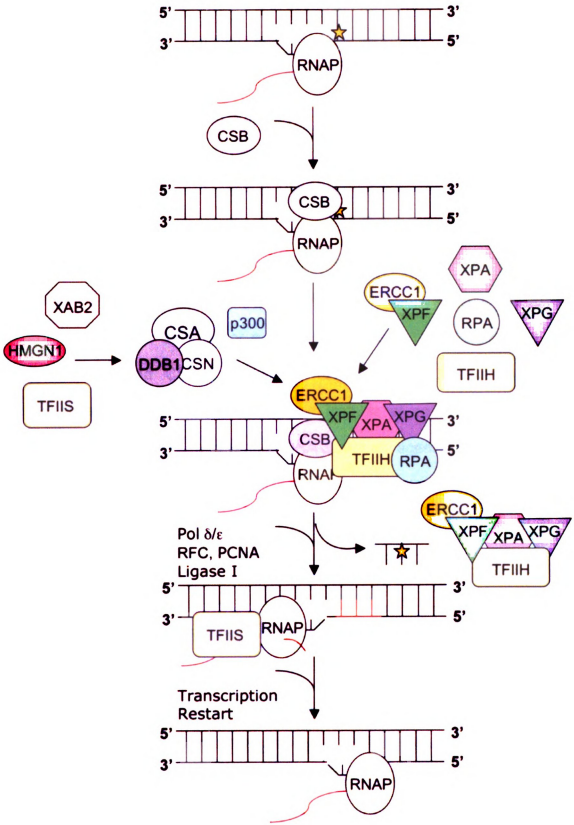
removed by RNAPII backtracking in the presence of the transcription factor, TFIIS. It has been speculated that TFIIS is recruited to a stalled RNAPII complex in a CS-dependent manner shifting the transcription bubble backwards allowing access to the DNA lesion [37]. After the DNA lesion is repaired, TFIIS can also stimulate cleavage of the extruded mRNA to reposition the 3' end by RNAPII allowing for transcription restart [58].

Based on these studies, a tentative model for TC-NER in humans has been suggested (Figure 4). If the RNAPII encounters a DNA lesion that it is unable to bypass during transcription, CSB forms a stable complex with stalled RNAPII. CSB facilitates chromatin remodeling by recruiting the histone acetyltransferase, p300 and also promotes recruitment of the pre-incision NER factors to the DNA damage site. CSB is also required for recruitment of the CSA-DDB1 E3-ubiquitin ligase/CSN complex. Once both CSB and the CSA complex are present, HMGN1, XAB2 and the TFIIS proteins are recruited to the site of DNA damage to facilitate further chromatin remodeling and transcription restart [37, 48].

5. Nucleotide Excision Repair Deficiency Diseases

At least three categories of disease result from impaired NER, i.e., xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD). Xeroderma pigmentosum is caused by defects in the mammalian NER system [59]. There are seven XP complementation groups, designated XP-A through

Figure 4 - The transcription-coupled nucleotide excision repair pathway in eukaryotes. The CSB protein interacts with the transcription machinery dynamically, and this interaction is stabilized in the presence of transcription-blocking DNA damage. CSB serves as the transcription-repair coupling factor by recruiting the pre-incision NER factors, RPA, XPA, XPG and the transcription factor TFIIH, as well as the histone acetyl transferase p300, and the CSA-DDB-CSN complex to the site of DNA damage. CSA facilitates further chromatin remodeling by recruiting HMGN1, XAB2 and TFIIS. These remodeling events enable cleavage on each side of the DNA lesions by the XPF-ERCC1 complex. Gap filling synthesis is then conducted by the Pol δ/ε complex, and the resultant DNA ends are ligated by DNA Ligase I. After the DNA lesion is repaired, TFIIS can stimulate cleavage of the extruded mRNA to reposition the 3' end allowing for transcription restart. This figure was adapted from references [37, 48].


5'
3'
3'
5'
RNAP
CSB
CSB
RNAP
XAB2
HMGN1
TFIIS
CSA
DDB1
CSN
p300
ERCC1
XPF
XPA
RPA
XPG
TFIIH
CSB
RNAP
Pol δ/ε
RFC, PCNA
Ligase I
TFIIS
Transcription
Restart

XP-G, that are the result of mutations in seven different proteins that are involved in NER (note that one additional complementation group, XP-V, results from mutations in DNA polymerase η and will be discussed in the upcoming section on translesion synthesis). Because NER is impaired in XP patients, they retain DNA damage and as a result, demonstrate severe photosensitivity and are at least a 1000 times more susceptible to UV-induced skin cancer.

Cockayne syndrome is a rare autosomal recessive disorder initially described in the 1930s by pediatrician Edward Cockayne [60]. Cockayne syndrome results from defects in either the CSA or much more frequently, the CSB protein [46]. Both the CSA and CSB proteins are required for TC-NER, and as a consequence, one hallmark of CS is the inability of cells to resume RNA synthesis following exposure to various DNA damaging agents [61]. Patients with CS exhibit a variety of symptoms, including dwarfism, impaired development of the nervous system, premature aging, and photosensitivity, but in contrast to XP patients, CS patients are not more susceptible to cancer [62].

Trichothiodystrophy is caused by mutations in the XPD, XPB, or TTDA proteins, all of which are subunits of the transcription factor TFIIH. Because TFIIH plays a role in transcription as well as in NER, it is unclear whether the phenotype of TTD patients results from defects in transcription or in DNA repair [45, 63]. Patients with TTD demonstrate developmental and neurological abnormalities similar to patients with Cockayne syndrome but in addition, exhibit the hallmark brittle hair

and nails and scaly skin, which result from a defect in synthesis of sulfur-rich proteins.

C. Mismatch Repair

DNA mispairs can occur as a result of several processes, most commonly by misincorporation of nucleotides by the replicative DNA polymerases during DNA synthesis (reviewed in [1]). If mismatches in DNA are not corrected, they can lead to mutations. The mismatch repair pathway (MMR) is responsible for correcting most DNA mispairs, thereby preventing the introduction of mutations. Unlike other DNA repair processes, where the target for repair, a DNA lesion, can be identified based on its abnormal structure, for mismatch repair the mismatched base is a normal constituent of DNA. As a result, the key component for avoiding mutations by mismatch repair is identifying which of the mispaired bases was erroneously generated. Inactivation of the mismatch repair system results in spontaneous mutation rates that are 50-1000 times higher than normal, indicating the importance of the MMR pathway for protecting against mutagenesis (reviewed in [64]).

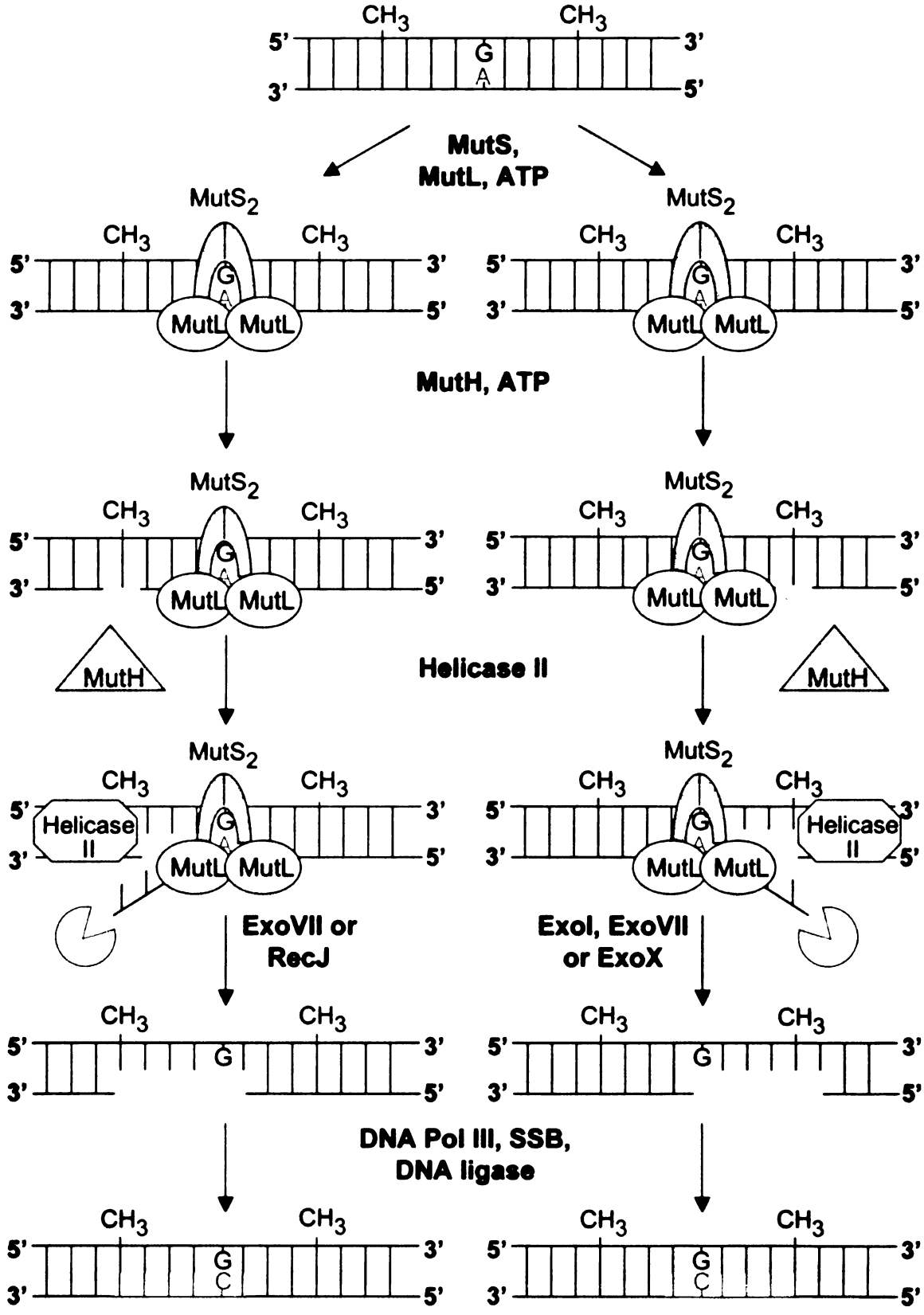
1. Mismatch Repair in Prokaryotes

The MMR pathway has been most extensively studied and is best characterized in *E. coli* (for review see [1, 64, 65]). As described above, the greatest barrier to

correctly repairing a DNA mismatch is directing repair to the newly synthesized daughter strand. In *E. coli*, the problem of strand discrimination is reconciled by use of methyl-directed mismatch repair. Because newly synthesized DNA in *E. coli* is subject to methylation at GATC sites, and because this methylation occurs slightly behind DNA synthesis, the difference in methylation status between the parent and daughter DNA strand permits strand discrimination [66].

The mismatch repair pathway in *E. coli* proceeds through four basic steps: incision of the unmethylated strand near the site of the mispair; excision of the DNA strand containing the mismatch; DNA synthesis to fill the resulting gap; and ligation (Figure 5). The first step of the MMR pathway requires recognition of the DNA mismatch. In *E. coli*, the "mismatch recognition factor" is a homodimer of the MutS protein, which recognizes and binds to single base DNA mispairs as well as small insertion/deletion loops generated by replication slippage [67, 68]. Once bound to the DNA mispair, MutS recruits a homodimer of MutL in an ATP-dependent manner [69]. After the $MutS_2$-$MutL_2$ complex has been formed, the MutH endonuclease is activated and nicks the unmethylated strand of hemimethylated DNA at a GATC site. The nicked DNA strand serves as a signal to direct the ensuing excision repair enzymes to remove the mismatch-containing portion of the unmethylated strand. Cleavage of the unmethylated DNA strand by MutH is bidirectional and as such, can occur either 5' or 3' of the DNA mispair [70, 71]. DNA helicase II recognizes the nicked strand and unwinds the DNA toward the mismatch [72]. Depending on the position of the DNA nick relative to

Figure 5 - The prokaryotic mismatch repair pathway. A homodimer of the MutS protein, recognizes and binds to single base DNA mispairs (indicated in red) as well as small insertion/deletion loops generated by replication slippage. Once bound to the DNA mispair, MutS recruits a homodimer of MutL. The $MutS_2$-$MutL_2$ complex recruits the MutH endonuclease, which nicks the unmethylated strand of hemimethylated DNA at a GATC site. The nicked DNA strand serves as a signal to direct the ensuing excision repair enzymes to remove the mismatch-containing portion of the unmethylated strand. Cleavage of the unmethylated DNA strand by MutH is bidirectional and can occur either 5' (right) or 3' (left) of the DNA mispair. DNA helicase II recognizes the nicked strand and unwinds the DNA toward the mismatch. Depending on the position of the DNA nick relative to the mismatch, the displaced portion of the nicked strand is degraded from the nick to slightly past the DNA mispair either by the 3'→5' exonucleases ExoI, ExoVII, or ExoX (right) or the 5'→3' exonucleases ExoVII or RecJ (left). The resulting DNA gap is then stabilized by single-stranded DNA binding protein, and filled by DNA polymerase III. The DNA ends are sealed by DNA ligase. This figure was adapted from reference [64].


CH3
CH3
5'
3'
G
A
MutS,
MutL, ATP
MutS2
MutL
MutH, ATP
MutH
Helicase II
ExoVII or
RecJ
ExoI, ExoVII
or ExoX
DNA Pol III, SSB,
DNA ligase
C

the mismatch, the displaced portion of the nicked strand is degraded from the nick to slightly past the DNA mispair, either by the 3'→5' exonucleases ExoI, ExoVII, or ExoX or the 5'→3' exonucleases ExoVII or RecJ [73, 74]. The resulting DNA gap is then stabilized by single-stranded DNA binding protein, and filled by DNA polymerase III. Finally, the DNA ends are sealed by DNA ligase completing the *E. coli* MMR pathway [75].

2. Mismatch Repair in Eukaryotes

The mismatch repair process is thought to be highly conserved from prokaryotes to eukaryotes with similarities including substrate specificity, bidirectionality, and nick-directed strand specificity (for review see [1, 64, 65]). Many of the eukaryotic MMR proteins have been identified based on their homology to the *E. coli* MMR proteins. In humans, five MutS homologs (MSH2-MSH6) and four MutL homologs (MLH1, MLH3, PMS1 and PMS2) have been identified, although the primary function of some of these proteins seems to be in processes other than MMR [76-85]. In humans, unlike in *E. coli*, the MutS and MutL homologs function as heterodimers. Human MSH2 can dimerize with either MSH6 to form MutSα [86, 87] or with MSH3 to form MutSβ [88]. Similar to their homologs in *E. coli*, both MutSα and MutSβ are mismatch recognition factors. MutSα recognizes single base mismatches as well as small insertion/deletion loops, whereas MutSβ seems to be involved only in recognition of larger insertion/deletion loops [87, 89]. Of the four MutL homologs, only MutLα, composed of a heterodimer of

MLH1 and PMS2, is required for MMR in humans [90]. In addition to the MutS and MutL homologs, several other factors have been demonstrated to be required for human MMR, including the exonuclease, Exo1 [91, 92]; the single-stranded binding protein, RPA [93, 94]; proliferating cellular nuclear antigen (PCNA) [95-97]; replication factor C (RFC) [98, 99]; DNA Polymerase δ [100]; and DNA ligase I [99].

Several studies have demonstrated that the initial steps of human MMR are similar to those in *E. coli*. Specifically, MutSα or MutSβ first binds to a DNA mismatch and then recruits MutLα in an ATP-dependent manner [97, 101-104]. In *E. coli*, after the $MutS_2$-$MutL_2$ complex has been formed, the MutH endonuclease is activated and nicks the unmethylated newly synthesized DNA strand at a GATC site, thereby conferring strand discrimination. However, in eukaryotic cells, strand discrimination must occur through a distinct mechanism because eukaryotes do not posses the Dam methylase enzyme [1]. Although the signals that direct strand discrimination in human cells are unknown, strand specificity is thought to be nick-directed because in human cell-free extracts nicked DNA is sufficient to direct strand specificity [105, 106].

In *E. coli*, the excision step of MMR requires the function of DNA helicase II and multiple exonucleases. In contrast, in human MMR there appears to be no requirement for a DNA helicase function [107-109], and only one exonuclease, Exo1, has been convincingly implicated thus far [64]. Similar to bacteria, in

humans, excision occurs both in a 5'→3' and a 3'→5' direction based on the placement of the strand discriminating nick, however in humans the two processes occur through distinct mechanisms. When a nick is placed 5' of the mispair, MutSα stimulates the 5'→3' exonuclease activity of ExoI, which begins excision at the 5' nick and continues until the mispair has been removed [89]. Termination of the exonuclease activity after removal of the mispair is dependent on RPA [89]. This 5'-directed excision reaction mechanism requires the activity of only three proteins, Mutsα, ExoI and RPA, although MutLα does enhance the mismatch dependence of the reaction [89]. In contrast, when the nick is placed 3' of the mispair, the activity of at least two other factors, RFC and PCNA are required [98]. In 3'-directed excision, RFC and PCNA suppress the 5'→3' exonuclease activity of ExoI and activate a cryptic 3'→5' exonuclease activity, resulting in removal of the mispair [98]. It has been suggested that the directionality of excision is regulated by the orientation in which PCNA is loaded [64]. In other words, a different face of the PCNA clamp would be oriented toward a mispair located 5' of the nick than would be oriented if the mispair was located 3' of the nick.

3. Mismatch Repair Deficiency Leads to Cancer

The primary role of the MMR pathway is to remove incorrectly paired bases or insertion/deletion loops generated during DNA synthesis, thereby offering protection against mutation. Deficiencies in MMR have been associated with an

increased rate of cancer development. Most commonly, defects in MMR have been associated with development of hereditary nonpolyposis colon cancer (for review see [1, 110]). Hereditary nonpolyposis colon cancer (HNPCC) or Lynch Syndrome [111] is an autosomal dominant disorder and is one of the most common cancer predisposition diseases of humans. Patients with HPNCC have an ~80% risk of developing colon cancer in their lifetime. Thus far, mutations in at least seven genes associated with MMR, including *MLH1*, *MSH2*, *MSH6*, *PMS1*, *PMS2*, *MLH3*, and *EXO1* have been linked to HNPCC. Mutations in mismatch repair genes are thought to contribute to HNPCC by causing an accumulation of deletion or insertion mutations in simple, repetitive microsatellite sequences, a hallmark of HNPCC.

II. DNA Damage Tolerance

As described above, many of the lesions generated in DNA can be repaired by excision of the damaged portion of the DNA. However, if DNA repair is slow or if the DNA damage is extensive, lesions may persist into DNA replication. The presence of lesions during DNA synthesis can cause replication to arrest, which can ultimately lead to cell death. To avoid the threat of cell death, cells have also evolved strategies for coping with the DNA lesions that remain during DNA replication. Because these mechanisms do not result in the physical removal of the DNA damage, but rather prevent the potentially lethal consequences of arrested replication, they are collectively referred to as DNA damage tolerance mechanisms. One such damage tolerance mechanism is translesion synthesis (TLS). Translesion synthesis is the process in which specialized DNA polymerases insert nucleotides opposite DNA lesions, thereby alleviating replication arrest. Depending on which specialized polymerase is involved, the type of DNA lesion being bypassed, and even the sequence context surrounding the DNA lesion, TLS can be either error-free or error-prone. In many organisms, error-prone TLS is a major source of mutations (reviewed in [112]).

In contrast to TLS, where the damaged portion of the DNA is used as a template to direct DNA synthesis, cells can also employ a second method of damage tolerance that makes use of a strand switch mechanism, whereby a homologous copy of the damaged DNA strand is temporarily used as an error-free template to

replicate past the DNA lesion. In this mechanism, synthesis across from the DNA lesion is not required, and thus this mechanism is often referred to as DNA damage avoidance. Because an undamaged, homologous strand of DNA is used as the template to direct DNA synthesis, damage avoidance is typically an error-free mechanism of damage tolerance (for review see [9]).

A. DNA Damage Tolerance Mechanisms in Prokaryotes

Although the existence of DNA damage tolerance mechanisms was recognized only relatively recently. Many of the important advances that led to their discovery were based on studies of the mechanism of mutagenesis in *E. coli* (reviewed in [1]). As it turns out, many of the mechanisms of DNA damage tolerance first elucidated in bacteria are conserved in eukaryotes, including humans. Because of the speed and ease with which bacterial systems can be manipulated, prokaryotes are still considered to be among the most valuable models available for studying DNA damage tolerance mechanisms. Moreover, the knowledge gained from such studies has fundamentally changed our understanding of the ability of an organism to sustain life in the face of consistent exposure to DNA damaging agents.

1. The SOS Response to DNA Damage

In 1974, Miroslav Radman hypothesized that *E. coli* possess a DNA repair system, which he called "SOS repair" (now more commonly known as the SOS response), that is repressed under normal physiological conditions, but that can be induced in response DNA damage [113]. The SOS response system was the first DNA damage-induced regulatory network to be characterized. It is now known that the *E. coli* SOS response system controls the expression of more than 40 genes than encode proteins required for a variety of DNA damage response mechanisms (for review see [1]).

Just as Radman suggested in 1974, the SOS response system in *E. coli* is repressed under normal physiological conditions by the LexA protein (see [1] for a more thorough review). In the absence of DNA damage or replication stress, the LexA protein binds to the operator sequences (SOS boxes) of genes controlled by the SOS response system and represses their transcription. However, if *E. coli* are exposed to DNA damaging agents or replication is interrupted, the SOS response system can be mobilized through activation of the RecA protein. The RecA protein becomes activated when it polymerizes along single-stranded regions of DNA that are generated by discontinuous DNA synthesis. These RecA nucleoprotein filaments stimulate LexA autodigestion, resulting in inactivation of the LexA repressor function and ultimately, LexA degradation. As the level of the LexA repressor decreases, the SOS genes begin to be derepressed. Genes whose operators are bound relatively weakly by LexA, are the first to be fully induced. If DNA damage and replication stress persist,

additional RecA nucleoprotein filaments are generated and RecA dependent cleavage of LexA continues. If the amount of LexA declines to a very low level, then even the genes whose operators are bound tightly by LexA are expressed. As the cell is able to complete DNA synthesis and eliminate single-stranded DNA gaps, the amount of activated RecA decreases and as a consequence, the amount of LexA repressor is elevated. As the amount of LexA accumulates, the SOS genes are once again repressed.

One important advantage of the *E. coli* SOS response is that the SOS genes are derepressed sequentially, based on how tightly the LexA repressor binds to their operator sequence (reviewed in [1]). This mechanism of chronological derepression of the SOS genes allows damage response mechanisms to be activated preferentially. For example, SOS-induced responses such as nucleotide excision repair, are induced first. Potentially mutagenic responses, such as translesion synthesis, are induced later, only as a final effort to rescue the cell from persistent DNA damage. Several other SOS-induced responses that destabilize the *E. coli* genome, such as adaptive mutagenesis, can be induced to offer long-term survival benefits by increasing the probability of acquiring a preferential genetic change.

2. Translesion Synthesis in Prokaryotes

It has long been known that induction of the SOS response system can be accompanied by a 100-fold increase in mutations [114]. However, for many years the source of this so-called SOS mutagenesis remained unknown. It is now recognized that the mechanistic basis for SOS mutagenesis is translesion synthesis. As described above, translesion synthesis allows the cell to avoid replication arrest by employing specialized polymerases to synthesized DNA past lesions that would typically block the major replicative polymerases. However, many of the TLS polymerases replicate DNA with a relaxed fidelity. As a result, the price of increased cell survival sometimes comes at the cost of increased mutagenesis. In *E. coli*, TLS is primarily conducted by two enzymes, which were first referred to as DinB and UmuC, but are now known as DNA polymerases IV and V.

a. DNA Polymerase V (UmuD'$_2$C)

In 1977, two independent laboratories screened for mutations in *E. coli* that could abolish UV-induced SOS mutagenesis. These screens identified mutations that could be mapped to three different loci in *E. coli*: the *recA*$^+$ locus and the *lexA*$^+$ locus (which were known regulators of the SOS response), as well as a previously uncharacterized locus called *umuC*$^+$ (UV-induced mutability) [115, 116]. It was later demonstrated that the *umuC*$^+$ locus is actually composed of two genes, *umuC*$^+$ and *umuD*$^+$, either of which when mutated eliminate UV and chemical-induced SOS mutagenesis in *E. coli* [117, 118].

Studies of the UmuD protein have indicated that in solution, it exists as a homodimer [119]. The UmuD homodimer is not active in mutagenesis, however interaction with RecA nucleoprotein filaments stimulates autodigestion of the N-terminal 24 amino acids of UmuD [120]. The resulting proteolytic fragment of UmuD, designated UmuD', is active in SOS-induced mutagenesis [121, 122]. This activated UmuD' homodimer binds a monomer of UmuC to form the UmuD'$_2$C complex [119]. The UmuD'$_2$C complex supports a polymerase activity and is, therefore, also referred to as DNA polymerase V (Pol V) [123, 124].

It is now recognized that *E. coli* DNA polymerase V belongs to the special subset of polymerases that conduct replication past fork-blocking DNA lesions, i.e. translesion synthesis. Translesion synthesis polymerases are typically much less faithful than the major replicative polymerases and, in addition, they lack 3'→5' proofreading activity. Therefore translesion synthesis is often associated with a high mutagenic potential. The idea that UV-induced SOS-dependent mutagenesis in *E. coli* is a direct result of error-prone TLS by Pol V was tested when Tang *et al.* [125] reconstituted translesion synthesis reactions *in vitro* using purified Pol V, and template primers that contained either UV-induced thymine-thymine cyclobutane pyrimidine dimers (T-T dimers) or thymine-thymine 6-4 pyrimidine-pyrimidone photoproducts (6-4 photoproducts). These investigators found that Pol V was able to bypass both T-T dimers and 6-4 photoproducts efficiently. Moreover, they found that the mutation spectrum generated by Pol V

in vitro was extremely similar to that which results from SOS-dependent UV-induced mutagenesis *in vivo*, confirming the role of error-prone TLS by Pol V in SOS-induced mutagenesis [125].

As described above, initial studies of the *umuDC* gene products indicated that only the proteolytic fragment of UmuD, i.e. UmuD', participates in mutagenic TLS, suggesting that UmuD is nothing more than an inactive precursor of UmuD' (reviewed in [1]). However, more recently Graham Walker's laboratory [126] demonstrated that expression of a non-cleavable form of the UmuD protein, along with UmuC, increases cell survival following exposure to UV radiation, despite the fact that UmuD is unable to participate in TLS. What is more, they showed that expression of these proteins causes a UV-induced delay in DNA replication. Taken together, these results suggest a role for UmuD along with UmuC in regulation of DNA damage-induced checkpoints.

Based on these more recent results, the Walker laboratory has proposed an intriguing DNA damage tolerance model where the products of the *umuDC* operon promote increased cell survival by two distinct and sequential mechanisms. Upon exposure to DNA damaging agents, RecA mediated cleavage of the LexA repressor results in derepression of the *umuDC* operon, resulting in an increase in the level of the UmuD and UmuC proteins. Together, the UmuD and UmuC proteins act to delay DNA synthesis and cell growth, allowing additional time for DNA repair to occur and ultimately increasing cell

survival. If DNA lesions persist, RecA promotes autocleavage of UmuD to form UmuD'. Formation of UmuD' switches the function of the *umuDC* gene products to their second role, which is translesion synthesis. Translesion synthesis past the remaining DNA lesions by the UmuD'$_2$C complex (Pol V) further promotes cell survival by allowing DNA replication to resume. This sequential response to DNA damage allows for an added measure of control by preferentially promoting accurate DNA repair before potentially mutagenic TLS [126].

b. DNA Polymerase IV (DinB)

Induction of the SOS response system contributes to increased mutagenesis in two ways termed targeted and untargeted mutagenesis. Targeted mutagenesis occurs as a result of an SOS-dependent increase in the frequency of error-prone replication of DNA lesions, i.e. TLS, and requires the function of Pol V and RecA as discussed above. On the other hand, SOS-dependent untargeted mutagenesis occurs in the absence of DNA damage and consists of two distinct pathways. One pathway of untargeted mutagenesis can be observed when an undamaged lambda phage is transfected into a host previously exposed to a DNA damaging agent. In such lambda phage untargeted mutagenesis, the number of mutations that occur in the undamaged phage increase as a consequence of being replicated in a host previously exposed to a DNA damaging agent. In other words, the lambda phage is replicated in an error-prone manner despite the absence of DNA lesions. In *E. coli*, untargeted mutagenesis of lambda phage is dependent upon the function of the *dinB* gene.

The *dinB* gene was originally identified in a screen for genes induced by DNA damage [127]. This same locus was subsequently identified by the Ohmori laboratory (and named dinP) as a putative SOS-inducible gene during an *E. coli* genome sequencing project [128]. Consistent with the requirement for DinB in untargeted mutagenesis, overexpression of the DinB protein in *E. coli* causes a dramatic increase in the frequency of spontaneous - 1 frameshift mutations (referred to as the DinB mutator phenotype).

Subsequent studies demonstrated that purified DinB protein possesses a polymerase activity [129]. Moreover, eliminating the polymerase activity of DinB by mutation, abolishes the DinB mutator phenotype in *E. coli*, indicating that the polymerase activity of DinB is required for its mutagenic function [129]. Based on these studies, as well as its homology to the UmuC family of proteins, the DinB protein was determined to be an *E. coli* TLS polymerase, which is named DNA polymerase IV [129].

Most TLS polymerases are characterized by their relatively low fidelity when replicating undamaged DNA, both as a result of misincorporation of nucleotides as well as their lack of 3'→5' exonuclease (proofreading) activity. Like other TLS polymerases, Pol IV is devoid of 3'→5' exonuclease activity [129]. However, steady state kinetic analysis of the frequency of misinsertions by Pol IV on undamaged DNA indicates that it is only four to fivefold less accurate when

replicating undamaged DNA than is *E. coli* Pol III, the major replicative DNA polymerase [125, 130]. These data indicate that, although Pol IV has been implicated in untargeted and spontaneous mutagenesis, it is a relatively faithful enzyme, at least for the insertion step, of DNA synthesis [131]. In contrast, Pol IV is much less stringent when extending primer termini. Pol IV is capable of elongating misaligned primer/template structures [129]. Because an increase in the frequency of -1 frameshifts is observed in both *dinB*-dependent untargeted mutagenesis, as well as in the DinB mutator phenotype, it is thought that Pol IV likely contributes to mutagenesis by extending misaligned primer termini [131-133].

Although the primary role of Pol IV seems to be for mutagenesis in the absence of DNA damage, Pol IV is also capable of performing TLS across a number of different types of DNA lesions *in vitro*, albeit with widely varying efficiencies. Specifically, Pol IV has been shown to be able to bypass 8-oxoguanine [131], O^6-methylguanine [131], abasic sites [125, 134], N-2-acetylaminofluorene and N-2-aminofluorene adducted guanines [135], cyclobutane pyrimidine dimers and 6-4 pyrimidine-pyrimidone photoproducts [125], cisplatinated guanine adducts [136], and benzo[a]pyrene diol epoxide adducted guanines (BPDE-G) [137]. However, whether or not the specificity of Pol IV *in vitro* reflects its *in vivo* action remains unclear because, although *in vivo* studies support a role for Pol IV in bypassing oxidative damages [138], alkylating DNA lesions [139] and BPDE-G adducts [137, 140], Pol IV does not seem to be required *in vivo* for TLS past abasic sites

[134]. Moreover, *dinB* mutant strains exhibit no clear phenotype with regard to mutagenesis following treatment with DNA damaging agents, therefore the role of Pol IV in targeted damage-induced mutagenesis is difficult to reconcile [131].

In addition to its roles in untargeted mutagenesis and TLS, Pol IV has also been implicated in other cellular functions. First, Pol IV has been shown to be important for adaptive mutagenesis. Adaptive mutagenesis refers to the ability of non-dividing cells to acquire advantageous mutations during periods of nutrient stress, which allow them to resume growth despite continued selective pressure. Pol IV is required for ~80% of adaptive mutations which, are typically -1 frameshift mutations, a hallmark of Pol IV-dependent mutagenesis [141]. A further requirement for Pol IV, along with Pol V, is in long-term survival and fitness. Interestingly, both *E. coli* TLS polymerases Pol IV and Pol V are upregulated during stationary phase even in the absence of DNA damage [142, 143]. More importantly, it has been demonstrated that cells lacking either Pol IV or Pol V are much less efficient at competing for energy resources than wild-type bacteria in stationary phase [142], indicating that these polymerases play an important role in promoting genetic diversity [144].

3. Damage Avoidance in Prokaryotes

In the early 1960's, Paul Howard-Flanders and his colleagues observed that *uvrA recA* double mutants of *E. coli*, which are defective in both nucleotide excision

repair and in recombination, are much more sensitive to UV radiation than either of the single mutants [145], reviewed in [146]. These observations were the first indication that the survival of cells exposed to DNA damaging agents is affected by cellular responses other than lesion repair. Shortly after this initial discovery, the Howard-Flanders laboratory also demonstrated that in *E. coli* defective in excision repair (*uvr* cells), DNA replication results in the production of newly synthesized daughter strands that are initially significantly smaller than those generated in unirradiated cells. They further observed that, after increasing lengths of time post-irradiation, these daughter strands are extended, ultimately reaching the length of those synthesized in unirradiated cells [147]. These data were interpreted to mean that, following UV radiation exposure, DNA replication occurs normally until a fork-blocking lesion is encountered, at which point, replication is temporarily delayed and then resumes somewhere beyond the lesion. This aborted DNA synthesis results in the production of a DNA gap in the newly synthesized daughter strand. The fact that DNA replication generates two identical sister molecules, led to the hypothesis that the daughter strand gap generated by aborted DNA synthesis could be filled by a recombination mechanism. This hypothesis was further supported by experiments from the same laboratory, which used density labels to provide direct evidence of exchanges between DNA strands during post-replicative gap filling [148].

Although this model describes a process often referred to as postreplicational repair, this mechanism does not result in the removal of DNA lesions, and therefore, does not constitute a mechanism of repair, but rather a mechanism of tolerance of the presence of DNA damage. This same process is also sometimes referred to as daughter strand gap repair, which is an appropriate designation with respect to the gaps themselves that are repaired by this mechanism (reviewed in [1]).

The mechanistic details and many of the proteins involved in prokaryotic damage avoidance remain uncertain. However, at least some of the proteins involved have been identified and are summarized in a recent review [146]. The function of the *recA* gene is absolutely required, and is likely involved in the pairing of the single-stranded DNA, generated as a result of aborted DNA synthesis, to its homologous DNA strand. About half of the DNA daughter strand gaps are repaired by a process that is dependent on the function of the *recombination protein F* (*recF*) gene, which most likely works in conjunction with the RecO and RecR proteins to direct loading of the RecA protein onto the gapped DNA. The remaining 50% of daughter strand gaps are filled by a process that is both *recF* and *recBC*-independent. The *recF*-independent pathway requires the 5'→3' exonuclease activity of DNA polymerase I. Because DNA polymerase I is known to be involved in joining Okazaki fragments that are formed during lagging strand synthesis, it has been suggested that daughter strand gaps generated in the lagging strand are selectively repaired in the

recF-independent pathway, whereas daughter strand gaps formed in the leading strand may be repaired by the *recF*-independent pathway.

Damage tolerance mechanisms involving recombination provide an alternative error-free strategy to the potentially mutagenic TLS discussed above. However, the fact that the RecA protein is required for both DNA daughter strand gap repair and for TLS by Pol V in bacteria, suggests that these two processes may not be completely independent of one another. Although advances have been made in understanding the mechanisms of damage tolerance in prokaryotes, much more remains to be learned.

B. Damage Tolerance Mechanisms in Eukaryotes

The damage tolerance responses employed by eukaryotes are very similar in strategy to those of prokaryotes. That is to say, in each case either translesion synthesis (TLS) or damage avoidance (DA) mechanisms are employed to allow replication past fork-blocking DNA lesions that would otherwise result in cell death. However, whereas in prokaryotes regulation of damage tolerance processes is primarily controlled by RecA, in eukaryotes the damage tolerance processes are controlled by a more sophisticated strategy of protein conjugation (reviewed in [149]).

Much of what is now known about the eukaryotic DNA damage tolerance mechanisms was originally determined using the budding yeast, *Saccharomyces cerevisiae*, as a model system. However, almost all of the proteins involved in damage tolerance in yeast have functional homologs in higher eukaryotes, suggesting that the mechanisms of DNA damage tolerance are conserved from lower to higher eukaryotes. In yeast, genetic analysis has led to the generally accepted view that the two pathways of eukaryotic damage tolerance, i.e. TLS and DA, are regulated by the function of the *RAD6* and *RAD18* genes. Consequently, mutation of either gene results in the most severe DNA damage-induced sensitivities of all the genes whose functions are known to be required for damage tolerance (reviewed in [149]).

The Rad6 protein is a ubiquitin-conjugating enzyme that forms a heterodimer with Rad18, which is a single-stranded binding protein with ATPase activity [150-152]. Recent studies have indicated that the target of ubiquitination by the Rad6-Rad18 complex is the processivity factor, proliferating cell nuclear antigen (PCNA) [153]. The current model for Rad6-Rad18-dependent regulation of DNA damage tolerance through covalent modification of PCNA is as follows: Rad18 recruits Rad6 to sites of single-stranded DNA gaps (sites of aborted DNA synthesis). The Rad6-Rad18 complex mono-ubiquitinates PCNA on lysine164, which promotes TLS [154]. Further ubiquitination of PCNA (conducted by the Mms2-Ubc13-Rad5 complex discussed below) promotes error-free DA (reviewed in [9]). Although this model suggests an elegant mechanism by which the

eukaryotic damage tolerance processes are regulated, more recent studies have challenged these currently accepted views. In particular, a 2006 study demonstrated, *in vitro*, that mono-ubiquitination of PCNA had no affect on the affinity of the TLS polymerases for PCNA, nor did it alter activity of the TLS polymerases examined [155]. Based on results such as these, it seems that there is still much more to learn about regulation of eukaryotic damage tolerance.

1. Translesion Synthesis in Eukaryotes

Genetic studies in *E. coli* clearly demonstrate that damage-induced mutagenesis is an active process that relies upon the induction of key components of the SOS regulatory system, including RecA and UmuDC (see above). Initially, it was thought that RecA and UmuDC were merely accessory proteins that were able to enhance the processivity of the high fidelity *E. coli* replicative polymerase III, thereby eliminating inhibition of DNA synthesis opposite a DNA lesion. The notion that cells contain specific specialized DNA polymerases, whose function is to carryout TLS was first suggested in 1996, when a heterodimer of the *S. cerevisiae* Rev3 and Rev7 proteins (both long implicated in mutagenesis) was shown to possess a polymerase activity, and to be capable of bypassing a UV-induced thymine-thymine cyclobutane pyrimidine dimer (T-T dimer) [156]. At nearly the same time, the *S. cerevisiae* Rev1 protein was found to possess a deoxycytidyl transferase activity that could promote DNA synthesis past an abasic site [157]. Although the *UmuDC* gene is homologous to the *Rev1* gene,

because neither was related to any of the known DNA polymerases (including the newly discovered heterodimer of Rev3 and Rev7 called Pol ζ), the idea that the *E. coli UmuDC* gene might encode a DNA polymerase was overlooked until 1999, when the UmuD'$_2$C complex was purified to homogeneity and found to demonstrate DNA polymerase activity [123, 124]. Shortly thereafter, a multitude of proteins homologous to UmuD'$_2$C and Rev1 were discovered and also found to be polymerases capable of DNA synthesis past replication-blocking DNA lesions. These translesion synthesis polymerases were initially referred to as the UmuC/DinB/Rev1/Rad30 superfamily, but have now, with the exception of Pol ζ, collectively come to be known as the Y-family of DNA polymerases [158]. To date, over 300 proteins that share homology to the Y-family of polymerases have been identified in eukaryotes and prokaryotes, including archaea [159]. In many cases each organism encodes more than one TLS polymerase. For example, *E. coli* have both Pol IV and Pol V (see above), whereas humans possess four Y-family polymerases, Pol η, Pol ι, Pol κ and Rev1 along with the B-family TLS polymerase, Pol ζ [158, 159].

Overall, the properties of polymerases involved in TLS differ quite dramatically from those of the classical replicative polymerases. For example, the major replicative polymerases are typically highly processive and highly accurate enzymes with active sites that have evolved with strict requirements for correct Watson-Crick base pairing. In addition, many of the classical replicative polymerases contain an intrinsic 3'→5' exonuclease activity that allows them to

remove and resynthesize any mispairs they may generate. In contrast, the TLS polymerases (particularly those from the Y-family) are generally poorly processive enzymes that copy DNA with low fidelity, both as a result of their less restrictive active sites and their lack of 3'→5' exonuclease activity (for review see [160]).

Perhaps the most striking difference between the classical and TLS polymerases is the property by which these enzymes were first characterized, their ability to synthesize DNA opposite lesions. The classical high-fidelity replicative polymerases are predominantly blocked by the presence of DNA lesions, as a consequence of their restrictive active sites. However, the open active sites of TLS polymerases are able to accommodate altered bases and as a result, these polymerases are highly specialized to synthesize DNA opposite distorting lesions [161-164]. Because DNA is constantly subject to damage from both endogenous as well as environmental sources, some of which escape the traditional repair processes, the TLS polymerases can provide the particularly useful advantage of overcoming lesion blocks by conducting replicational bypass across from such DNA lesions. Nevertheless, because of the poor fidelity of the TLS polymerases, the protection of cells from premature replication arrest sometimes comes at the cost of generating mutations (reviewed in [160]).

a. DNA Polymerase ζ

DNA polymerase ζ (Pol ζ) consists of two subunits, a catalytic subunit called

Rev3, as well as an accessory subunit called Rev7. The *REV3* gene, designated *rev* for "reversionless", was initially identified in yeast during a screen for mutants defective in UV-induced reversion of the highly UV-revertible, ochre suppressible *arg4-17* allele [165]. Several years later, a similar type of UV-induced reversion study was used to identify *REV7* in yeast [166].

The *S. cerevisiae REV3* gene was cloned in 1989 and nucleotide sequence analysis revealed that, despite being non-essential for yeast viability, the *REV3* gene encodes a predicted protein with sequence similarity to the B-family of DNA polymerases, which also includes the replicative polymerases α, δ, and ε [167]. It was somewhat surprising therefore that when assayed for polymerase activity, purified Rev3 protein demonstrated only a very weak and unstable activity *in vitro* [156]. The apparent lack of polymerase activity of Rev3 alone was however, thought to be indicative of a requirement for additional subunits to provide enhanced activity and stability to the Rev3 polymerase [156]. Yeast two-hybrid assays demonstrated that Rev7 was capable of associating with Rev3, suggesting that Rev7 was a subunit of a Rev3-containing DNA polymerase [156]. Indeed, upon addition of Rev7, the polymerase activity of Rev3 increased 20 to 30-fold [156]. Because the Rev3/Rev7 complex was the sixth eukaryotic polymerase to be discovered, it was named DNA polymerase ζ [156]. It should be noted that native Pol ζ has never been directly purified from *S. cerevisiae* or from any other species. Therefore, It remains unknown whether or not additional subunits are required for maximum polymerase stability or activity. [168].

Compared to other B-family polymerases, such as Pol δ or Pol ε, yeast Pol ζ is a poorly processive enzyme [156, 169]. Polymerase ζ also lacks 3'→5' exonuclease activity, meaning that, unlike Pol δ or Pol ε, Pol ζ is unable to proofread any mispairs generated during DNA synthesis [156]. Overall, on undamaged DNA, yeast Pol ζ incorporates nucleotides with moderate fidelity. That is to say, Pol ζ, which generates errors at rates of 1.3×10^{-3}, is much less accurate when copying undamaged DNA than related eukaryotic B-family polymerases δ, ε and α (which generate errors at rates of 1.3×10^{-5}, 2×10^{-5}, and 9.6×10^{-5} respectively), but is substantially more accurate than TLS polymerases from the Y-family, such as Pol η or Pol κ (which generate errors at rates of 3.5×10^{-2} and 5.8×10^{-3} respectively) [170]. Although lack of 3'→5' exonuclease activity no doubt partially contributes to the reduced fidelity of Pol ζ, even exonuclease deficient B-family polymerases, such as Pol α, incorporate nucleotides with a higher fidelity (~9.6×10^{-5}) than Pol ζ. Therefore the ability of Pol ζ to discriminate between nucleotides during DNA synthesis, although substantially better than that of Y-family polymerases, is intrinsically poorer than that of related B-family polymerases [170].

Perhaps one of the most remarkable characteristics of Pol ζ is the unusual propensity with which it extends from DNA mispairs [169, 171]. Compared to Pol

α, which is also a B-family polymerase lacking 3'→5' exonuclease activity, yeast Pol ζ is anywhere from twofold to more than 1,200-fold more efficient at extending from terminal mispairs, depending on the sequence context and type of mismatch extended [169]. In fact, in some cases Pol ζ is nearly as efficient at extending from terminal mispairs as it is in extending from correctly paired termini [169]. Based on these data, as well as the fact that Pol ζ is quite adept at extending from abnormal primer ends resulting from the presence of DNA lesions (discussed below), it has been suggested that the primary function of Pol ζ in TLS is not to insert nucleotides opposite the lesions, but rather to extend from atypical primer termini generated by other TLS polymerases [171].

In keeping with the relaxed fidelity and with the unique ability of Pol ζ to extend from DNA mispairs, this polymerase is responsible for the majority of spontaneous mutations in yeast. The function of Pol ζ was first implicated in spontaneous mutagenesis when a yeast antimutator strain was isolated and the antimutator locus was determined to be a *REV3* allele [172]. Based on data from that study, as well as a study by Roche et al. [173], it has been estimated that Pol ζ is responsible for between 50% to 75% of all spontaneous mutations that arise in yeast [172, 174]. Furthermore, Pol ζ has been implicated in spontaneous mutagenesis that arises from a variety of circumstances, including those that result from transcription [175], double-strand break repair [176], and deficiency in excision repair [173].

In addition to its involvement in spontaneous mutagenesis, Pol ζ is also unique among B-family polymerases in that it is capable of replicating past fork-blocking DNA lesions. The ability of Pol ζ to perform such translesion synthesis was first demonstrated in 1996, when the Lawrence laboratory showed that Pol ζ can replicate past a UV-induced thymine-thymine cyclobutane pyrimidine dimer (T-T dimer) in primer extension assays *in vitro* [156]. However, the ability of Pol ζ to replicate past a T-T dimer *in vitro* apparently does not translate to an *in vivo* requirement for this polymerase in TLS past such lesions, because subsequent work by the same laboratory demonstrated that the frequency of TLS past T-T dimers was unaltered in yeast strains lacking Rev3, the catalytic subunit of Pol ζ [177]. It is now clear that DNA Pol η, not Pol ζ, is chiefly responsible for bypass of T-T dimers *in vivo* (see below).

Despite the fact that Pol ζ is not required for bypass of T-T dimers, yeast *rev* mutants nevertheless show reduced levels of UV-induced mutagenesis, suggesting a role for Pol ζ in error-prone TLS past UV-induced DNA lesions. Therefore, the role of yeast Pol ζ in bypassing the less abundant, but more mutagenic UV-induced thymine-thymine pyrimidine-pyrimidone (6-4) photoproduct (6-4 photoproduct) has also been examined extensively. Results from studies of the ability of yeast Pol ζ to perform TLS past 6-4 photoproducts *in vitro* have been somewhat conflicting. A report by Johnson et al. indicated that Pol ζ is unable to incorporate nucleotides opposite the 3'T of a 6-4 photoproduct, but efficiently and accurately extends from nucleotides inserted opposite the 3'T

by Pol η (which preferentially misinserts a G residue) [178]. These results suggest that Pol ζ contributes to UV-induced mutagenesis only by extending from a mispaired primer terminus generated by another TLS polymerase [178]. However, in a study by Guo et al., Pol ζ was determined to be efficient at nucleotide insertion opposite both residues of a 6-4 photoproduct, inserting an A, T and more rarely a G opposite the 3'T and primarily inserting the correct A opposite the 5'T [179]. Results from this study, therefore, suggest that Pol ζ is directly responsible for generating mutations opposite 6-4 photoproducts by inserting the incorrect nucleotides opposite the 3'T of this UV-induced DNA lesion.

The contribution of Pol ζ, as well as Pol η, to TLS past 6-4 photoproducts *in vivo* is perhaps best demonstrated in experiments where yeast strains lacking one or the other of such polymerases are transformed with plasmid constructs containing a single defined lesion and then assayed for the frequency with which they complete lesion bypass. Unfortunately, like with *in vitro* studies, the results of these *in vivo* studies are equally conflicting. For example, a study by Bresson and Fuchs demonstrated that the mutagenic bypass of a 6-4 photoproduct resulted exclusively from incorporation of a G opposite the 3'T and furthermore, that mutagenic bypass of this lesion was essentially suppressed in yeast strains lacking Pol η (the role of Pol ζ was not tested) [180]. These results support the *in vitro* studies performed by Johnson et al., which indicate that Pol η is responsible for mistakes opposite 6-4 photoproducts and that Pol ζ contributes to UV-induced

mutagenesis only by extending from such mistakes [178]. In contrast, a similar study conducted in the Lawrence laboratory demonstrated that only 15% of the total mutagenic TLS past a 6-4 photoproduct resulted from misincorporation opposite the 3'T of this lesion and that C, T and G were all misincorporated (although G was misincorporated most frequently) [181]. In this study, Pol η deficiency only reduced the mutagenic bypass of the 6-4 photoproduct by 7%, indicating a much more limited role for Pol η in the mutagenic bypass of this DNA lesion [181]. More recently, Abdulovic and Jinks-Robertson conducted an elegant study in which yeast mutant strains deficient in Pol ζ, Pol η, or both polymerases were used to examine the relative contribution of each of these enzymes to UV-induced cell killing and mutagenesis. What is more, these studies were conducted in the presence and in the absence of a photolyase that specifically removes cyclobutane pyrimidine dimers (CPDs), allowing them to distinguish between the contributions of CPDs and 6-4 photoproducts to overall survival and mutagenesis [182]. Results from these experiments indicate a role for Pol ζ, but not Pol η in TLS past UV-induced 6-4 photoproducts. Such results are consistent with the studies conducted by the Lawrence laboratory and in direct contradiction to those conducted by Bresson and Fuchs, which suggested a major role for Pol η in the bypass of this lesion [182].

Although the exact function of Pol ζ in TLS past 6-4 photoproducts, i.e. whether it is required for the insertion step, the extension step, or both steps, is still a matter for debate, a clear role for Pol ζ as a TLS extender has been indicated in

replication past abasic sites. Independent studies, conducted by Nelson et al. [157], Yuan et al. [183], and Haracska et al. [184], each demonstrated that, *in vitro*, Pol ζ is unable to insert nucleotides opposite an abasic site. However, all three studies demonstrated that Pol ζ is able to extend from a nucleotide inserted opposite this lesion by another polymerase. Although the polymerase performing the insertion step differed in each study (Rev1, Pol η or Pol δ respectively), all three groups agreed that the requirement for Pol ζ in TLS past abasic sites is strictly a reflection of the ability of this enzyme to extend from atypical primer termini that present a block to other polymerases.

The ability of yeast Pol ζ to bypass a variety of DNA lesions that result from chemical mutagens has also been examined. For example, *in vivo* experiments using *rev* mutant strains transformed with plasmids containing an N-2-acetylaminofluorene adducted guanine (AAF-G) indicate an absolute requirement for Pol ζ in TLS past this lesion. *In vitro* studies, however, demonstrated that yeast Pol ζ is unable to insert nucleotides opposite an AAF-G, but is able to efficiently extend from an AAF-G overlapped by a primer [179]. Taken together, these data indicate that, as with abasic sites, Pol ζ is required only for the extension step of TLS past AAF-induced DNA lesions and that the insertion step is likely performed by another TLS polymerase.

The role of yeast Pol ζ in bypassing benzo[a]pyrene adducted guanine (BPDE-G) has also been examined both *in vitro* and *in vivo*. Reports of the ability of Pol ζ to

bypass BPDE-G *in vitro* vary from complete blockage [185] to unassisted accurate bypass [186]. However, the latter results may not be relevant with respect to *in vivo* conditions, because the reactions were conducted with high levels of Pol ζ and the time allowed for lesion bypass was greater than 90 min [169]. Experiments using site specifically modified plasmids and a yeast *rev3* mutant strain demonstrated that the frequency of TLS in such cells is reduced to 16% of that in wild-type cells, indicating a requirement for Pol ζ in TLS past BPDE-adducted guanines *in vivo*. Nevertheless, the exact function of Pol ζ in such TLS remains unclear [187].

In addition to being deficient in mutagenesis induced by UV, the yeast *rev3* and *rev7* mutant strains are also deficient in mutagenesis induced by a variety of other DNA damaging agents, including ionizing radiation [188, 189], 4-nitroquinoline-1-oxide [190], methyl methane sulfonate [191], and ethyl methane sulfonate [192]. The fact that the *rev* mutant strains exhibit similar phenotypes when they are exposed to these damaging agents as they do when exposed to UV, suggests that Pol ζ is required for TLS past DNA lesions induced by these damaging agents as well.

Homologs of the yeast *REV3* gene have been identified in many eukaryotes, including *Drosophila melanogaster* [193], *Arabidopsis thaliana* [194], mouse [195], chicken [196], and, of particular interest, humans [197, 198]. The human homolog of the *S. cerevisiae REV3* gene encodes a predicted protein of 3,130

amino acid residues, which is twice the size of the yeast Rev3 protein (1,504 amino acids). The predicted human Rev3 protein shares three regions of sequence homology with the yeast Rev3 protein, a 340 residue amino-terminal domain that is 29% identical to the yeast protein, a small, central 55 residue Rev7 binding domain that is 29% identical, and a carboxy-terminal 850 residue DNA polymerase domain that shares 39% identity with yeast Rev3 [197]. It has been suggested that the large, nonhomologus regions of the hRev3 protein are indicative of additional or more diverse functions for the human protein compared to the yeast counterpart [197]. The fact that in yeast, Rev3 is not essential for viability, but that disruption of *Rev3* in mice results in embryonic lethality [199-201], supports the idea that Rev3 has additional, crucial roles for in higher eukaryotes.

Similar to the yeast Rev3 protein, the human Rev3 protein contains a polymerase domain placing it in the B-family [197]. However, to date, the hRev3 protein has never been successfully expressed or purified, presumably as a result of its large size (353 kDa) and low cellular levels. Therefore, formal demonstration of Rev3 polymerase activity has not yet been achieved, and *in vitro* primer extension assays using reconstituted hPol ζ are lacking. Nevertheless, both human and mouse cells expressing high levels of *hREV3* antisense RNA demonstrate a lower frequency of UV-induced mutations than their appropriate control strains [202, 203]. In addition, by using photolyases, the Yasui laboratory demonstrated that reduction of *hREV3* mRNA renders nucleotide excision repair deficient

human cells sensitive to UV-induced 6-4 photoproducts, but not to CPDs [204]. Together, these data indicate that, as in yeast, Rev3 is required for the mutagenic TLS of UV-induced 6-4 photoproducts in human cells, and suggest that the functions of Pol ζ are conserved from yeast to higher eukaryotes [197, 202, 205].

The human homolog of the yeast Rev7 protein was initially identified using a yeast two-hybrid assay with a fragment of hRev3 as bait [206]. Human Rev7 is a 211 amino acid protein with a predicted molecular weight of 24 kDa. At the amino acid level, hRev7 shares 23% identity and 53% similarity to the yeast Rev7 protein. The Rev7 protein contains a moderately conserved domain (HORMA) that has also been found in hMAD2, a mitotic spindle assembly checkpoint protein and in scHop1, a protein involved in meiotic-synaptonemal-complex assembly [206, 207]. Although the exact function of this domain is unknown, it has been suggested that the HORMA domain is involved in recognizing chromatin states resulting from the presence of DNA adducts, double-strand breaks, or failure of DNA to attach to the spindle, and that it acts as an adaptor that recruits proteins involved in DNA repair [207].

The hRev7 protein also shares 23% identity to and 54% similarity with the mitotic spindle assembly checkpoint protein, hMad2 [206]. Rev7 has been shown, in *Xenopus* extracts, to associate with activators of the anaphase-promoting complex, CDH1 and CDC20, and to inhibit cell progression from metaphase to

anaphase [208, 209]. What is more, in addition to interacting with hRev3, a strong interaction has also been demonstrated between hRev7 and hMAD2, suggesting that hRev7 plays a role in the mitotic spindle assembly checkpoint [206]. However, overexpression of hRev7 in human osteosarcoma cells did not lead to cell cycle arrest [206] and decreasing the level of hRev7 protein in nasopharyngeal carcinoma cells had no obvious effects on cell proliferation, cell cycle distribution, or mitotic checkpoint control [210]. Therefore, if a role for hRev7 in mitotic checkpoint control exists, it remains uncharacterized.

Although the most well characterized function of Pol ζ is in TLS, Pol ζ has been implicated in at least two additional cellular processes. First, a requirement for Pol ζ in somatic hypermutation [211] has been demonstrated both in human and mouse cells [212, 213]. Somatic hypermutation refers to the programmed process whereby a high level of mutations are introduced within the gene segment that encodes the variable region of an antibody, and is one mechanism through which antibody diversity is generated (for review see [214]). In the first phase of SHM, activation-induced deaminase (AID) catalyzes targeted deamination of deoxycytidine residues in DNA, generating U:G mispairs. Subsequent excision of uracil, by a uracil DNA glycosylase, generates abasic sites, which ultimately lead to nucleotide substitution mutations. It is thought that the role of Pol ζ in SHM is in processing these abasic sites. As described above, although Pol ζ is unable to insert nucleotides opposite abasic sites, it is required from the extension of nucleotides inserted opposite these DNA lesions by other

enzymes [157, 183, 184]. In particular, it is believed that Pol ζ contributes to the base substitution mutations of C:G base pairs by extending from nucleotides inserted opposite abasic sites by Rev1 [214].

Finally, increasing evidence has indicated that Pol ζ is involved in homologous recombination (HR)-mediated repair of double-strand breaks. Polymerase ζ was first associated with double-strand break repair in yeast, when it was found to be responsible for elevated rates of mutations near sites of recombinational DNA double-strand break repair [176, 215]. More recently, chromatin immunoprecipitation experiments demonstrated that Pol ζ, together with Rev1, is enriched at areas surrounding a double-strand break generated by homothallic endonuclease [216]. Recruitment of Rev1, and presumably the Pol ζ/Rev1 complex, was determined to be dependent on the kinase activity of the yeast ATR homolog, Mec1, suggesting a model whereby the Pol ζ/Rev1 complex is recruited to the vicinity of a double-strand break by proteins phosphorylated in a Mec1-dependent fashion [216].

Data from vertebrate cells are also consistent with a role for Pol ζ in HR-mediated double-strand break repair. Chicken B lymphocyte DT40 cells with disrupted *REV3* alleles (*REV3-/-*) exhibit a threefold higher frequency of spontaneous chromosomal aberrations compared to wild-type cells and demonstrate reduced frequencies of gene targeting [196]. In addition, *REV3-/-* cells were not only more sensitive to ionizing radiation than wild-type cells in G_1

and early S-phase (likely due to impaired TLS), but were also more sensitive to ionizing radiation than wild-type cells in G_2 phase, and were slightly more sensitive than a *RAD54-/-* clone defective in HR [196]. In addition, studies of mouse cell lines lacking Rev3 suggest that, similar to chicken, Rev3 is employed in double-strand break repair in mice. Disruption of Rev3 in mice causes embryonic lethality [199-201]. Moreover cells from *REV3 (-/-)* embryos demonstrated a significant increase in double-stranded DNA breaks as well as chromatid and chromosome aberrations [217].

b. Rev1

Rev1, like Rev3 and Rev7, was discovered during a search for yeast mutants defective in UV-induced mutagenesis [165]. The *REV1* gene was cloned in 1989 and sequence analysis showed that an amino-terminal portion of Rev1 was 25% identical and 42% similar to UmuC, the catalytic subunit of *E. coli* Pol V [218]. At the time the function of the UmuC protein was still largely uncharacterized. However, these two proteins are now both known to be founding members of the Y-family of polymerases [158].

The yeast Rev1 protein has at least two distinct cellular functions. The first function is as a template-dependent deoxycytidyl transferase [157]. During the original biochemical study, the yeast Rev1 protein was found to preferentially insert a dCMP (but not dAMP, dTMP or dGMP) in a template dependent manner, i.e. opposite a template G. Even more surprisingly, Rev1 was found to be more

efficient at inserting a dCMP opposite a template abasic site than opposite a template G [219]. This subsequent finding was particularly relevant in light of the fact that in *S. cerevisiae* a C is incorporated opposite 60%-85% of abasic sites that are bypassed *in vivo* [220].

Structural analysis of the polymerase domain of *S. cerevisiae* Rev1, in complex with a template primer and an incoming dCTP has provided interesting insight into the mechanism by which Rev1 incorporates strictly C residues but does so in a template-dependent manner [221]. In the structure of this ternary complex, the templating G is flipped out of the DNA helix by a leucine residue that protrudes into the DNA. The templating G forms two hydrogen bonds with the main-chain amides of nearby methionine and glycine residues. This pattern of hydrogen bonding is such that any templating base other than a G would pose unfavorable steric constraints. The evicted template G is replaced by an adjoining arginine residue that can form hydrogen bonds with an incoming dCTP. Again, the pattern of hydrogen bonding is such that binding to any other dNTP would be unfavorable. Thus the mechanism for DNA polymerization by Rev1 is one in which the specificity for both the templating base and the incoming nucleotide are governed by the protein and not by the DNA [221].

Evidence of the existence of a second function of Rev1 in TLS, that is distinct from the deoxycytidyl transferase activity, was first demonstrated in a study by Nelson et al. [177], where they showed *in vivo* that the function of yeast Rev1 is

required for bypass of a UV-induced 6-4 photoproduct. A requirement for Rev1 in TLS past a 6-4 photoproduct is not particularly surprising in light of the fact that the *REV1* gene was initially identified in yeast as a loci involved in UV-induced mutagenesis. However, because Rev1 possesses only a deoxycytidyl transferase activity, and because incorporation of a C occurs only very rarely across from 6-4 photoproducts, these data also suggest the existence of a second, non-catalytic function for Rev1 in TLS.

Further evidence for the existence of a second function of Rev1 in TLS, was demonstrated when Otsuka et al. assayed Rev1 for the ability to perform TLS past an abasic site *in vivo* [222]. In these experiments yeast *rev1* mutant strains carrying a *cyc1-31* mutation were transformed with lesion-containing oligonucleotides that have the *CYC1+* sequence spanning the mutation site. Transformants are only obtained if the oligonucleotide is incorporated into DNA and then used as a template for TLS. Therefore these assays allow for a measurement of the frequency of TLS past a single defined lesion at a specified genomic location. As expected based on *in vitro* studies [219], these experiments showed that Rev1 is essential for bypass of an abasic site. However, when similar studies were conducted in a *rev1* mutant strain that produces a protein lacking deoxycytidyl transferase activity, TLS past an abasic site still occurred with about 50% of the frequency in the wild-type strain. These data suggest that although the transferase activity of Rev1 contributes to bypass of abasic sites, a

function of Rev1 distinct from its transferase activity is also important for TLS [223].

The human homolog of the yeast *REV1* gene has also been identified [219, 224]. The *hREV1* gene encodes a 1,251 amino acid protein that is similar in size to the yeast Rev1 protein (985 amino acids). The human Rev1 protein shares four regions of high sequence homology with the yeast protein. Two amino-terminal domains, including a BRCA1 C-terminal (BRCT) domain that is 35% identical, and an internally conserved region of unknown function that is 29% identical to the yeast Rev1 protein, a polymerase domain that shares 31% identity with that of the yeast protein, and a carboxy-terminal ubiquitin binding motif that shares 21% identity with yeast Rev1 [224, 225].

In addition to possessing significant sequence homology, the cellular functions of the yeast and human Rev1 homologs also appear to be highly conserved. Like its homolog in *S. cerevisiae*, the human Rev1 protein, possesses a deoxycytidyl transferase activity that is highly specific and template dependent [224]. Moreover, similar to its yeast counterpart, hRev1 is able to utilize this deoxycytidyl transferase activity to bypass abasic sites *in vitro* [224]. However, the physiological relevance of this reaction in humans is questionable because unlike in yeast, where dCMP is predominantly incorporated opposite abasic sites, in human cells dAMP is the most commonly incorporated nucleotide [226].

The enigmatic second, so-called non-catalytic function of Rev1 also seems to be conserved from yeast to humans. In a study using chicken DT40 cells lacking Rev1, the deoxycytidyl transferase domain (but not the C-terminal domain) of human Rev1 was determined to be dispensable for DNA damage tolerance [227]. In addition, human fibroblasts expressing high levels of *hREV1* antisense RNA exhibited greatly reduced levels of UV-induced mutagenesis compared to control cells, indicating that Rev1 is required for most mutations induced by UV radiation in human cells [219]. Like yeast Rev1, human Rev1 is unable to perform TLS opposite either of the two most common UV-induced DNA lesions *in vitro* and furthermore, incorporation of a C opposite these DNA lesions occurs very rarely. These data suggest that, as in yeast, a second transferase-independent role for Rev1 in TLS exists in humans.

Rev1 contains a highly conserved BRCT domain in its N-terminal region that appears to be of some importance for the non-catalytic function of this enzyme. Studies using cells obtained from mice with a targeted deletion in the Rev1 BRCT domain demonstrated that deletion of this domain results in a complete loss of UV-induced mutations in the *HPRT* gene [228]. The reduction of UV-induced mutagenesis was accompanied by loss of transversions at T-T dimer sites, indicating the importance of the Rev1 BRCT domain in TLS past UV-induced T-T dimers. Interestingly, however, a deletion and point mutation analysis of the Rev1 protein indicated that the BRCT domain is dispensable for transferase activity *in vitro* [229]. Together, these data suggest that the

requirement for the Rev1 BRCT domain in TLS is unrelated to the catalytic function of this enzyme.

More recent evidence suggests that the BRCT domain is required for interaction between Rev1 and PCNA. As described above, if DNA damage occurs that blocks replication, mono-ubiquitination of PCNA can be used as a mechanism to activate TLS [153, 154]. Studies by Guo et al. [230], demonstrated that Rev1 can bind directly to PCNA and that this interaction is enhanced by mono-ubiquitination of PCNA. Moreover, they found that deletion or inactivation of the Rev1 BRCT domain by mutation abolishes the interaction between Rev1 and PCNA. Interestingly, deletion or inactivation of the Rev1 BRCT domain also eliminates targeting of Rev1 to replication foci in undamaged cells, but only slightly reduces such targeting in cells that are UV-irradiated. Similar studies conducted in the same laboratory showed that the two conserved ubiquitin-binding motifs located at the C-terminus of Rev1 also mediate enhanced association with mono-ubiquitinated PCNA [231]. In contrast to results obtained by deleting the Rev1 BRCT domain, deletion of the ubiquitin-binding motifs had no effect on constitutive nuclear targeting to Rev1, but completely abolished nuclear targeting in the presence of UV radiation. These data suggest that localization of Rev1 to sites of replication-blocking DNA lesions is most efficient when both the BRCT domain and the ubiquitin-binding motifs are fully functional.

In addition to interacting with mono-ubiquitinated PCNA, both mouse and human Rev1 have been shown to interact with Pol η, Pol ι, Pol κ, and the accessory subunit of Pol ζ, Rev7, through the same carboxy-terminal 100 amino acid region [232, 233]. However, this interaction, at least with respect to Pol κ, had no effect on the polymerase activity of either enzyme. These data suggest that competitive binding to Rev1 plays an important regulatory role for TLS in mammals. Taken together with the fact that Rev1 can be localized to sites of DNA damage through interaction with mono-ubiquitinated PCNA, it is tempting to speculate that the second, as of yet undefined function of Rev1 in TLS is as a structural scaffold that coordinates other TLS polymerases at sites of DNA damage.

c. DNA Polymerase η

Shortly after identification of the Rev1 protein in eukaryotes, a search of the *S. cerevisiae* genome identified a gene with significant homology to the eukaryotic Rev1 protein. When this gene was disrupted in yeast, they were rendered more sensitive to UV radiation and as a result, this new gene was designated *RAD30* [234]. Subsequent studies of the Rad30 protein demonstrated that unlike Rev1, which possesses only a deoxynucleotidyl transferase activity, Rad30 is a bona fide polymerase, able to incorporate all four dNTPs onto a primer in a template-specific manner [235]. Because Rad30 was the seventh eukaryotic polymerase to be discovered it was renamed DNA polymerase η (Pol η) [235].

Studies of the yeast *rad30* mutant strain demonstrated that deletion of *RAD30* in yeast results in a 10-fold increase in UV-induced mutagenesis. This was the first indication that the product of the *RAD30* gene, Pol η, might participate in an error-free mechanism of DNA repair [234]. This hypothesis was further refined when the purified Pol η protein was shown to be both efficient and accurate at bypassing thymine-thymine cyclobutane pyrimidine dimers (T-T dimers) *in vitro,* indicating that Pol η participates in tolerance of UV-induced DNA lesions by performing error-free TLS past T-T dimers [235].

Cells from patients with the variant form of the disease xeroderma pigmentosum (XP-V) are proficient in nucleotide excision repair (unlike cells from patients with the classical forms of xeroderma pigmentosum), but are defective in replication of DNA containing UV-induced lesions [236]. Pol η possesses the ability to carry out error-free TLS past UV-induced T-T dimers *in vitro.* This finding raised the possibility that lack of the human homolog of Pol η is the cause of the disease xeroderma pigmentosum variant in humans [235]. This prediction was soon confirmed by two independent groups who demonstrated that XP-V cell lines harbor mutations in the *POLH* gene, which encodes human Pol η [237, 238]. The discovery that mutations in the *POLH* gene, that encodes Pol η, are responsible for the variant form of the disease xeroderma pigmentosum added further significance to the discovery of Pol η.

Structural studies of the catalytic core of *S. cerevisiae* Pol η have proven to be particularly useful for understanding the mechanism by which TLS polymerases are able to incorporate nucleotides opposite bulky DNA lesions [163]. Although there is very little similarity between the amino acid sequence of Pol η and those of the classical polymerases, Pol η retains the characteristic right hand architecture of a DNA polymerase, including the palm, fingers and thumb domains. Similar to the classical polymerases, the palm domain of Pol η harbors the catalytic triad, viz., Asp, Asp-Glu active site residues. In fact, the core of the palm domain of Pol η is nearly superimposable upon the core of the palm domain of classical DNA polymerases. The fingers domain, which is typically thought to mediate nucleotide selectivity, is small and stubby, compared to other DNA polymerases. In addition, Pol η lacks the equivalent of helices "O" and "O1" that are thought to play a role in fidelity enhancement. The thumb domain, that mediates DNA binding and polymerase processivity, is similarly small and stubby compared to the thumb domain of other polymerases. Unlike the classical polymerases, Pol η contains a fourth domain, called the polymerase-associated domain, which sits alongside the fingers domain [163]. This additional domain increases the DNA binding surface area of Pol η. Although the crystal structure of Pol η was determined in the absence of DNA, the similarity between the palm domain of Pol η and that of other DNA polymerases, allowed both a template-primer and an incoming nucleotide to be modeled into the Pol η DNA binding site. The active site of Pol η was found to be much more open then the active sites of other DNA polymerases. Taken together, these data suggest that TLS

polymerases are able to accommodate bulky DNA lesions into their DNA binding pockets because they possess more open and extended active sites than the classical high-fidelity polymerases [163].

Polymerase η is most well know for its remarkable ability to catalyze error-free TLS past UV-induced T-T dimers. Structural and kinetic studies have suggested, surprisingly, that Pol η is able to incorporate both residues of a T-T dimer into its active site simultaneously, making it particularly efficient at conducting TLS past this distorting DNA lesion [163, 239]. The fact that Pol η possesses such an open active site provides an explanation for the low fidelity of this polymerase when replicating undamaged DNA (discussed further below), but seems inconsistent with the error-free nature of Pol η when replicating past UV-induced T-T dimers. It is currently thought that unlike the classical replicative polymerases that govern fidelity primarily by the geometric complementarity of the incoming nucleotide, Pol η relies on Watson-Crick-type hydrogen bonds to achieve accurate DNA synthesis. The effect of hydrogen bonds on the replication fidelity of Pol η was directly tested *in vitro* by using a thymine analog, diflourotoluene, that is nearly identical to thymine in shape, but lacks the ability to form hydrogen bonds with adenine [240]. Pol η was unable to insert any nucleotide opposite a template containing diflourotoluene, whereas insertion by the *E. coli* Klenow enzyme was unhindered. Thus, unlike the classical replicative polymerases, the fidelity of Pol η (and likely Y-family polymerases in general) is governed largely based on

hydrogen bonds between the templating base and the incoming nucleotide rather than by its size or shape [240].

Although the ability of Pol η to bypass UV-induced T-T dimers accurately and efficiently has been well established [239, 241, 242], the ability of Pol η to bypass a variety of other types of DNA lesions has also been studied extensively *in vitro*. Unlike with T-T dimers, Pol η is unable to replicate past a UV-induced, 6-4 photoproduct. However, Pol η is able to catalyze misinsertion of a G opposite the 3'T of a 6-4 photoproduct with limited efficiency [241]. Because 6-4 photoproducts are formed more commonly at 5'-TC-3' and 5'-CC-3' sites, it is thought that the ability of Pol η to insert a G opposite the 3' nucleotide of a photoproduct might contribute to the overall error-free bypass of 6-4 photoproducts [243]. Pol η is also significantly inhibited by both abasic sites [184, 244, 245] and N-2-acetylaminofluorene adducted guanines *in vitro* [244]. In contrast, Pol η is readily able to bypass an 8-oxoguanine lesion, although reports of the insertion specificity by Pol η across from this lesion are conflicting. In one study, Pol η was reported to insert both A and C residues with similar efficiencies [245]. However, in another study Pol η was found to replicate past an 8-oxoG lesion in a primarily error-free manner, misinserting an A only a small proportion of the time [246]. It is likely that these discrepancies result from the influence of sequence context on insertion specificities. Finally, with some DNA lesions, Pol η promotes primarily error-prone TLS. For example, Pol η has been shown to predominantly insert an A residue opposite benzo[a]pyrene adducted guanines

[245, 247]. Because G→T is the most common type of base substitution induced by BPDE in mammalian cells, it has been speculated that error-prone TLS by Pol η is the cause of this base substitution mutation [245, 247].

When compared to the classical replicative polymerases, both yeast and human Pol η have reduced fidelity when copying undamaged DNA and in addition, both yeast and human Pol η lack 3'→5' proofreading exonuclease activity [239, 248, 249]. As a result, Pol η is at least 100-times less accurate than the major replicative polymerases during the nucleotide incorporation step DNA synthesis [250]. Although it seems counterintuitive that the benefits of possessing Pol η, i.e. lesion bypass, outweigh the mutagenic potential of this polymerase, human Pol η is much more processive when synthesizing DNA opposite T-T dimers than it is when copying undamaged DNA [242]. This suggests a mechanism whereby Pol η switches to a less processive mode when it encounters an undamaged template, presumably excluding this polymerase from active DNA synthesis [242].

In addition to its function in TLS, Pol η has also been shown to play a role in other cellular processes, including somatic hypermutation (SHM) [211] and homologous recombination. Somatic hypermutation, an important process through which antibody diversity is achieved by hypermutation of the antibody variable region, can be divided into two phases, a first phase where base substitution mutations are induced in C:G base pairs, and a second phase where

A:T base pairs are the primary target for mutation. It is during this second phase, predominated by mutagenesis of A:T base pairs, that Pol η plays a critical role. Clear evidence for the requirement of Pol η in SHM was demonstrated by studies of humans and mice lacking this polymerase, which showed a striking reduction of the number of mutations at A and T sites within antibody variable genes [251, 252]. The exact mechanism by which Pol η generates mutations at A:T sites during SHM remains to be determined [214].

Polymerase η has also been reported to be involved in homologous recombination (HR). In a study conducted in the West laboratory [253], they demonstrated that, *in vitro*, Pol η (but not Pol δ) was able to extend DNA from D-loop recombination intermediates where the invading strand serves as a primer. Moreover, these investigators showed that this D-loop extension activity is diminished in extracts from XP-V cells, which lack Pol η. Using a completely different approach, Shunichi Takeda's laboratory evaluated HR-dependent double-strand break repair by introducing an artificial SCneo HR substrate DNA construct carrying an I-SceI restriction site into chicken DT40 cells that lack Pol η. Double-strand breaks induced by transient expression of I-SceI are repaired by gene conversion from an upstream donor homologous sequence, generating a functional neomycin resistance gene. Therefore, the number of HR events can be determined by counting the number of neomycin-resistant colonies. When cells lacking Pol η were assayed for the efficiency with which I-SceI-induced double-strand breaks were repaired by HR they were shown to be

10-fold less efficient than wild-type cells. Furthermore, the gene conversion deficiencies exhibited by cells lacking Pol η could be rescued by transfection of wild-type Pol η, but not by transfection of a catalytically dead mutant of Pol η, indicating that the activity of this polymerase is required for these gene conversion events [254].

d. DNA Polymerase ι

Only a few months after the discovery of human Pol η, a second homolog of the *S. cerevisiae RAD30* gene, initially designated *RAD30B*, was identified in humans [255]. Like Pol η, the Rad30B protein was also shown to possess DNA polymerase activity, and was therefore designated DNA polymerase ι (Pol ι) [256]. Unlike Pol η, which is distributed ubiquitously throughout eukaryotes, Pol ι is found only in higher eukaryotes, beginning with *Drosophila*. Interestingly, the enzymatic properties of *Drosophila* Pol ι are much more comparable to those of human and yeast Pol η than they are to human Pol ι [257]. Based on these observations, it is thought that Pol ι likely arose as a genetic duplication of Pol η, just prior to the emergence of insects, and that further selective pressures have resulted in the evolution of two distinct polymerases in higher eukaryotes [258].

Similar to other TLS polymerases, Pol ι is a distributive enzyme that lacks exonuclease activity and is highly error-prone when copying undamaged DNA [256]. However, the misincorporation frequency and specificity of Pol ι is unparalleled even with respect to other TLS polymerases [250]. The fidelity of Pol

ι is uniquely template dependent [256]. On primed single stranded DNA in vitro, Pol ι exhibits the highest efficiency and fidelity opposite a template A, incorporating the incorrect nucleotide at a reasonable frequency of ~1 x 10^{-4} [256]. In contrast, Pol ι is extremely inaccurate when synthesizing DNA opposite a template T, exhibiting a much higher error rate of ~7 x 10^{-1} [256]. In fact, within certain sequence contexts, Pol ι incorrectly inserts a G opposite a template T up to 11 times more frequently than it inserts the correct A residue, meaning the fidelity of Pol ι can vary up to 10^{5}-fold merely as a result of which templating base is being replicated [159, 171, 256, 259]. With undamaged DNA, Pol ι is just as efficient at extending from a mispaired primer terminus as it is at generating a mispair [260], making Pol ι one of the most error-prone DNA polymerases discovered to date [256].

Overall, the structure of Pol ι is similar to that of other polymerases, typified by a right hand architecture consisting of palm, fingers and thumb domains. Like other Y-family polymerases thus far characterized, Pol ι contains the unique polymerase associated domain, and thumb and finger domains that are shorter than those of the classical polymerases. However, in contrast to all other known polymerases, including fellow members of the Y-family, during DNA synthesis, the active site of Pol ι employs Hoogsteen base pairing, rather than the typical Watson-Crick base pairing [261]. In such Hoogsteen base pairing, the templating base is driven to the *syn* conformation by bulky residues in the fingers domain,

forcing the incoming nucleotide to form hydrogen bonds with the Hoogsteen edge, rather than the Watson-Crick edge, of the templating base [261].

The revelation that Pol ι employs Hoogsteen base pairing, rather that the typical Watson-Crick, has provided an interesting explanation for the ability of Pol ι to be relatively accurate when synthesizing DNA opposite a template A, but be highly error-prone when inserting opposite a template T. Opposite a template A in *syn* conformation, only an incoming *anti* T can make two favorable hydrogen bonds. In contrast, a template T lacks a Hoogsteen edge, which would prevent hydrogen bonding, thus making base pairing ambiguous in this context [261].

DNA polymerase ι, like its paralog Pol η, is also proficient at bypassing fork-blocking DNA lesions. However, the lesion bypass specificity of Pol ι is somewhat different than that of its ancestor Pol η. Perhaps the most notable difference between the two enzymes concerns the proficiency and fidelity with which they are able to bypass UV-induced DNA lesions. Unlike Pol η, which is characterized by its ability to bypass T-T dimers both efficiently and accurately, Pol ι bypasses T-T dimers in a more limited and inaccurate manner [262, 263]. With regard to a 6-4 photoproduct, Pol ι performs relatively efficient but inaccurate nucleotide incorporation (although it is unable to perform the subsequent extension steps), whereas Pol η is primarily blocked by this UV-induced DNA lesion [262, 263]. More recently, convincing evidence of a role for Pol ι in mutagenic TLS past UV-induced DNA lesions has been indicated from

studies using mice carrying a naturally occurring nonsense mutation in the *Poli*. These mice are characterized by elevated UV-induced mutation frequencies and are more susceptible to certain types of UV-induced cancers [264, 265]. In fact, studies have indicated that in xeroderma pigmentosum variant cells, which lack Pol η, mutagenic TLS of UV-induced DNA lesions by Pol ι is responsible for the elevated frequency of UV-induced mutations that ultimately leads to their malignant transformation [266].

In addition to DNA lesions induced by UV, the efficiency and accuracy with which Pol ι is able to replicate past a variety of other types of DNA lesions has also been examined *in vitro*. It is generally agreed upon that Pol ι is able to efficiently insert any of the four nucleotides opposite an abasic site, but is not able to perform the subsequent extension reaction [171, 258, 267]. Likewise, it is generally accepted that Pol ι is relatively efficient at incorporating a C opposite an N-2-acetylaminofluorene adducted guanine, but further extension is negligible [258, 267]. However, reports of the ability of Pol ι to bypass 8-oxoguanine lesions in primer templates varies somewhat between laboratories. In a study by Zhang et al., they found that 8-oxoguanine presented a severe block to TLS by Pol ι, which could only be overcome by adding increasing amounts of Pol ι to the reaction [267]. Whereas a study by Vaisman et al., demonstrated only a very modest reduction in TLS past an 8-oxoguanine adduct [258]. Both groups found that on the whole, Pol ι favors insertion of the correct C residue opposite an 8-oxoguanine lesion [258, 267]. Although benzo[a]pyrene adducted guanines

present a strong block to TLS by Pol ι, insertion opposite benzo[a]pyrene and benzo[*c*]phenanthrene adducted adenines is both efficient and accurate (although the subsequent extension requires another polymerase). Because Pol ι is also very accurate at replication of an undamaged template A, it has been suggested that Pol ι has evolved to protect organisms from mutations induced by damaged adenosines in a manner similar to which Pol η protects us from mutations induced by damaged thymines [250].

Interestingly, Pol ι has also been shown to possess an intrinsic 5' deoxyribosephosphate lyase activity. In other words, Pol ι is able to remove a 5' deoxyribose phosphate group from DNA [268]. In reconstituted reactions containing uracil-DNA glycosylase, apurinic/apyrimidinic endonuclease, and DNA ligase I, Pol ι can use its dRP lyase and polymerase activities to repair G:U and A:U pairs in DNA [268]. Furthermore, in cell extracts devoid of Pol β, addition of purified Pol ι restores base excision repair of substrates containing uracil [269], suggesting that Pol ι has a role in a specialized form of base excision repair of uracil-containing substrates.

e. DNA Polymerase κ

DNA polymerase κ (Pol κ) was originally identified in humans as a homolog of the *E. coli* DinB (Pol IV) protein, which was discovered based on its involvement in the UV-induced, untargeted mutagenesis of bacteriophage lambda, and later shown to be a specialized DNA polymerase [270-272]. The DinB subfamily is the

most widely distributed, found in eukaryotes, prokaryotes, as well as archaea. However, it is clearly not essential for life as it is conspicuously absent from the genomes of *Saccharomyces cerevisiae* and *Drosophila melanogaster* [158].

Polymerase κ, like other TLS polymerases, lacks 3'→5' proofreading exonuclease activity [273-275], and similarly, is much less accurate than the classical replicative polymerases when replicating undamaged DNA. For example, in an M13 mutagenesis forward assay, DNA synthesis by Pol κ produced 760 errors per 10^5 nucleotides, whereas synthesis by Pol δ produced only three errors per 10^5 nucleotides [274]. Polymerase κ is also less accurate than the *S. cerevisiae* B-family TLS polymerase ζ, which generated 130 errors per 10^5 nucleotides [170], but is the most accurate of the human Y-family polymerases, as Pol η [248] and Pol ι [276] produced 3,700 and 72,000 errors per 10^5 nucleotides, respectively. Based on structural analysis, it has been suggested that the higher fidelity of Pol κ compared to other Y-family polymerases results from a more constrained active site [277].

In addition to being relatively inaccurate during DNA synthesis, Pol κ is also quite proficient at extending from mispaired primer termini. In fact, Pol κ extends from each of the 12 possible DNA mismatches more efficiently than it produces the mispairs. These results suggest that Pol κ, like Pol ζ, contributes to spontaneous mutagenesis by extending from mispairs generated by other DNA polymerases

[275, 278]. In keeping with this characteristic, upregulation of Pol κ has been shown to elevate the frequency of spontaneous mutations tenfold in mouse cells and seven to ninefold in human cells [270, 279].

The specificity of lesion bypass of human Pol κ has been studied extensively *in vitro* using primer extension assays. These experiments have demonstrated that, Pol κ is not able to bypass either UV-induced T-T dimers or 6-4 photoproducts [272, 280, 281]. Additionally, Pol κ is unable to bypass cisplatin adducts [273, 281] and is either inefficient or unable to bypass abasic sites [272, 280, 281]. Polymerase κ is, however, able to bypass DNA adducts induced by acetylaminofluorene, as well as 8-oxoguanine adducts, but does so primarily in an error-prone manner [135, 273, 280, 281].

Although Pol κ has the potential to introduce mutations during error-prone translesion synthesis, it has been shown, also *in vitro*, to bypass thymine glycol, a biologically important form of oxidative base damage, in an error-free manner by inserting the correct base, adenine across from this lesion [282]. Moreover, Pol κ bypasses lesions resulting from benzo[a]pyrene diol epoxide (BPDE), an active metabolite of the environmental carcinogen benzo[a]pyrene, in an error-free manner [280, 283, 284].

In addition to a requirement for incorporation of nucleotides opposite DNA damage, Pol κ has also been implicated in the extension step of TLS. For

example, although Pol κ cannot insert nucleotides opposite the 3'T of a T-T dimer, it has been shown to efficiently extend from a G placed opposite the 3'T of this lesion [278]. Polymerase κ has also been shown to efficiently extend from a variety of nucleotides placed opposite an O^6-methylguanine lesion or an 8-oxoguanine lesion by Pol δ [285]. What is more, Pol κ efficiently extends from an A residue misincorporated opposite a BPDE adducted guanine by Pol η, which is unable to extend from this lesion-containing terminus alone [286].

There have been fewer studies reported which examine the role of Pol κ *in vivo*. The Ohmori laboratory developed mouse embryonic stem cells defective in Pol κ and found that such cells were not only hypersensitive to the cytotoxic effects of BPDE, they also accumulated more BPDE-induced mutations than the parental cell strains [287]. In addition, when mouse embryonic fibroblasts (MEFs) generated in the Livneh laboratory were assayed for their ability to bypass a site specific BPDE lesion in a plasmid based TLS assay, they found that Pol κ was responsible for approximately two-thirds of lesion bypass events and that such events occurred in primarily an error-free manner [288]. Together these data indicate, in accordance with *in vitro* data, that Pol κ plays an important role in the accurate bypass of DNA lesions induced by benzo[a]pyrene.

Both the mouse and human *POLK* genes contain two xenobiotic responsive elements (XRE) in their promoters [289, 290]. Xenobiotic responsive elements are binding sites for polycyclic aromatic hydrocarbons (PAH), such as

benzo[a]pyrene, that when bound result in upregulation of specific PAH-inducible genes. One such gene is the *CYP1A1* gene, which encodes cytochrome P450. Cytochrome P450 is a protein that metabolizes benzo[a]pyrene and other PAHs into compounds, which are more easily excreted from cells. However, metabolism can also inadvertently result in the activation of some PAHs into electrophilic forms, such as benzo[a]pyrene diol epoxide, that are highly reactive to DNA. The presence of these XRE sequences in the *POLK* gene may also be indicative of a requirement for Pol κ in TLS past DNA lesions induced by PAHs.

Interestingly, the bacterial homolog of Pol κ, Pol V is also able to bypass BPDE adducted guanines in an error-free manner *in vitro*, indicating that the ability to perform TLS past BPDE-induced DNA lesions is conserved from *E. coli* to mammals [137]. However, such a role in *E. coli* cells is likely not physiologically relevant because *E. coli* do not posses an enzyme that activates benzo[a]pyrene into BPDE. Moreover, it is unlikely that mammalian Pol κ is conserved strictly for bypassing BPDE-induced DNA lesions [291]. More recently, it was found that Pol κ is able to bypass estrogen-derived DNA adducts [292]. Furthermore, Pol κ is highly expressed in human testis and ovaries [271] and the adrenal cortex of mice [293] where steroid hormones are produced. Based on these data, it has been suggested that the cognate lesions for TLS by Pol κ are steroid-derived DNA adducts, especially those that are structurally similar to BPDE and are formed at the N^2 position of guanine [291].

In vivo experiments with mouse embryonic stem cells lacking Pol κ also demonstrated that such cells are moderately sensitive to UV radiation [287]. This finding was particularly intriguing in light of the fact that *in vitro*, Pol κ is unable to bypass T-T dimers or 6-4 photoproducts [272, 280, 281]. Polymerase κ is, however, able to extend from a G or A placed opposite the 3'T of a T-T dimer [278]. Initially the increased sensitivity of cells lacking Pol κ to UV radiation was attributed to a role for Pol κ in the extension step of TLS past UV-induced T-T dimers [287]. However, more recent evidence has indicated that such an increase in UV-induced sensitivity might be attributed to a role for Pol κ in nucleotide excision repair (NER). In a study by Alan Lehmann's group [294], they showed that both recovery of RNA synthesis after UV irradiation (associated with transcription-coupled NER) and unscheduled DNA synthesis (a measure of global genome NER), are substantially reduced in MEFs lacking Pol κ. In addition they demonstrated that DNA repair synthesis and photoproduct removal are reduced in cells lacking Pol κ compared to wild-type. These data suggest that Pol κ is involved in the repair synthesis step of NER. However, it remains unclear how employing a polymerase with such a reduced fidelity would be beneficial in a process that should be primarily error-free.

2. Damage Avoidance in Eukaryotes

The eukaryotic damage avoidance pathway is much less well understood than eukaryotic TLS. Relatively recently, however, some of the key factors involved in

this pathway have been identified in *S. cerevisiae*. One such factor is Mms2. The *MMS2* gene was first identified in a yeast mutant strain sensitive to methyl methane sulfonate, and functional complementation analyses placed the product of the *MMS2* gene in the *RAD6* pathway (discussed above) [295, 296]. Yeast *mms2* mutants are much less sensitive to DNA damaging agents then either the *rad6* or *rad18* mutants are, therefore it is commonly accepted that, the function of the *MMS2* gene is required for only one of the two *RAD6*-mediated damage tolerance pathways [296]. Based on the fact that the *mms2* mutant demonstrates a high frequency of spontaneous mutations that can be completely abolished by inactivating the *REV3*-mediated TLS pathway, and that a *rev3/mms2* double mutant (but neither of the single mutants) is as sensitive to DNA damaging agents as a *rad18* single mutant is, the function of the *MMS2* gene has been implicated in the error-free DA pathway [296, 297].

The *MMS2* gene encodes a protein that is homologous to a ubiquitin conjugating enzyme, but lacks the active site cysteine residue required for ubiquitin conjugation [296]. Such ubiquitin enzyme variants have previously been hypothesized to function as dominant-negative regulators of ubiquitin conjugation. Based on the epistatic relationship between *mms2* and *rad6*, it was believed for sometime that the function of Mms2 was to modulate the ubiquitin conjugating activity of Rad6. However, direct evidence to support such a theory has not yet been established (reviewed in [149]). On the other hand, co-purification assays and yeast two-hybrid screens have demonstrated that Mms2

forms a stable complex with the ubiquitin conjugating enzyme, Ubc13 [298, 299]. Moreover, the *ubc13* yeast mutant is phenotypically indistinguishable from the *mms2* mutant. Based on these data, Mms2 is now believed to modulate the activity of Ubc13 in the DA pathway [300]. The Mms2-Ubc13 complex has been shown to catalyze the formation of lysine63-linked poly-ubiquitin chains [298]. Studies have further demonstrated that the cognate E3 for Mms2-Ubc13 is Rad5, a DNA helicase protein with ATPase activity [301, 302]. Rad5 interacts with both Ubc13 and Rad18 and thus provides a physical link between the *RAD6* and DA pathways [303].

In a recent review by Andersen et al., a model demonstrating how the functions of the Mms2 and Ubc13 proteins might be used for initiation of DA, was proposed [9]. In this model, Rad18 recruits Rad6 to segments of single-stranded DNA that are bound by PCNA, such as sites of unrepaired DNA damage. The DNA-bound PCNA is then mono-ubiquitinated by the Rad6-Rad18 complex, which either directly or indirectly, promotes TLS. The Mms2-Ubc13 complex is recruited to DNA-bound PCNA through interaction with Rad5. The presence of the Mms2-Ubc13-Rad5 complex results in further addition of ubiquitin residues linked through lysine63. Finally, such poly-ubiquitination of PCNA promotes the error-free damage avoidance pathway.

Although significant advances have been made concerning the processes by which DA is initiated, the actual mechanism of error-free DA is much less well

understood. Thus far, two possible mechanisms of DA have been suggested, namely replication fork regression and template switching [9]. Replication fork regression, refers to a mechanism whereby the replication fork migrates backward until the original template strands are re-annealed and the newly synthesized daughter strand is expelled. Such a process can result in the generation of a so-called "chicken foot structure" that can serve as a homologous recombination intermediate. Evidence supporting the use of a fork regression mechanism in DA comes from studies by Blastyak et al. [304], who showed that yeast Rad5 exhibits a DNA helicase activity that can facilitate fork regression.

Despite some evidence of Rad5-mediated fork regression, it is most commonly accepted that the mechanism employed by the error-free DA pathway is template switching (reviewed in [9]). In template switching, when a high fidelity replicative polymerase is blocked by a DNA lesion, this polymerase temporarily uses a segment of DNA homologous to that containing the lesion (for example the newly replicated daughter strand) as a template to replicate around the damaged DNA. The template switch mechanism of DA is supported by the studies of Li et al. [305], who demonstrated that human fibroblasts strains with reduced levels of the human homolog of the yeast Mms2 gene, hMms2, were virtually unable to use an allelic gene copy as a template for replication past UV-induced DNA lesions. These results are consistent with the notion that error-free damage avoidance occurs through a mechanism template switching.

Although the mechanism through which error-free damage avoidance is achieved is still unclear, this process is apparently highly conserved throughout all eukaryotes. Homologs of all the proteins known to be involved in DA, including Mms2, Ubc13 and Rad5, have been found in plants, mammals and other higher eukaryotes (see [9] and references therein). Such a high level of conservation is likely a reflection of the importance of error-free damage avoidance process.

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CHAPTER II

hRev7, putative subunit of hPolζ, plays a critical role in survival, induction of mutations, and progression through S-phase, of $UV_{(254nm)}$-irradiated human fibroblasts

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ABSTRACT:

Translesion synthesis (TLS) refers to mechanisms by which specialized DNA polymerases incorporate nucleotides opposite fork-blocking lesions and extend replication until standard replicative polymerases take over. The first eukaryotic TLS polymerase discovered, *S. cerevisiae* Polζ, consists of catalytic subunit Rev3 and non-catalytic subunit Rev7. Human homologs of these two proteins have been identified. Studies by Lawrence, Maher, and colleagues comparing $UV_{(254nm)}$-irradiated human fibroblast cell strains expressing high levels of *hRev3* antisense to their normal parental strains demonstrated that there was no difference in cell survival, but that the frequency of UV-induced mutations in the derivative strains was 10-fold lower than that of the parental strains, indicating that hRev3 plays a critical role in such mutagenesis. To examine the role of hRev7 in TLS, we generated human fibroblasts expressing *hRev7* siRNA, identified two derivative cell strains with significantly reduced levels of hRev7, and compared them to their parental strain and a vector control for cell survival, induction of mutations, and ability to traverse the cell cycle following exposure to UV radiation. Cells with reduced hRev7 were ~2-times more sensitive to UV-induced cytotoxicity than the controls, indicating that unlike hRev3, hRev7 plays a protective role for cells exposed to UV radiation. When these cell strains were assayed for the frequency of mutations induced by UV in their *HPRT* gene, cell stains with reduced hRev7 were 5-times less sensitive to UV-induced mutagenesis than control strains. In addition, when these four strains were

synchronized at the G1/S border, released from the block, UV-irradiated, and allowed to traverse the cell cycle, the rate of progression through S-phase of the cell strains with reduced hRev7 was significantly slower than that of the control strains. These data strongly support the hypothesis that hRev7 is required for TLS past UV-photoproducts, and together with hRev3, comprise hPolζ.

INTRODUCTION:

Human cells are continually exposed to endogenous and exogenous DNA damaging agents, many of which create fork-blocking lesions. If DNA replication past such lesions cannot take place, this can lead to cell death, nevertheless replication past such lesions can result in mutations. Because mutations play a crucial causal role in the development of cancer, it is important to examine processes that produce them.

Human cells have efficient, error-free repair pathways for excising DNA fork-blocking lesions from either strand of their DNA. They also possess cell cycle checkpoints [1], some of which, when activated, provide additional time for excision repair to occur before the replicative polymerases encounter fork-blocking lesions, such as UV-induced pyrimidine dimers. In spite of these protective processes, replication forks still encounter lesions. Cells have evolved damage tolerance mechanisms to cope with such lesions, viz., translesion synthesis and damage avoidance pathways. Such methods of dealing with fork-blocking damage have been, and continue to be actively examined. Overviews summarizing in detail such areas of research can be found in reference [2].

Translesion synthesis in both prokaryotes and eukaryotes involves specialized DNA polymerases capable of incorporating nucleotides directly across from fork-blocking DNA lesions. This insertion step can be error-free or error-prone,

depending upon 1) the type of DNA lesion encountered, 2) the specialized polymerases involved, and 3) the sequence context surrounding the site of the damage. Insertion of a nucleotide or nucleotides by one or other such polymerases is followed by extension, i.e., the addition of nucleotides beyond the site of the blocking lesion. This latter step also involves TLS DNA polymerases. Such extension beyond the damage is necessary if the high fidelity replicative DNA polymerases are to resume their function. Thus, TLS is a two-step process whereby specialized DNA polymerases, with relaxed fidelity, incorporate and/or extend nucleotides at sites of fork-blocking DNA damage, allowing DNA replication to continue, but often introducing mutations.

Reports and summaries of the discovery of many translesion synthesis polymerases, first in *S. cerevisiae*, and later in mammalian cells, can be found in the cited references (see for example, [3-6]). However, many aspects still remain to be clarified. Polζ was found using *S. cerevisiae* cells whose specific mutated phenotypes could not be reverted to wild type by exposure to mutagenic agents. Genes that complemented the deficiencies in such strains of *S. cerevisiae*, i.e., allowed them to revert, were identified and subsequently shown to code for proteins that allow replication past fork-blocking DNA damage [7, 8]. For example, the yeast Rev3 protein was found to exhibit polymerase activity in primer extension assays *in vitro*. The addition of yeast Rev7 to such assays enhanced the polymerase activity of Rev3 over 20-fold. Together, Rev3 and Rev7 were recognized as constituting yeast Polζ [9].

Genes coding for the human homologs of yeast Rev3 [10-13] and Rev7 [14] were subsequently identified. By using antisense directed against *hRev3* mRNA, Lawrence, Maher, and their colleagues [10, 15] demonstrated that hRev3, the putative catalytic subunit of hPolζ, is critically involved in generating UV-induced mutations in diploid human fibroblasts. These results indicate that hRev3 is essential for a mutagenic process involving DNA lesions that interfere with replication, just as yeast Rev3 is. The hRev3 protein of human cells, a predicted 353 kDa molecule [10], has not yet been isolated, but the non-catalytic subunit, hRev7, a much smaller molecule, has been isolated [14].

The present study was carried out to test the hypothesis that hRev7, the putative noncatalytic subunit of hPolζ, is also involved in human cell mutagenesis. For such a study, an approach similar to that used for investigating the role of hRev3 was employed, but instead of using antisense RNA to block expression of the target protein, siRNA against hRev7 was used to reduce the level of this protein in human fibroblasts. The fact that antibodies capable of detecting very low levels of hRev7 protein were available allowed us to identify independent cell strains in which the level of hRev7 protein had been greatly reduced by siRNA. Comparing the results obtained using these cell strains with those obtained using their parental human fibroblasts and a vector control strain allowed us to demonstrate that hRev7, the noncatalytic subunit of hPolζ, plays a role in the survival of UV-irradiated human cells, and has a significant role in UV-induced mutagenesis.

Using these human cell strains, we also demonstrated that reduction in the expression of hRev7 impedes the cells' ability to progress through S-phase.

MATERIALS AND METHODS:

Cell culture-- Cells were grown in Eagle's minimum essential medium, supplemented with 0.2 mM L-aspartic acid, 0.2 mM L-serine, 1 mM sodium pyruvate, 10% supplemented calf serum (HyClone), 100 units/ml penicillin, 100 µg/ml streptomycin, 1 µg/ml hydrocortisone and 1 µg/ml tetracycline.

Cell strains-- The parental human cell strain used for these studies, designated MSU-1.2.9N.58, was derived from the cell strain MSU-1.2, a spontaneous derivative of the infinite life span cell strain MSU-1.1, whose origin from the foreskin-derived from a normal neonate and subsequent acquisition of an unlimited life span in culture has been described [16]. MSU-1.2 cells are near-diploid, chromosomally-stable, and grow vigorously as a result of expressing their endogenous gene for platelet-derived growth factor.

Derivation of cell strains with reduced hRev7-- Oligonucleotides designed to target *hRev7* mRNA were annealed to a complementary oligonucleotide according to the manufacturer's protocol (Ambion). Using T4 DNA ligase (New England Biolabs), annealed-oligonucleotides were ligated into the pSilencer3.1 vector (Ambion), which includes the gene coding for puromycin resistance, and purified. The parental MSU-1.2.9N.58 cells were transfected with such siRNA vectors, using Lipofectamine (Invitrogen) according to the manufacturer's protocol, and stable transfectants were selected and maintained in medium

containing 1 μg/ml puromycin.

Preparation of nuclear protein extracts and Western blot analysis-- Subconfluent monolayers of cells were washed with ice-cold phosphate buffered saline (PBS), scraped from the 150-mm-diameter dishes 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. 10% NP-40 (62 μl) was added to each sample of lysed cells, and the samples were vortexed for 10 sec, and centrifuged for 30 sec at 10,000 RPM, 4°C. 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 nuclei 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 for 5 min at 16,000 RPM, 4°C. The supernatants, which contained the nuclear proteins, were saved. Protein was quantified using the Bradford method (Pierce). Protein lysates were subjected to gel electrophoresis using 14% SDS-polyacrylamide, transferred to a PDVF Immobilon membrane (Millipore), and probed with a 1:600 dilution of a custom-made (Bethyl) rabbit polyclonal antibody raised against the C-terminal 19 amino acids of the human Rev7 protein. 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 (Santa Cruz) and a 1:10,000 dilution of anti-rabbit secondary antibody (Santa Cruz).

Assay of UV cytotoxicity-- The cytotoxic effect of $UV_{(254nm)}$ radiation was determined by assaying the colony-forming ability of the cells as described [17]. 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 h for attachment. The cells were rinsed twice with PBS, the excess PBS was removed, and the cells were irradiated with the designated doses of $UV_{(254nm)}$ as described [17]. Immediately after irradiation, the cells were given fresh culture medium. The culture medium was renewed 24 h after irradiation and again after 7 days. After 14 days, the resulting clones were stained with crystal violet. Cell survival was determined by comparing the cloning efficiency of the irradiated cells with that of the sham-irradiated control cells. Cell survival at each dose was expressed as a percent of the cloning efficiency of the unirradiated control cells for each cell strain.

Assay for frequency of UV-induced mutations-- The mutagenic effect of UV radiation in each strain was determined from the frequency of cells that lost expression of the *HPRT* gene and, therefore, were resistant to 6-thioguanine. As described [17], sufficient sets of cells plated at densities of 0.5-1.5 x 10^6 cells per 150-mm-diameter dish, were used, to ensure at least 1 x 10^6 surviving target

cells per dose. Cells were allowed 12 h for attachment, then rinsed twice with PBS, UV-irradiated at the designated doses, and immediately covered with fresh culture medium. The culture medium was renewed 24 h after irradiation, and the cells were allowed to replicate for 4 days. Cells were then detached using trypsin, pooled, plated at densities of 0.5-1.0 x 10^6 cells per 150-mm-diameter dish, and allowed to grow exponentially for 4 additional days in order to deplete the pre-existing wild-type HPRT protein. Cells were then detached using trypsin, and plated at a density of 500 cells/cm^2 in medium containing 40 μM 6-thioguanine (TG) to select for cells lacking functional HPRT protein. At the same 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. The medium on these cells was renewed after seven days. After 14 days, the colonies that had formed 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 for each cell strain were calculated by subtracting the background frequencies in the sham-irradiated control populations that accompanied each experiment.

Hypoxanthine phosphoribosyltransferase (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 TG-resistant mutant

clones were isolated by treating with trypsin, and each independent mutant was subjected to reverse transcription, followed by 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 coding region of *HPRT*. Only base substitutions at adjacent pyrimidines that resulted in an amino acid change were considered UV-induced mutations.

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 h for attachment. Cells were then re-fed with complete culture medium containing lovastatin at a final concentration of 60 μM to synchronize the cells in early G1 phase. After 12 additional hours of incubation, the medium containing lovastatin was removed, the cells were washed twice with PBS, and culture medium containing aphidicolin at a final concentration of 2 μg/ml and mevalonic acid at a final concentration of 6 mM was added to the dishes for 12 h to synchronize the cells at the G1/S border. The cells were released from the aphidicolin/mevalonic acid block by rinsing twice with PBS, and immediately irradiated with the designated doses of UV as described [17]. 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.

Statistical methods -- To compare the slopes of the curves in Fig. 2A and 2B, a regression model was used. The slopes indicate the effect of UV on the survival (A) of the cells' colony-forming ability and on their frequency of UV-induced mutants (B). The data were taken from a series of experiments that were treated as blocks in the regression analysis.

For comparing the types of mutations induced (see Table 1), the sparse categories, i.e., C→G and T→G, were collapsed for the chi-square analysis. They showed no statistically significant difference between the clones and the comparison group (P-value = 0.60). Other categories were collapsed to reduce the number of table cells analyzed with small expected frequencies.

RESULTS:

Efficient reduction of hRev7 protein using siRNA-- The parental cell strain, designated MSU-1.2.9N.58, was transfected with a vector expressing an siRNA targeted against *hRev7* and also carrying the gene for puromycin resistance to allow selection of transfectants. As a control, we similarly transfected the parental cells with a vector containing this selectable marker and an siRNA that has no significant homology to any human gene sequence. Puromycin-resistant clones were isolated and expanded. Nuclear protein extracts from the parental cells, vector control transfectants, and from candidate transfectants that received the siRNA against *hRev7* were prepared and analyzed by Western blotting for their level of expression of hRev7. Figure 1 shows a representative Western blot. Lane 1 shows hRev7 protein from the parental cell strain (P), migrating as expected for a 24 kDa protein. Lane 2 shows hRev7 protein from the vector control cell strain (VC). Lanes 3 and 4 were loaded with protein from two clones, designated 2-2 and 2-6, that had been transfected with a vector expressing *hRev7* siRNA. No hRev7 protein could be observed in the latter two clones (Fig. 1). However, when the Western blots were allowed ≥ 24 h of exposure, a very low level of hRev7 protein could be detected on the blots (data not shown). The morphology of clones 2-2 and 2-6 did not differ from that of the parental or the vector control cells. Neither did the rate of growth in culture of these two cell strains differ from that of their parent or the vector control cells (data not shown).

Fig. 1 - Western blot analysis of hRev7 protein levels in the cell strains. A polyclonal antibody against hRev7 was used to analyze the level of hRev7 protein in nuclear lysates extracted from the parental cells (P), the vector control cells (VC), and two derivative clones expressing a transfected siRNA targeted against *hRev7*, viz., clones 2-2 and 2-6. The arrowhead on the right indicates the location of a 22 kDa marker. Ku80 was used as the loading control.

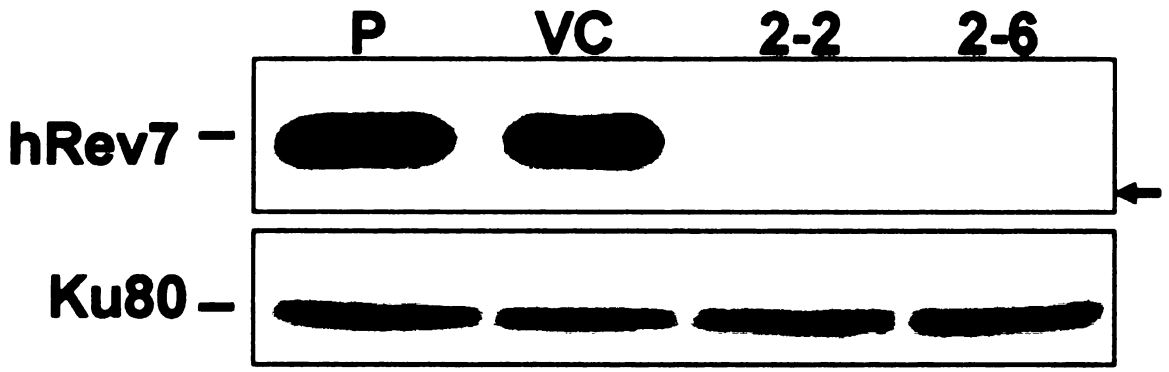

Fig. 1

Effect of reduction of the level of hRev7 protein on the sensitivity of the cell strains to the cytotoxic effect of $UV_{(254nm)}$ radiation– To examine the sensitivity of these cell strains to the cytotoxic effect of UV, we irradiated the parental cells, the vector control cells, and the two cell clones virtually devoid of hRev7 protein and assayed them for survival of colony-forming ability. As shown in Fig. 2A, the parental cell strain and the vector control strain exhibited identical sensitivity to the cytotoxic effect of $UV_{(254nm)}$ radiation. The dose required to reduce their survival to 37% that of unirradiated cells was 12 J/m^2. The survival of clones 2-2 and 2-6 were identical to each other, but both were 1.7 times more sensitive to UV-induced cell killing than the control cells. The dose required to reduce their survival to 37% that of unirradiated cells was only 8.5 J/m^2. These differences were shown to be statistically significant. The data indicate that hRev7 plays a protective role in survival of cells exposed to UV irradiation.

Effect of reduced hRev7 protein on the frequency of UV-induced mutations- The frequency of mutations induced by UV in the *HPRT* gene of these four cell strains was determined using resistance to 6-thioguanine as an indicator of cells with a mutation in their *HPRT* gene. As shown in Fig. 2B, the frequency of mutations induced by UV in the parental cells and vector control cells expressing hRev7 were identical. The dose that reduced their survival to 37%, i.e., 12 J/m^2, induced an *HPRT* mutation frequency of ~135 x 10^{-6}. In contrast, the two clones

Fig. 2 – Effect of reduced expression of hRev7 on the survival of cell strains exposed to $UV_{(245nm)}$ radiation and on the frequency of UV-induced mutations. **(A)** Clones 2-2 and 2-6 (closed symbols), which have greatly reduced levels of expression of hRev7 protein, along with their parental strain and a vector control strain (open symbols) were UV-irradiated and assayed for cell survival as determined by colony forming ability. Some data points have been offset slightly to make them visible. The lines represent least squares lines. **(B)** The frequency of UV-induced mutations in the *HPRT* gene of these four cell strains was determined by resistance to 6-thioguanine. The frequency of 6-thioguanine-resistant cells was calculated using the cloning efficiency of cells at the time of selection, which averaged ~44% for the parent and the vector control cells, and ~25% for clones 2-2 and 2-6. Induced frequencies were calculated by subtracting the background frequencies observed in sham-irradiated populations. For the vector and parental control cells, these values were always $<11 \times 10^{-6}$. For clone 2-2, they ranged from $0\text{-}18 \times 10^{-6}$, with the majority being $0\text{-}4 \times 10^{-6}$. For clone 2-6, they ranged from $8\text{-}25 \times 10^{-6}$, with the majority being $8\text{-}11 \times 10^{-6}$. Some data points have been offset slightly to make them visible. The lines represent least squares lines.

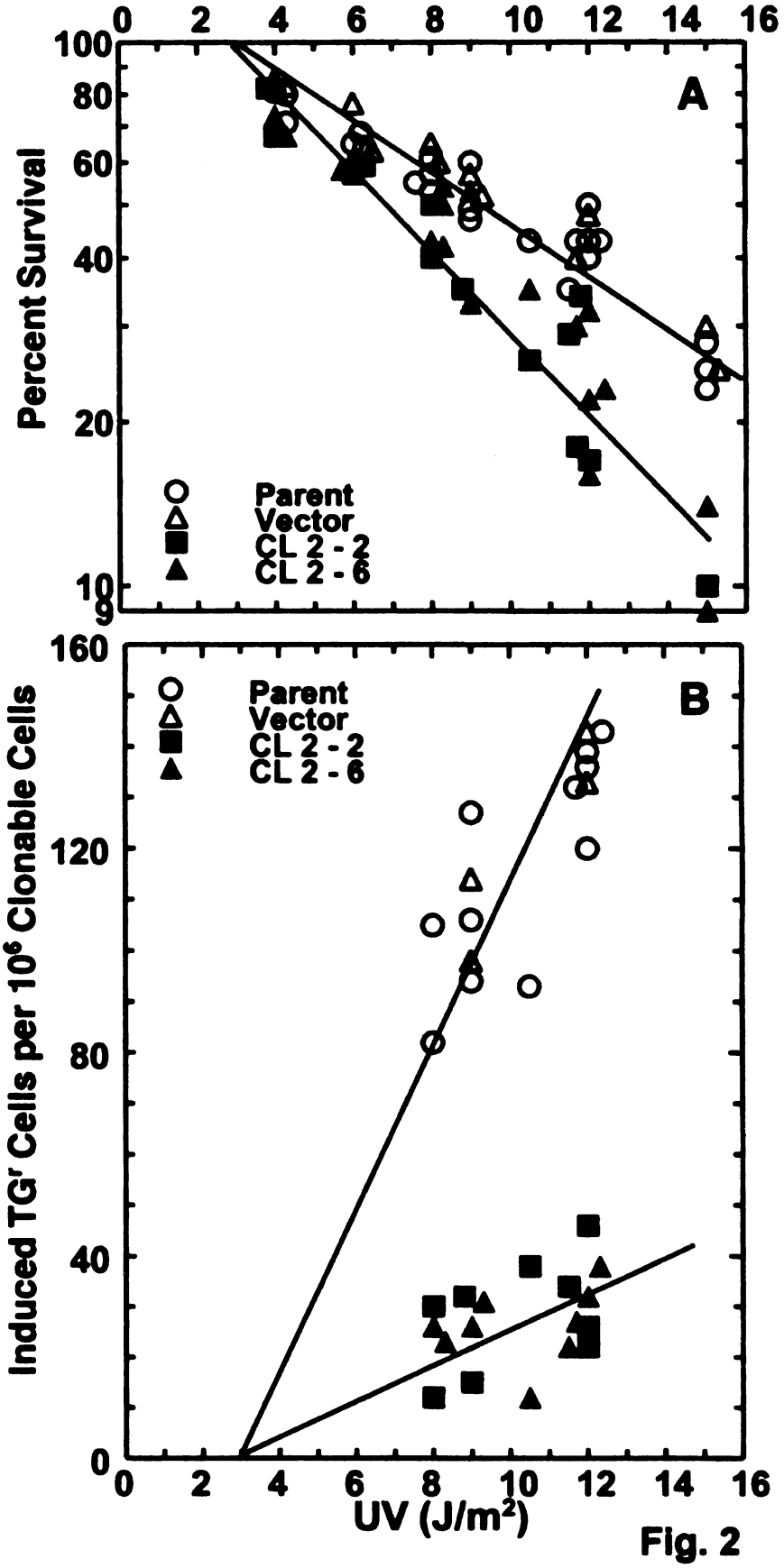
A
Percent Survival
100
80
60
40
20
10
0
2
4
6
8
10
12
14
16
Parent
Vector
CL 2 - 2
CL 2 - 6
B
Induced TG^r Cells per 10^6 Clonable Cells
160
120
80
40
0
UV (J/m^2)

Fig. 2

with greatly reduced expression of hRev7 protein showed a statistically significant decrease in the frequency of $UV_{(254nm)}$-induced mutations (P-value for comparison of slopes). The frequency of mutants induced by 12 J/m^2 in clones 2-2 and 2-6 was only 32 x 10^{-6} clonable cells. At a dose that reduced their survival to 37%, i.e., 8.5 J/m^2, the frequency of induced mutants was ~20 x 10^{-6}, significantly lower than the 135 x 10^{-6} seen in the two control cell strains. This significant reduction in the frequency of UV-induced mutations in cells with reduced hRev7 indicates that hRev7 plays an important role in UV-induced mutagenesis in human fibroblasts.

Effect of decreased hRev7 protein expression on kinds of UV-induced mutations– The kinds of base substitutions induced by UV in the coding region of the *HPRT* gene of cells expressing or not expressing hRev7, as determined by nucleotide sequencing, are shown in Table 1. The data report kinds of mutations induced in the two clones extremely deficient in expression of hRev7 (clones 2-2 and 2-6) and those induced in the comparison group (parent and vector control). [A table detailing the kinds and showing the context of 95 independent $UV_{(254nm)}$-induced mutations in *HPRT* of these two sets of human skin fibroblasts can be found in the Supplement]. There was no statistically significant difference in the kinds of mutations induced in the parental and vector control cells compared to those induced in the two derivative cell strains, virtually devoid

Table 1 – Effect of reduced expression of hRev7 on the types of base substitution mutations induced by $UV_{(245nm)}$ radiation. Cell clones with reduced hRev7 (clone 2-2 and 2-6), their parental strain and a vector control were treated with UV and the types of base substitutions induced in the *HPRT* genes of these cell strains was determined by subjecting 6-thioguanine resistant colonies to reverse transcription PCR and then sequencing the resulting cDNA. The number of each of the six possible types of UV-induced base substitution mutations that occur at sites at pyrimidines (also expressed as a percentage of the total number of base substitutions) is indicated. Only independent base substitutions at adjacent pyrimidines that resulted in an amino acid change were considered UV-induced mutations.

TABLE 1. Kinds of UV-induced base substitutions in the *HPRT* gene of cells with normal or greatly decreased levels of hRev7

Base changes	Parent and vector control		Clone 2-2 and clone 2-6	
C→T	25	(47.2%)	19	(45.2%)
T→C	6	(11.3%)	2	(4.8%)
T→A	8	(15.1%)	11	(26.2%)
C→A	10	(18.9%)	7	(16.7%)
C→G	4	(7.5%)	1	(2.4%)
T→G	0		2	(4.8%)
Total	53	(100%)	42	(100%)

of hRev7 protein. These data indicate that reducing the level of hRev7 results in a decrease in the frequency of UV-induced mutations without altering the types of base substitutions generated.

Evidence that reduction of expression of hRev7 protein in human fibroblasts results in a UV-induced delay in traversing S-phase -- To assess potential effects of decreased hRev7 on cell cycle progression after UV irradiation, the parental and vector control cell strains and the two clones with greatly reduced hRev7 were synchronized at the G1/S border as described, released from the block, and immediately UV-irradiated with 12 J/m^2, the dose determined to reduce the survival of normal cells to ~37% and that of the hRev7-deficient cells to ~20% (see Fig. 2A). Independent synchronized populations of these UV-irradiated cells were assayed by flow cytometry immediately following release from the G1/S block (0 h), or after 10 h or 16 h. As shown in Fig. 3A, at the time of release from the replication block (0 h), all four strains were synchronized at the G1/S border. Ten h later, the parental (P) and vector control cells (VC) were predominantly in S-phase and G2 phase. In contrast, after 10 h, cell strains 2-2 and 2-6 were still predominantly in G1 and S-phase, i.e., their cell cycle progression was greatly delayed, compared to that of the two control strains. The data from cells assayed 16 h following UV irradiation show that the cell strains with reduced hRev7 were still delayed in S-phase, compared to their parental strain and the vector control cell strain.

Fig. 3 - Flow cytometry analysis of UV-irradiated cells. (A) Cell strains with reduced levels of hRev7 (2-2 and 2-6), their parental strain (P), and a vector control transfectant (VC) were synchronized at the G1/S border as described, and UV-irradiated immediately after release from synchrony. The cell strains were assayed by flow cytometry for DNA content at 0 h, 10 h, and 16 h post-irradiation. **(B)** These four cell strains were UV-irradiated while growing asynchronously and were assayed by flow cytometry for DNA content at 0 h and 10 h post-irradiation. **(C)** As a control, clones 2-2, 2-6, and their parental strain were synchronized at the G1/S border, as above, released from synchrony, but not exposed to UV, and analyzed by flow cytometry at 0 h, 4 h, and 6 h after release from the G1/S block.

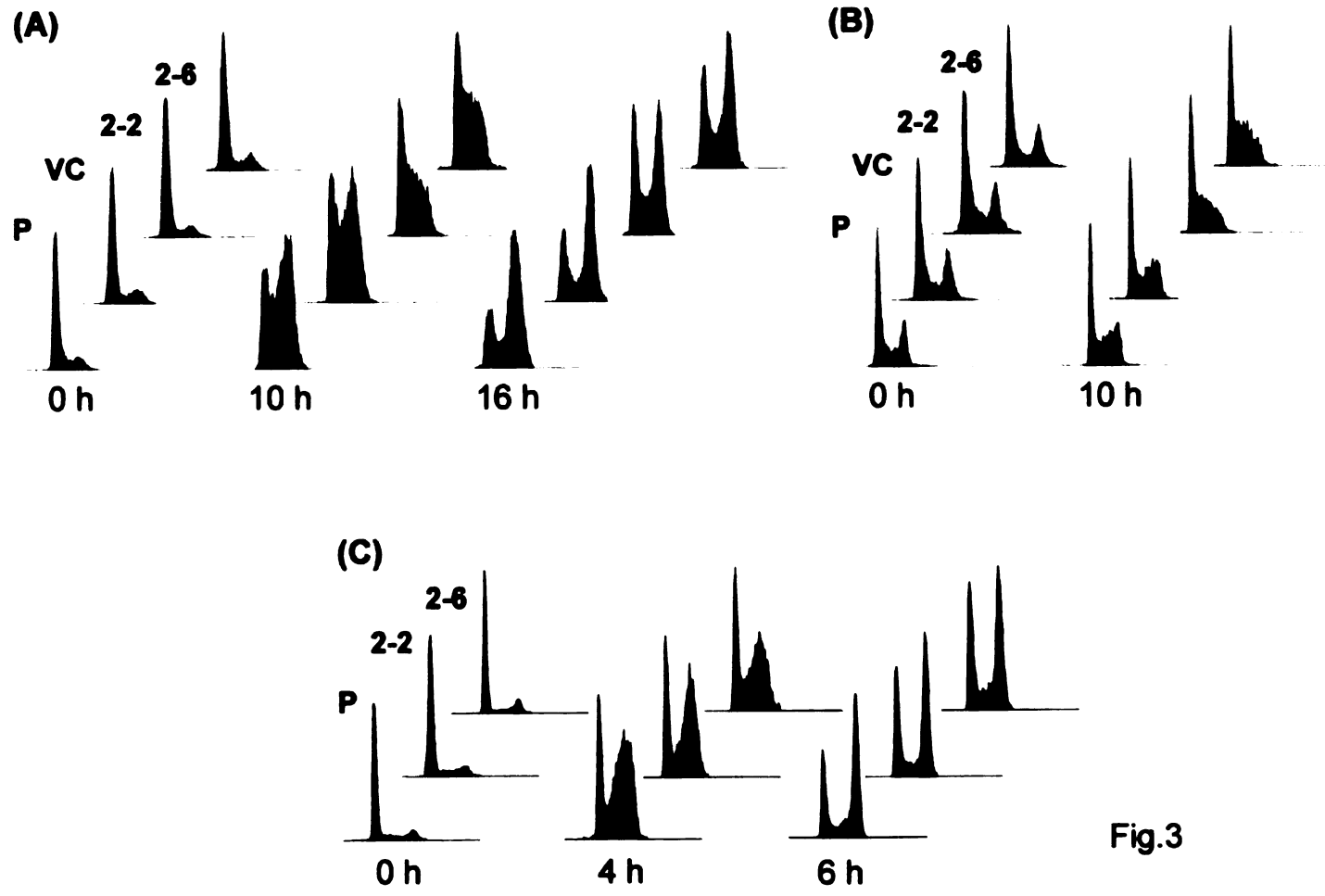

Fig.3

These four cell strains were similarly irradiated with 12 J/m^2 while growiing asynchronously and assayed by flow cytometry. As shown in Fig. 3B, UV-irradiation of asynchronously-growing cells with decreased expression of hRev7 also delayed their progression through S-phase compared to control cells. As a control, we synchronized the parental and the derivative strains with reduced hRev7, released them from the block, as described above, but did not expose them to UV radiation. The subsequent flow cytometric analysis (Fig. 3C) demonstrated that all three unirradiated cell strains proceeded through the cell cycle at an equal rate. Taken together, the data in Fig. 3 indicate that cells with reduced hRev7 replicate their DNA more slowly in the presence of UV-induced DNA damage.

DISCUSSION:

A role for hRev7 as the non-catalytic subunit of Polζ was suggested by its homology to the yeast Rev7 protein, as well as its physical interaction with human Rev3 in a yeast-two-hybrid assay [14]. The results of our study establish a functional role for hRev7 in UV-induced mutagenesis, and what is more, a role in the survival of colony-forming ability. The fact that at every dose of UV radiation, the cells with greatly decreased levels of hRev7 protein exhibited ~5-fold lower frequency of induced mutations than the control cell strains is strong evidence that in human cells the hRev7 protein plays a role in error-prone TLS past UV-induced DNA photoproducts, as does yeast Rev7.

The mutagenesis data from the present study, using human fibroblasts that have greatly reduced levels of hRev7 protein, confirm and greatly strengthen the results obtained previously in this laboratory using similar cell lines and antisense against *hRev3* [10, 15]. The ~5-fold decrease in UV-induced mutation frequency observed in cells virtually devoid of hRev7 is similar to the decrease in frequency observed previously in this laboratory using human fibroblasts expressing antisense *hRev3* [10, 15]. These data strongly support the hypothesis that hRev3 and hRev7 function in the same pathway, most likely as catalytic and non-catalytic subunits, respectively, of human polymerase ζ.

Our results demonstrating that human fibroblasts with reduced hRev7 are more sensitive to the cytotoxic effects of UV-radiation than the control strains (Fig. 2A) support the results obtained by Cheung et al., which demonstrated that nasopharyngeal carcinoma cell lines with reduced hRev7 are also more sensitive to the cytotoxic effects of specific DNA damaging anticancer drugs than the control cells [18]. The fact that reduction of hRev7 results in increased sensitivity to the cytotoxic effects of various DNA damaging agents in both normal and cancer cells, underscores the importance of hRev7 for protecting human cells from DNA damage.

In addition to sharing a high degree of amino acid sequence similarity to the yeast Rev7 protein, hRev7 also has a high degree of similarity to the mitotic checkpoint protein hMAD2, and thus is also referred to as hMAD2B [14, 19]. In fact, hRev7 has been shown to interact with hMAD2 and furthermore, to inhibit the anaphase-promoting complex by binding to activators Cdh1 and Cdc20 in *Xenopus* extracts, suggesting a role for hRev7 in regulating the mitotic checkpoint [14, 20, 21]. One might hypothesize that the sensitivity to the cytotoxic effects of UV we observe in our human fibroblasts reflects interference with the mitotic checkpoint response of UV-irradiated cells. However, Cheung et al., [18] found that reducing hRev7 in nasopharyngeal carcinoma cells had no effect on their mitotic checkpoint response. Therefore, we consider it unlikely that the increased sensitivity to the cytotoxic effects of UV-radiation that we observe in human fibroblasts with reduced hRev7 results from an aberrant mitotic

checkpoint response. Nevertheless, further experimentation could be conducted to specifically determine whether or not hRev7 affects the mitotic checkpoint of human fibroblast cells in the presence of DNA damage.

In addition to decreasing the survival of UV-irradiated human fibroblast strains, reduced levels of hRev7 also resulted in a marked decrease in their rate of progression through the DNA synthesis phase of the cell cycle (Fig. 3). This suggests that for human cells with reduced expression of hRev7, and therefore with reduced hPolζ, the presence of DNA damage during S-phase presents a major problem for DNA replication. If cells with decreased expression of hRev7 were simply unable to resume DNA replication as a result of impaired TLS, this situation would be expected to result in replication fork breakdown and ultimately lead to cell death. It is always possible that replication arrest due to impaired TLS accounts for the increase in sensitivity to the cytotoxic effects of UV that we observe when cell strains with reduced hRev7 are irradiated. However, previous results in our laboratory using human cells expressing antisense against *hRev3*, the catalytic subunit of hPolζ, demonstrated that such cells did not differ from the parental cells in sensitivity to the cytotoxic effects of UV [15]. Taken together, these results suggest that in our clones with reduced hRev7, factors other than impaired TLS contribute to the observed increase in sensitivity to the cytotoxic effects of UV.

In 2005, a study by Bi et al. [22], demonstrated mouse embryonic fibroblasts

lacking Polκ were unable to recover from a benzo[a]pyrene diol epoxide (BPDE)-induced S-phase checkpoint and, in addition, were more sensitive to the cytotoxic effects of BPDE than were the wild-type cells. The fact that we found a clear indication of a UV-induced delay in S-phase, and an increase in sensitivity to the cytotoxic effects of UV in cell strains with reduced hRev7, suggests that there is a similar requirement for hRev7 in recovery from a UV-induced S-phase checkpoint as there is for Polκ in a BPDE-induced S-phase checkpoint.

In summary, our mutagenesis data demonstrate that hRev7, like hRev3, is required for TLS past UV photoproducts and is causally involved in producing the mutations that result from such TLS. These data strongly support the hypothesis that hRev7, together with hRev3, comprise hPolζ. In addition, our data demonstrate a requirement for hRev7 in protecting human fibroblasts from the cytotoxic effects of UV-induced DNA damage that was not found in human fibroblast cells expressing antisense against *hRev3*. This suggests that the protective role of hRev7 for cells exposed to UV radiation is independent of the requirement for hRev7 in hPolζ-dependent TLS.

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CHAPTER III

hRev7, the accessory subunit of hPol ζ, protects human fibroblasts from the cytotoxic effect of benzo[a]pyrene diol epoxide, but is not essential for translesion synthesis past DNA damage induced by this agent.

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ABSTRACT:

DNA polymerase zeta (Pol ζ), comprised of hRev3 and hRev7, is a specialized DNA polymerases that, unlike the classical replicative polymerases, is able to replicate past fork-blocking DNA lesions (i.e. to carry out translesion synthesis). The catalytic subunit of hPol ζ, hRev3, has been shown to play a critical role in the induced mutagenesis of human cells. However, much less is known about the role of hRev7, the accessory subunit of hPol ζ, in such mutagenesis. Recently, we generated human fibroblasts with significantly reduced levels of hRev7 protein and used such cells to demonstrate that hRev7 is required to protect cells from the cytotoxic and mutagenic effects of $UV_{(254nm)}$ radiation. To determine the effect of hRev7 on the biological effects of benzo[a]pyrene diol epoxide (BPDE) cell strains with reduced levels of hRev7 were compared to their parental strain and a vector control for cell survival, induction of mutations, and ability to progress through the cell cycle following exposure to BPDE. We determined that like with UV, cell strains with reduced hRev7 were more sensitive to the cytotoxic effects of BPDE than the control strains, and that these cell strains progressed through S-phase at a much slower rate than the control cells following BPDE treatment. But, our surprise, the frequency of BPDE-induced mutations in the cells with reduced hRev7 did not differ from those induced in their parental and vector control strains, indicating the hRev7 is not required for translesion synthesis past BPDE-induced DNA damage. We also found that cell strains with reduced hRev7 were more sensitive than their

parental and vector control strains to the cytotoxic effect of ionizing radiation, cisplatin, and *N*-methyl-*N*-nitrosourea, indicating that hRev7 also protects cells from these DNA damaging agents and suggesting that hRev7 is generally required to protect cells from the cytotoxic effect of DNA damaging agents. Taken together, these results indicate that the protective role hRev7 plays for the survival of cells exposed to DNA damaging agents is independent of its role in translesion synthesis.

INTRODUCTION:

Human cells undergo countless rounds of DNA replication, which must be very accurate to preserve critical genetic information. To maintain such a significant level of accuracy, the classical replicative polymerases have evolved highly selective active sites that only accommodate nucleotides when they are correctly paired to the DNA template. In addition, many of these DNA polymerases possess 3'→5' proofreading exonuclease activity, which removes nucleotides that are incorrectly incorporated during replication, allowing an additional attempt at accurate DNA synthesis. As a consequence of their stringency, the classical replicative polymerases cannot tolerate fluctuations in the DNA structure, including those that result from DNA damage. Nevertheless, DNA is continually subjected to a variety of insults, from both endogenous and environmental agents that generate DNA damage. Much of this damage is excised by DNA repair mechanisms before replication occurs. However, if repair is slow or the DNA damage is extensive, DNA lesions may persist during replication. If the high fidelity replicative polymerase complex encounters a DNA lesion that blocks elongation, potentially fatal stalling or arrest of replication will occur.

To avoid replication arrest, mechanisms may be employed that enable DNA lesions to be tolerated without their physical removal. One such mechanism is translesion synthesis (TLS). Translesion synthesis involves the use of specialized DNA polymerases to replicate past the DNA lesions until the high

fidelity replicative polymerases are able to resume DNA synthesis. Several novel DNA polymerases have been discovered, whose primary function is likely to carry out TLS. These TLS polymerases typically contain active sites that are less restrictive, making them better able to accommodate distortions in DNA (see for example [1-4]). Although TLS polymerases demonstrate the unique ability to synthesize past replication-blocking DNA lesions, enabling the cell to survive such DNA damage, they are also characterized by relaxed nucleotide selectivity and lack of 3'→ 5' proofreading exonuclease activity. As a result, protection of cells from replication arrest can come at the cost of introducing mutations in DNA, which can result in the development of cancer.

More than 300 polymerases involved in TLS have been discovered in eukaryotes, bacteria and archaea [5]. The first TLS polymerase to be identified in eukaryotes was DNA polymerase zeta (Pol ζ) [6]. DNA polymerase ζ was initially characterized in the budding yeast, *Saccharomyces cerevisiae*, and is composed of two subunits, a catalytic subunit, called Rev3, as well as an accessory subunit, Rev7 [6]. Studies using yeast *rev* mutant strains have demonstrated that Pol ζ is responsible for the majority of both spontaneous [7, 8] and DNA damage-induced mutations that occur in this organism [9-14], suggesting that this polymerase participates in error-prone TLS past a extensive array of DNA lesions (reviewed in [15]).

Human homologs of the yeast *REV* genes have been identified [16, 17]. The transcript of the human *REV3* gene encodes a 353 kDa protein, which is about twice the size of the yeast protein [18]. Presumably because of the large size and low cellular levels of hRev3, the protein has never been expressed or isolated and therefore, *in vitro* studies using human Pol ζ are lacking [19]. However, human cells expressing high levels of *hREV3* antisense RNA have been reported to demonstrate a lower frequency of both ultraviolet (UV) and benzo[a]pyrene diol epoxide (BPDE)-induced mutations than the control cells, indicating that, as in yeast, hRev3 is required for induced mutagenesis and suggesting that the functions of Pol ζ are conserved from yeast to humans [18, 20].

To investigate the role of hRev7 in TLS, we recently generated two human fibroblast cell strains in which the levels of hRev7 protein were significantly reduced by small interfering RNA (siRNA) [21]. When cell strains with reduced hRev7 were UV-irradiated, their rate of progression through S-phase was considerably slower, and their cell survival was significantly reduced, compared to control strains. In addition, the frequency of UV-induced mutations in cell strains with reduced hRev7 was five times lower than normal. These data showed that like hRev3, hRev7, presumably as a part of human Pol ζ, plays a role in UV-induced mutagenesis of human cells.

To determine whether hRev7 is similarly involved in the tolerance of mutations induced by benzo[a]pyrene diol epoxide, the reactive form of the widespread

environmental carcinogen benzo[a]pyrene, cells strains with reduced levels of hRev7 were compared to their parental strain and a vector control for their response to the biological effects of BPDE. Our results show that cell strains with reduced hRev7 progress through the cell cycle at a slower rate than control strains after exposure to BPDE, and are also more sensitive to its cytotoxic effect. These data indicate that hRev7 is required for protecting cells from BPDE-induced killing. We also found that cell strains with reduced hRev7 are more sensitive to the cytotoxic effects of ionizing radiation (IR), cisplatin, and *N*-methyl-*N*-nitrosourea (MNU), suggesting that hRev7 has a generalized role in protecting cells from an assortment of DNA damaging agents.

Our results further demonstrate, that the frequency of mutations induced by BPDE in cells with reduced levels of hRev7 does not differ from the frequency induced in the control cell strains, indicating that hRev7 is not required for TLS past BPDE-induced DNA lesions. These results are particularly surprising in light of the fact that previous studies in our laboratory [20] demonstrated that hRev3, the catalytic subunit of hPol ζ, is required for mutagenic TLS past BPDE-induced DNA lesions. However, after careful re-evaluation of our previous data, we now believe that neither hRev3 nor hRev7 are required TLS past BPDE-induced mutagenesis.

MATERIALS AND METHODS:

Cell culture-- Cells were routinely cultured in Eagle's minimal essential medium supplemented with L-aspartic acid (0.2 mM), L-serine (0.2 mM), sodium pyruvate (1 mM), supplemented calf serum (SCS) (Hyclone, Logan, UT) (10% (v/v)), penicillin (100 U/ml), streptomycin (100 µg/ml), tetracycline (1 µg/ml) and hydrocortisone (1 µg/ml) (culture medium). The cells were cultured in a 37°C humidified incubator with 5% CO_2, 95% air.

Cell strains-- The human fibroblast cell strains used as the parental strains in this study, designated MSU-1.2.9N or MSU-1.2.9N.58 (9N and 9N.58 for short), were derived from the infinite life span, telomerase positive, near-diploid, karyotypically stable, MSU-1.2 lineage of cells established in the Carcinogenesis Laboratory [22].

Cell strains 2-2 and 2-6, which have significantly reduced levels of hRev7 protein, as well as the vector control strain, VCA, were derived from the parental strain, 9N.58, by McNally et al., as described [21]. The two additional cell strains with reduced hRev7 that were used in this study, designated 2.5 and 3.2, as well as the vector control, V1.1, were derived from the same parental strain, i.e. 9N.58, using the methods previously described [21]. Briefly, the pSilencer3.1/hRev7 vector, which expresses siRNA targeted to *hRev7* and contains puromycin as a selectable marker, or the appropriate vector control, pSilencer3.1, were

transfected into the parental strain 9N.58 using Lipofectamine^{TM} (Life Technologies, GibcoBRL) according to the manufacturer's instruction. Forty-eight h post-transfection, the cells were selected in culture medium containing 1 μg/ml puromycin. This medium was renewed every 5-7 days for 2 weeks. When puromycin-resistant colonies formed, they were isolated, expanded, and analyzed by Western blot for the level of expression of hRev7 protein. Cell strains 2-2, 2-6, 2.5 and 3.2 were determined to express significantly reduced levels of hRev7 protein (Figure 1 and [21]).

Cell strains expressing high levels of *hRev3* antisense (6I and 12C) were generated by Li et al., as described in reference [20]. In short, the pKS-2 plasmid [18], which expresses *hRev3* antisense RNA under control of the TetP promoter (Tet off system) and contains puromycin as a selectable marker, was transfected into the parental strain using LipofectAMINE according to the manufacturer's instructions (Life Technologies, GibcoBRL). Forty-eight h after the transfection, cells were selected with puromycin (1 μg/ml). When puromycin resistant colonies formed, they were isolated and expanded. Clones 6I and 12C were determined to express high levels of *hRev3* antisense RNA by Northern blot analysis [20].

Western blot analysis-- Western blot analysis was performed as described [21]. Briefly, to obtain nuclear protein extracts, subconfluent monolayers of cells were washed with ice cold phosphate buffered saline (PBS), and cells were removed from 150 mm-diameter dishes by scraping into 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). Cells were incubated on ice for 15 min and then lysed by adding 62 μl of 10% Nonidet P-40 and vortexing for 10 sec. Cell lysates were centrifuged for 30 sec at 10,000 RPM, 4°C and the supernatant, containing the cytoplasmic proteins, was removed. Cell pellets were then washed once with 1 ml of Lysis Buffer A containing Nonidet P-40. Nuclear protein extracts were obtained by incubating cell pellets 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 vortexing every 5 min for a total of 15 min. The nuclear extracts were centrifuged for 5 min at 16,000 RPM, 4°C and the supernatants, containing the nuclear proteins, were saved. The protein concentration was measured using a Coomassie (Bradford) Protein Assay Kit (Pierce). For Western analysis, 75 μg of nuclear protein was separated by gel electrophoresis using 14% SDS-polyacrylamide gels and electrotransferred to a polyvinylidene fluoride membrane (Millipore). The membrane was blocked for 1 h at room temperature in 5% non-fat milk diluted in tris-buffered saline containing 0.1% Tween-20, and incubated with primary antibodies against hRev7 (1:600, Bethyl) or Ku80 (1:10,000, Santa Cruz) at 4°C overnight. The following day, the membrane was incubated for 1 h at room temperature with horseradish peroxidase–conjugated, goat anti-rabbit secondary antibody (1:10,000, Sigma) and signals were visualized using the SuperSignal Chemiluminescent substrate (Pierce).

Exposure of cells to chemical mutagens-- When treating with chemical mutagens, the number of DNA lesions generated is dependent upon the cell density at the time of treatment. Therefore, both for survival and for mutagenesis studies, cells in exponential growth were detached from the dishes using trypsin and plated in 150 mm-diameter dishes approximately 16 h prior to treatment, such that the density of cells at the time of treatment would be as near 10,000 cells/cm^2 as possible. Following the 16 h attachment period, the culture medium was removed from each dish, cells were rinsed twice with (PBS), and then covered in Eagle's minimal essential medium [for MNU treatments, Eagle's minimal essential medium buffered with 15mM HEPES (pH 7.2)]. Immediately prior to treatment, BPDE (Midwest Research Institute) or MNU (Sigma) were dissolved in anhydrous dimethylsulfoxide (DMSO), and the designated doses were delivered by micropipette. To ensure that all cells were exposed to the same concentration of DMSO, regardless of the dose of BPDE or MNU, appropriate amounts of DMSO were added to dishes (including the untreated control cells) to equal the total amount of DMSO delivered to cells treated with the highest dose. Cisplatin (American Pharmaceutical Partners Inc.), which was supplied in an aqueous saline solution (1 mg/ml), was delivered to the cells directly by micropipette. Cells were exposed to BPDE or cisplatin for 1 h or to MNU for 30 min, at 37°C in a humidified 5% CO_2 incubator. At the end of the exposure period the medium containing the mutagen was removed, cells were rinsed twice with PBS, and covered with fresh culture medium. Induced cytotoxicity and mutagenesis assays were performed as described below.

Exposure of cells to ultraviolet radiation-- The method for exposing cells to $UV_{(254\ nm)}$ radiation differs somewhat from the method for exposure to chemical agents because, unlike what happens with chemical treatments, the number of DNA lesions generated by exposure to UV radiation is not dependent on cell density. For UV radiation, 12 h prior to UV exposure, cells in exponential growth were trypsinized and plated at cloning densities (100-500 cells per 100 mm-diameter dish) for assaying cell survival, or at $1x10^6$ cells per 150 mm-diameter dish for mutagenesis assays. Immediately prior to UV exposure, the culture medium was removed from each dish and the cells were washed twice with PBS. The cells were then UV-irradiated for the appropriate amount of time to result in the designated doses of UV as described previously [23] and replenished with fresh culture medium. The culture medium was renewed the following day and UV-induced cytotoxicity and mutagenesis assays were conducted as described below.

Exposure of cells to ionizing radiation-- On the day of treatment, exponentially growing cells were detached from dishes with trypsin and diluted to 200,000 cells/ml in culture medium containing 2% supplemented calf serum. Cells were irradiated as described [24] in 50 ml polypropylene tubes on ice using a U.S. Nuclear ^{60}Co variable flux, sealed source irradiator with a dose rate of 1.378 Gy/min.

Cell survival assay-- The procedures for determining the cytotoxic effects of DNA damaging agents by colony forming ability differ slightly based upon the particular type of DNA damaging agent used. For chemical mutagens, the cells were exposed to the appropriate agent at a density of 10,000 cells/cm^2 as described above. Immediately following treatment, the cells were rinsed with PBS, and detached from the dishes using trypsin. Trypsinized cells were then diluted and plated into four 100 mm-diameter dishes for each dose at cloning densities (i.e. the densities necessary to obtain approximately 50 surviving colonies per 100 mm-diameter dish depending on the expected cytotoxicity). After 7 days cells were provided with fresh culture medium, and after 14 days they were stained with crystal violet. To determine sensitivity to the cytotoxic effect of a particular agent (expressed as percent survival), the cloning efficiencies of cells exposed to the mutagen were normalized to the cloning efficiency of the untreated control cells.

For UV, cells were plated at cloning densities 12 h prior to exposure and then UV-irradiated the as described. Cells were provided with fresh culture medium after 1 day after UV-irradiation and again after 7 days. Fourteen days after UV-irradiation cells were stained with crystal violet. As with chemical mutagens, the survival of colony-forming ability of cells exposed to UV was determined by normalizing the cloning efficiency of treated cells to the cloning efficiency of the untreated cells.

To determine the cytotoxicity induced by ionizing radiation, cells were detached using trypsin and irradiated in suspension as described earlier. Immediately after irradiation, each cell suspension was diluted appropriately into fresh culture medium and plated into 4, 100 mm-diameter dishes for each dose at cloning densities. The culture medium was renewed after 7 days and the cells were stained after 14 days. The survival of the irradiated cells was calculated by normalizing the cloning efficiency of the irradiated cells to that of the control cells.

Mutagenesis assay-- To determine the frequency of induced mutations in the *hypoxanthine phosphoribosyl transferase (HPRT)* gene, assays were performed as described [23]. In short, a sufficient number of target cells were plated into 150 mm-diameter dishes to ensure that after treatment, the number of surviving cells was large enough to result in at least 40, 6-thioguanine (TG) resistant clones. Because exposure to either BPDE or UV causes a high frequency of induced mutants, it is sufficient to have approximately $0.8x10^6$ surviving cells. Following exposure to the mutagenic agent, cells were maintained in exponential growth for an 8-day expression period to allow any wild-type HPRT protein to be depleted. After the 8-day expression period, cells were trypsinized and diluted to 2,500 cells/ml in culture medium. To determine the cloning efficiency of the cells at the time of selection, a small portion of the cell suspension (2 ml) was diluted further and plated into 4, 100 mm-diameter dishes at cloning densities. To assay for TG-resistance, the remainder of the cell suspension was selected with TG, at

a final concentration of 40 μM, and then cells were plated at a density of 25,000 cells per 100 mm-diameter dish. All dishes were supplied with fresh culture medium (with or without TG as appropriate) after 7 days, and stained with crystal violet after 14 days. The observed mutation frequency was corrected by the cloning efficiency of the unselected cells. The induced mutation frequency was calculated by subtracting corrected frequencies observed in untreated control cells from the corrected mutant frequencies of the treated cells.

Analysis of the cell cycle progression of BPDE or UV treated cells by flow cytometry-- Cells in exponential growth were detached from the dishes using trypsin, and plated in 100 mm-diameter dishes such that the density of G_1/S-synchronized cells at the time of treatment would be as near 10,000 cells/cm^2 as possible. For the cell strains analyzed, approximately 140,000 cells were required for each 100 mm-diameter dish. Cells were allowed 16 h to attach and then arrested in G_1 phase using lovastatin (Sigma) at a final concentration of 60 μM. Twelve h later, cells were rinsed twice with PBS and replenished with fresh culture medium containing aphidicolin (Sigma) at a final concentration of 2 μg/ml and mevalonic acid (Sigma) at a final concentration of 6 mM to synchronize them at the G_1/S border. After an additional 12 h, cells were washed twice with PBS to release them from synchrony and immediately treated with BPDE or UV as described above. Every 4 h for the first 24 h post-treatment, a set of cells were detached using trypsin, harvested by centrifugation and fixed in 80% ethanol.

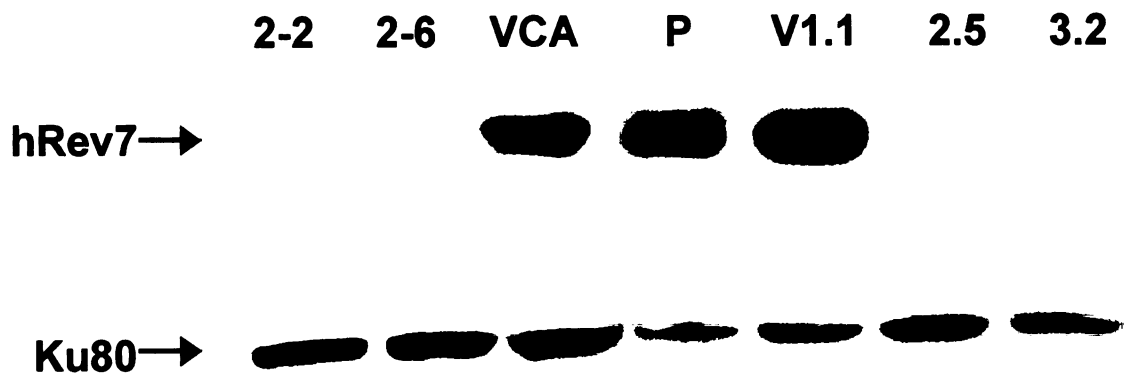
Prior to flow cytometry, cells were washed in PBS containing 1% SCS and then incubated in a propidium iodide solution (PBS, 1 mg/ml propidium iodide, 10% Triton X-100, 0.5 mM EDTA, 10 mg/ml RNase A) for 1 h. Cells were analyzed for DNA content by flow cytometry at the Flow Cytometry Core Facility at Michigan State University.

CHAT selection-- To eliminate the background frequency of 6-thioguanine resistant cells, they were plated in culture medium containing CHAT (20 μM deoxycytidine, 100 μM hypoxanthine, 0.4 μM aminopterin, 30 μM thymidine) and maintained in exponential growth for 3 weeks. Following the selection period, cells were grown for several days in medium containing CHT (CHAT medium excluding aminopterin) and then for several days in the appropriate culture medium.

RESULTS:

Effect of reduced hRev7 protein on the survival of human fibroblasts exposed to BPDE and on the frequency of BPDE-induced mutations-- Using siRNA, we previously generated two derivatives of the human fibroblast strain 9N.58 with significantly reduced levels of hRev7 protein [21]. These derivative strains were designated 2-2 and 2-6. Those two cell strains, along with two additional 9N.58-derived cell strains with reduced hRev7 protein (designated 2.5 and 3.2), as well as their appropriate controls, were used in our current study (Figure 1). To determine whether reducing the level of hRev7 protein alters the response of human fibroblast cells to the cytotoxic or mutagenic effects of BPDE, two cell strains with significantly reduced levels of hRev7 protein, (clones 2-2 and 2-6) were assayed along with their parental strain and a vector control transfectant for their sensitivity to the cytotoxic effect of BPDE as measured by survival of colony-forming ability (Figure 2A). These data show that, whereas the vector control strain demonstrated a BPDE-induced cytotoxicity that was very similar to that of the parent strain, each of the cell strains with reduced hRev7 protein was considerably more sensitive to BPDE-induced cytotoxicity. Specifically, 80% of the parent and vector control cells survived after being exposed to 0.07 μM BPDE, but only 50% of cells with reduced hRev7 survived following exposure to the same dose. These results, which show that cells with reduced hRev7 are more sensitive to the cytotoxic effect of BPDE than control

Figure 1 - Western blot analysis of the level of hRev7 protein. The level of hRev7 protein in nuclear extracts obtained from cell strains transfected with a vector expressing hRev7 siRNA (clones 2-2, 2-6, 2.5 and 3.2), their parental strain (P), or cell strains transfected with a vector control expressing an siRNA with limited homology to known sequences in the human genome (VCA and V1.1) was analyzed by Western blotting. Ku80 was used as a loading control. Note that the level of hRev7 protein in cell strains expressing hRev7 siRNA is significantly lower than the level of hRev7 protein in the parent or vector control strains.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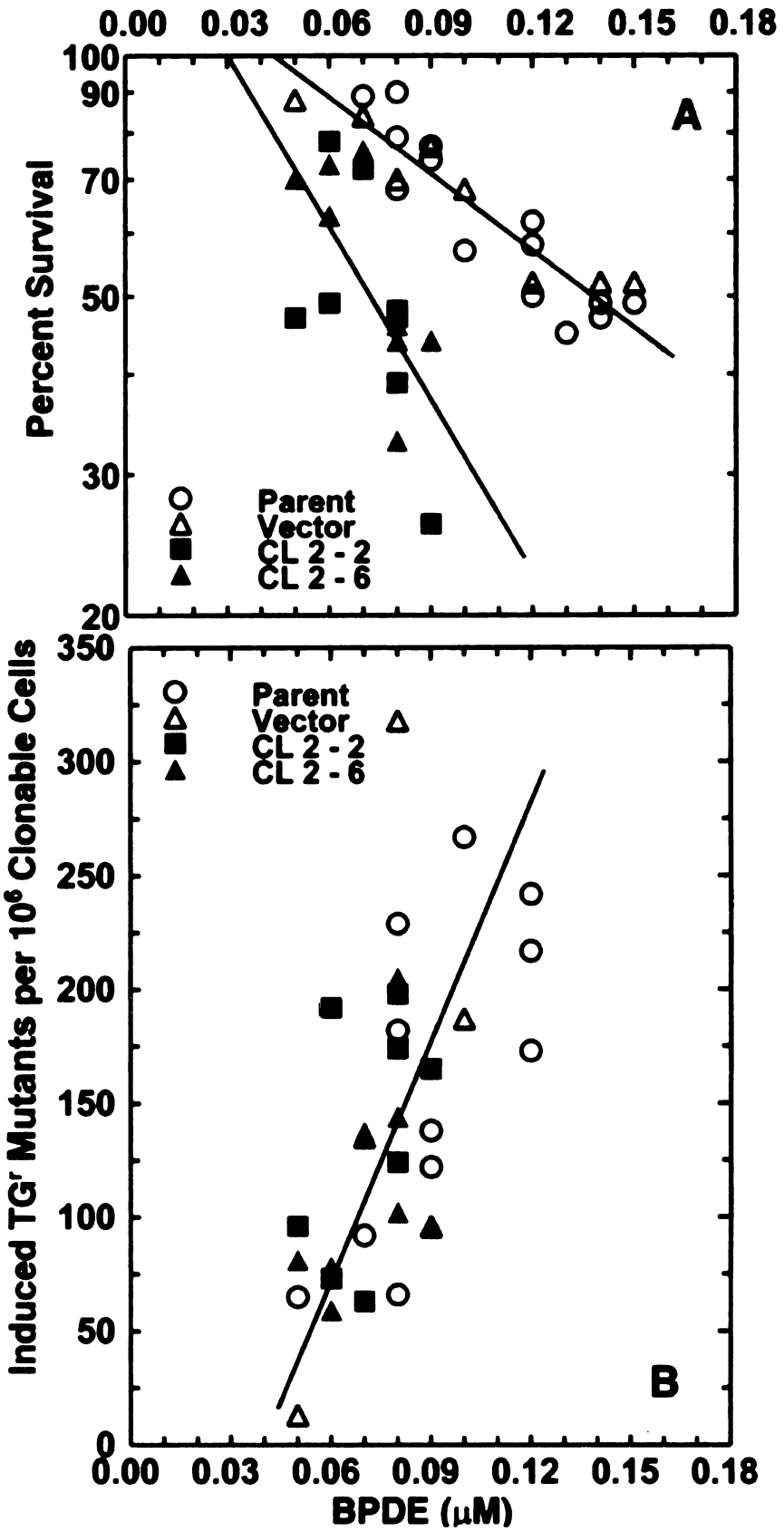
cells, indicate that the hRev7 protein plays a protective role for cells following exposure to BPDE.

Because cell strains with reduced hRev7 protein were more sensitive to the cytotoxic effect of BPDE, we were interested to determine whether the frequency of BPDE-induced mutations in these cell strains also differed from those of the control strains. Therefore, cell strains with reduced hRev7 were compared to their parental cell strain as well as to the vector control strain for the mutagenic effects of BPDE. To our surprise, the BPDE-induced mutation frequencies of cell strains with reduced expression of hRev7 protein did not differ significantly from those of their parental cell strain or from those of the vector control cell strain (Figure 2B). These data suggest that hRev7 is not essential for TLS past BPDE-induced DNA lesions.

Effect of reduced hRev7 on cell cycle progression following BPDE treatment-- Our laboratory previously demonstrated that decreasing the level of hRev7 protein in human fibroblasts rendered them more sensitive to the cytotoxic effect of UV and resulted in impaired progression through S-phase following UV-irradiation [21]. We hypothesized that this UV-induced delay in cell cycle progression contributed to the increased UV-induced cytotoxicity that we observed. In the present study we found that, like with UV, cell strains with reduced hRev7 are more sensitive to the cytotoxic effect of BPDE than control

Figure 2 - Reducing the level of hRev7 in human fibroblasts renders them more sensitive to the cytotoxic effect of BPDE, but does not affect their frequency of BPDE-induced mutations. Cell strains with reduced hRev7, designated clones 2-2 and 2-6 (closed symbols), the parental strain (open circles) and the vector control strain (open triangles) were treated with BPDE and assayed for **(A)** cell survival and **(B)** the frequency of mutations induced in the *HPRT* gene. The solid lines represent the least squares regression for the data.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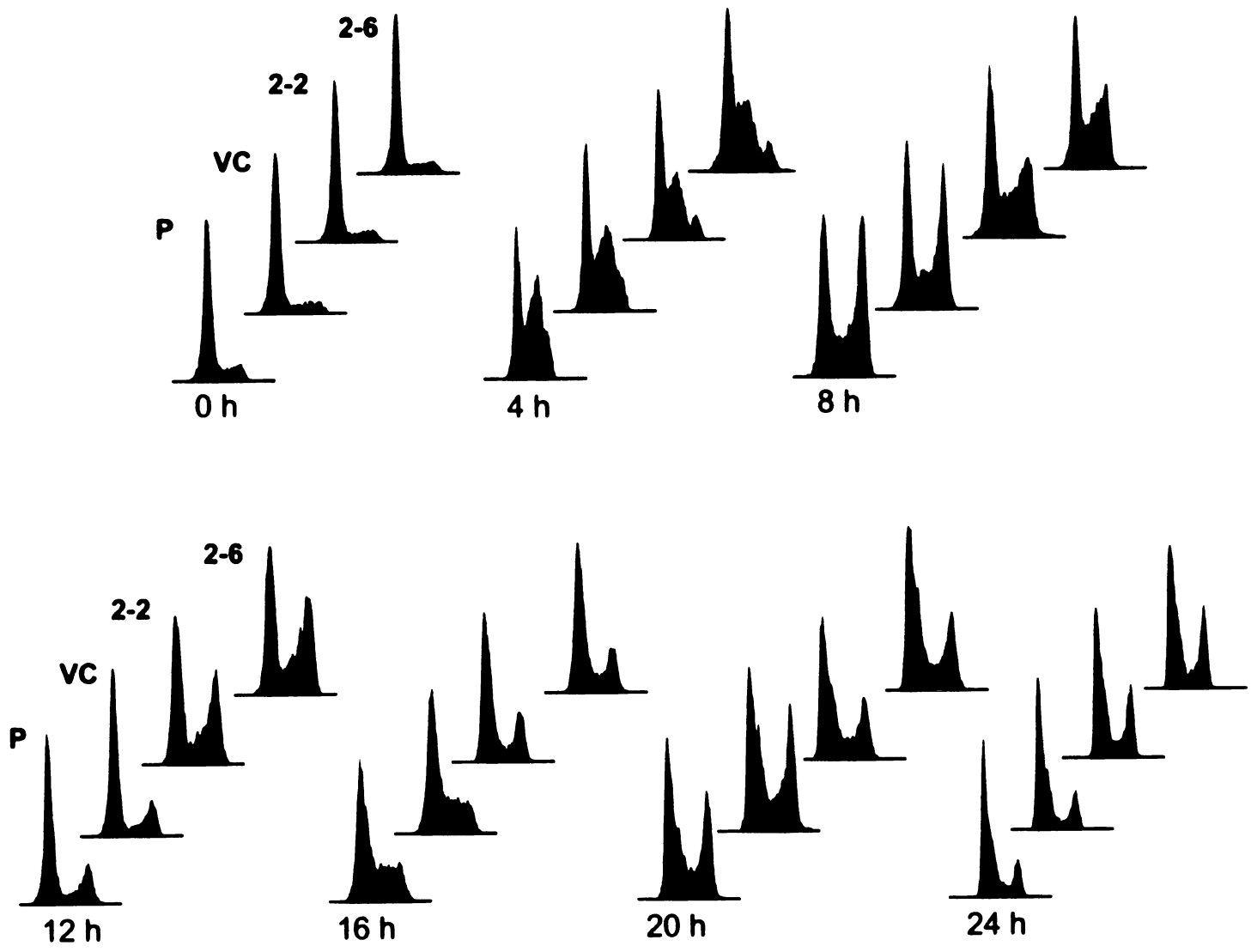
strains. Therefore, we also examined the ability of these cell strains to progress through the cell cycle following BPDE treatment.

To determine the effect of decreased expression of hRev7 protein on the rate of cell cycle progression of BPDE-treated cells, the cell strains with reduced hRev7 (2-2 and 2-6), their parental cell strain (P) and the vector control strain (VC) were synchronized at the G_1/S border, released from synchrony, and exposed to BPDE for 1 h. At the end of BPDE exposure, populations of each of the four cell strains were harvested every 4 h for 24 h and analyzed by flow cytometry to determine the percentage of cells in each stage of the cell cycle. Figure 3 shows the resulting DNA histograms obtained from the sets of cells harvested at various times after BPDE treatment. Immediately after release from synchrony and just prior to BPDE treatment (0 h), the majority of cells in each of the four cell strains were at the G_1/S border, indicating that each of the four cell strains synchronized equally well. After 4 h, most of the cells previously synchronized at the G_1/S border had progressed into S-phase regardless of the level of hRev7 protein, indicating that cells released from the block and entered S-phase at similar rates post-BPDE treatment. Eight h after BPDE treatment, many of the synchronized control cells had moved through S-phase and entered into the G_2 phase, whereas significantly fewer of the cells with reduced hRev7 had completed S-phase, indicating that following BPDE treatment, cell strains with reduced hRev7

Figure 3 - Reducing the level of hRev7 in human fibroblasts results in a BPDE-induced delay in progression through the cell cycle. Cell strains with reduced levels of hRev7 (2-2 and 2-6), their parental cell strain (P), and a vector control transfectant (VC) were synchronized at the G_1/S border and treated with BPDE for one hour immediately after release from synchrony. Cells were harvested, fixed, and stained with propidium iodide for analysis of DNA content by flow cytometry. The distribution of cells in each phase in the cell cycle 0 h, 4 h, 8 h, 12 h, 16 h, 20 h, and 24 h post-BPDE treatment are depicted.

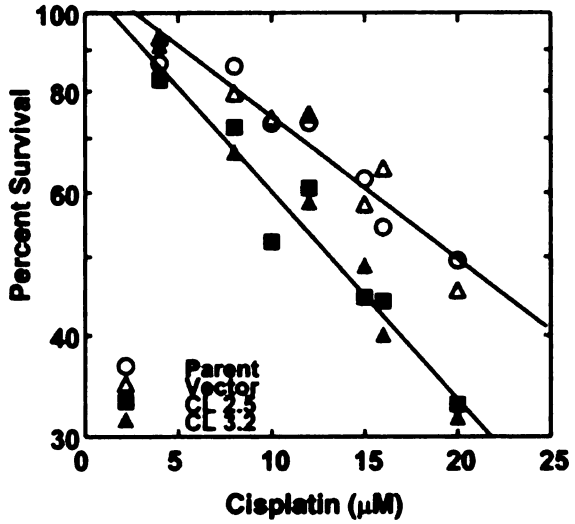

2-6
2-2
VC
P
0 h
4 h
8 h
2-6
2-2
VC
P
12 h
16 h
20 h
24 h

progressed through S-phase at a slower rate than the two control strains. After 12 h, the control cells had divided and cycled back into G_1. In contrast, cells with reduced hRev7 were still primarily in S and G_2 phase i.e., compared to control cells, cells with reduced hRev7 were still delayed in cell cycle progression. Sixteen h post-BPDE treatment, the control strains had progressed through G_1 and back into S-phase, whereas the cell strains with reduced hRev7 were primarily in G_2 and G_1, a distribution similar to that of the control cells 4 h earlier. After 20 h, although only a proportion of the cells remained synchronized, the synchronized control cells were moving through S-phase and into G_2, but the cells with reduced hRev7 were delayed in moving into S phase and G_2 phase, indicating that, even after entering a second cell cycle, cells with reduced hRev7 progressed somewhat slower than the control cells. Twenty-four h post-BPDE treatment, the synchrony of all four cell strains was lost. Taken together, the data presented in Figure 3 indicate that, after BPDE treatment, cells with reduced hRev7 progress more slowly through the cell cycle than the control strains.

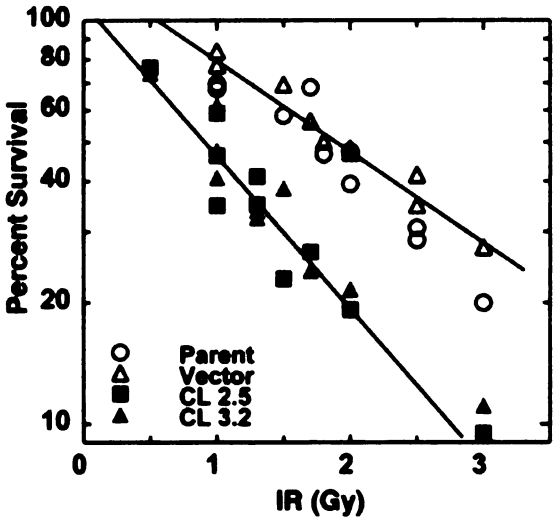
Effect of reduced hRev7 on the survival of cells exposed to DNA damaging agents-- Cell strains with reduced hRev7 are sensitive to the cytotoxic effects of both UV and BPDE, which generate structurally distinct types of DNA lesions. Therefore, we examined whether the cell strains with reduced hRev7 also differed from the control strains in their response to the cytotoxic effects of other

Figure 4 - Reducing the level of hRev7 in human fibroblast cells renders them more sensitive to the cytotoxic effects of a variety of different types of DNA damaging agents. Cell strains 2.5 and 3.2, which have significantly reduced hRev7 protein, (closed symbols) were compared to their parental strain and the vector control strain (open symbols) for their sensitivity to the cytotoxic effects of **(A)** the DNA cross-linking agent cisplatin, **(B)** ionizing radiation (IR), and **(C)** the alkylating agent *N*-methyl-*N*-hydroxyurea (MNU). The solid lines represent the least squares regression for the data.

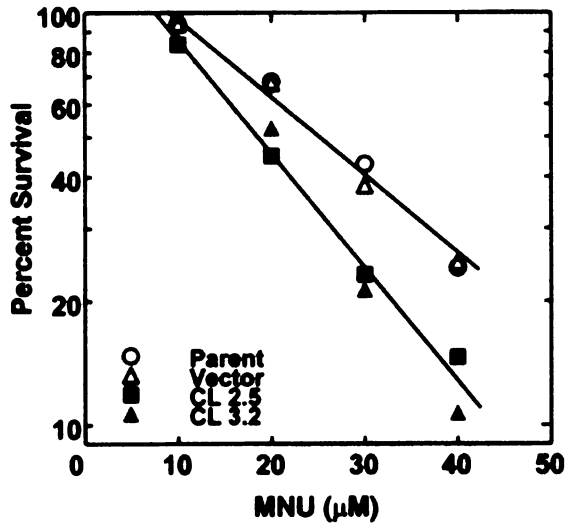
A


100
80
60
40
30
Percent Survival
0
5
10
15
20
25
Cisplatin (μM)
Parent
Vector
CL 2.5
CL 3.2

B


100
80
60
40
20
10
Percent Survival
0
1
2
3
IR (Gy)
Parent
Vector
CL 2.5
CL 3.2

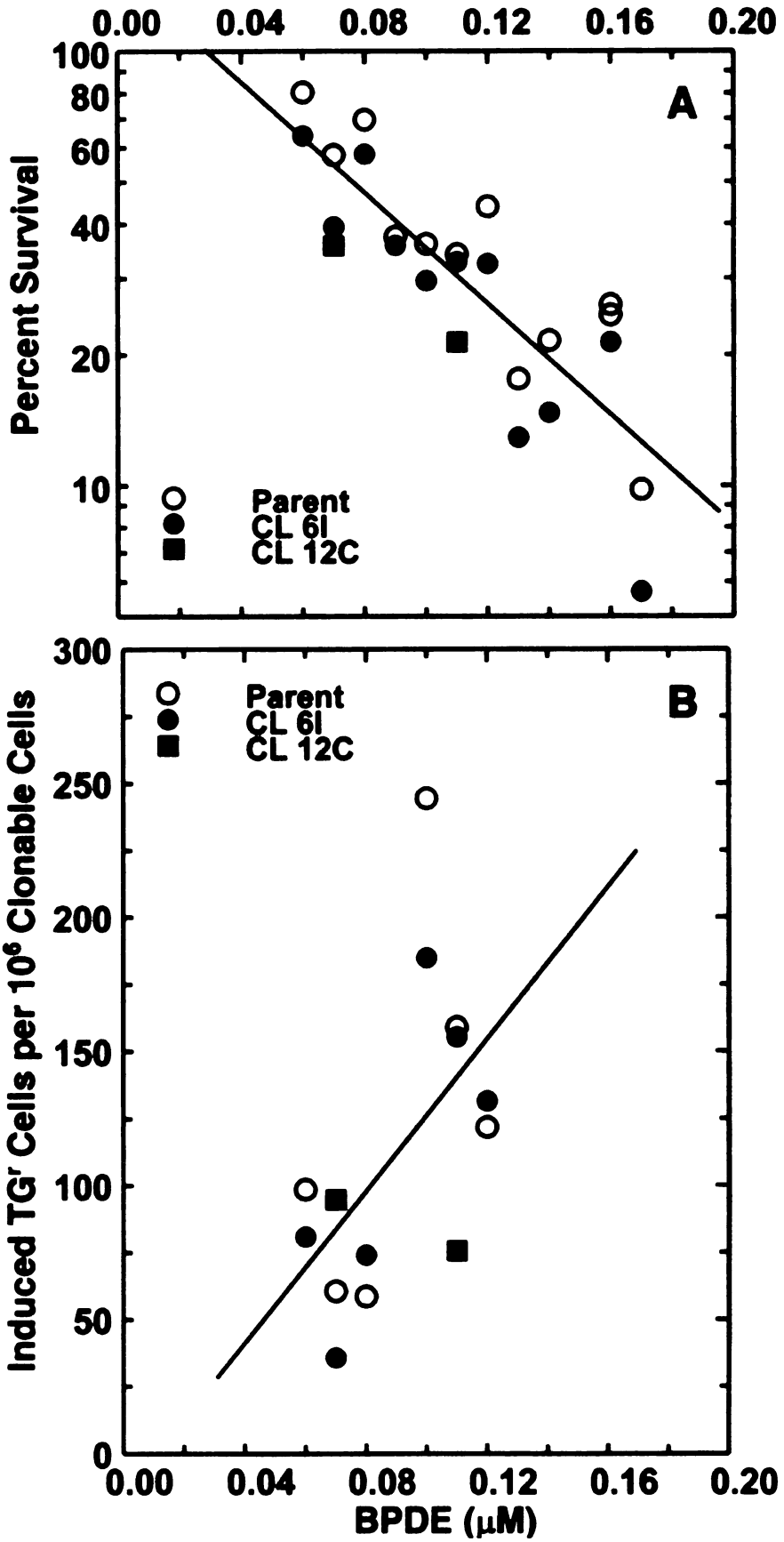
C


100
80
60
40
20
10
Percent Survival
0
10
20
30
40
50
MNU (μM)
Parent
Vector
CL 2.5
CL 3.2

types of DNA lesions by exposing them to ionizing radiation; the DNA cross-linking agent, cisplatin; and the alkylating agent, MNU. As shown in Figure 4, cell strains with reduced hRev7 were more sensitive to cell killing by each of these three DNA damaging agents, indicating that hRev7 is required to protect human fibroblasts from a variety of distinct types of DNA damage.

Effect of hRev3 antisense expression on BPDE-induced cytotoxicity and mutagenicity-- A previous study in our laboratory [20], using human fibroblasts expressing *hREV3* antisense RNA, appeared to demonstrate that hRev3, the catalytic subunit of hPol ζ, is required for the mutagenic bypass of BPDE-induced DNA lesions [20]. Therefore, it was quite unexpected that reducing hRev7, the accessory subunit of hPol ζ, had no effect on the frequency of BPDE-induced mutations. Recently, the reasons for this discrepancy have become clear. In our previous study, cell strains expressing high levels of *hRev3* antisense were derived from the human fibroblast strain designated 9N. During the course of this study, the parental strain, 9N, began to accumulate a large number of TG-resistant cells. In an effort to eliminate this high TG-resistant background frequency, a new strain, designated 9N.58, was cloned from 9N and used as the control strain for this study. Unfortunately, we have now learned that, 9N.58 has a much higher BPDE-induced mutation frequency than 9N does. As a result, 9N.58 is not a suitable comparison for cell strains derived from 9N. To accurately determine the effect of high levels of expression of *hRev3* antisense on the survival of cells exposed to BPDE and on the frequency of BPDE-induced

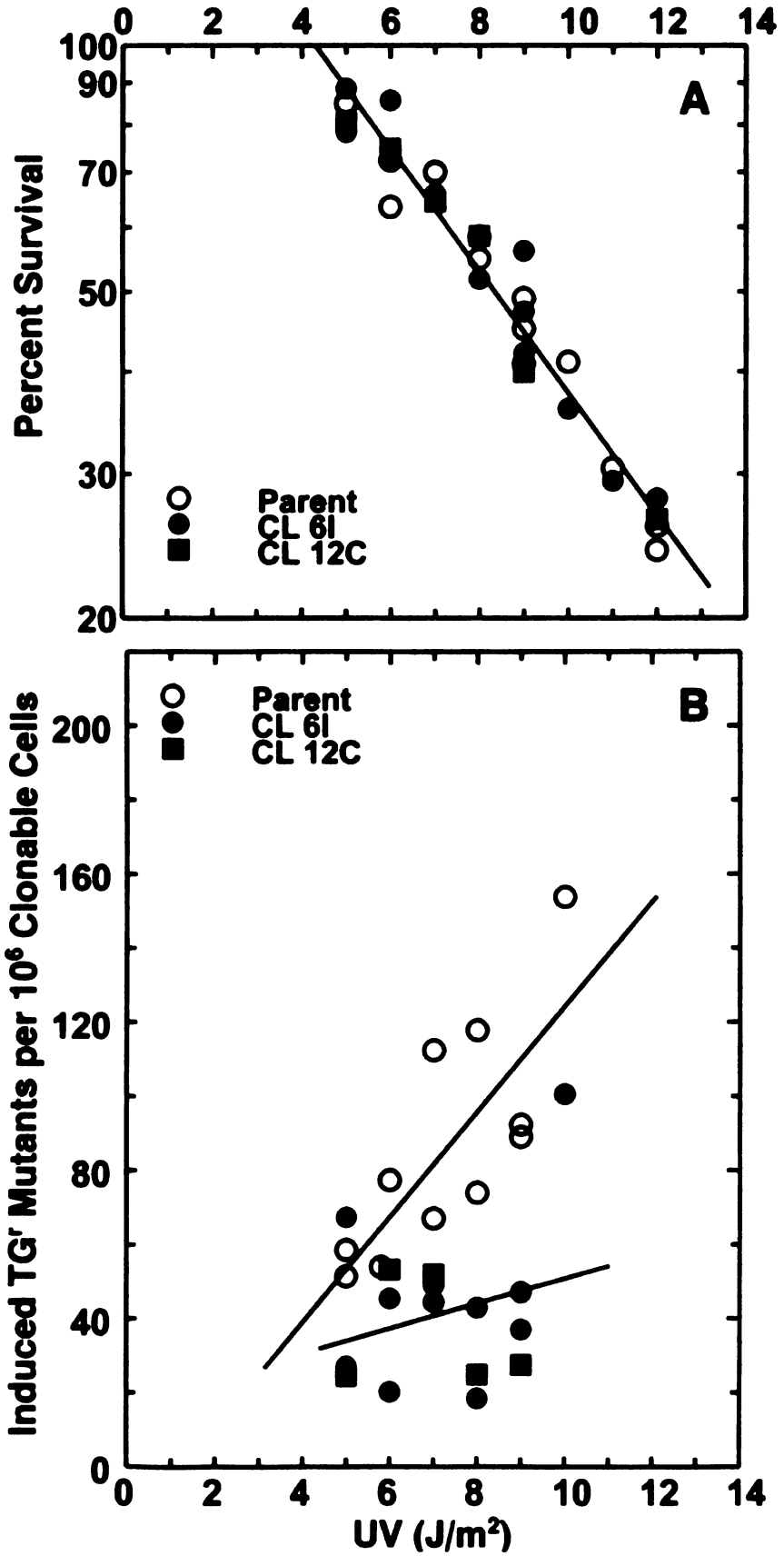
Figure 5 - Expression of high levels of *hRev3* antisense does not affect the cell survival or induced mutation frequency of human fibroblasts treated with BPDE. Cell strains expressing high levels of *hRev3* antisense, designated clones 6I and 12C (closed symbols), and their parental strain, 9N, (open circles) were treated with BPDE and assayed for **(A)** cell survival and **(B)** induced mutation frequency. The solid lines represent the least squares regression for the data.


0.00
0.04
0.08
0.12
0.16
0.20
100
80
60
40
20
10
Percent Survival
A
Parent
CL 6I
CL 12C
300
250
200
150
100
50
0
Induced TG^r Cells per 10^6 Clonable Cells
B
Parent
CL 6I
CL 12C
BPDE (μM)

mutations, the original parental strain, 9N, was CHAT selected to eliminate the TG-resistant background. This newly selected, low-background parental strain was then compared to derivative strains 6I and 12C, which express high levels of *hRev3* antisense, for BPDE-induced cytotoxicity and mutagenicity. As shown in Figure 5A, neither of the cell strains expressing high levels of *hRev3* antisense differed from their parental strain in sensitivity to the cytotoxic effect of BPDE. These results are consistent with data obtained previously using 9N.58 as the control strain, and indicate that hRev3 is not required to protect cells from BPDE-induced cell killing. However, when cell strains expressing high levels of *hRev3* antisense were compared to their appropriate parental strain for their frequency of BPDE-induced mutations (Figure 5B), in contrast to results obtained previously by Li et al., there were no significant differences in the number of BPDE-induced mutations. These results indicate that hRev3 is not required for BPDE-induced mutagenesis in human fibroblasts.

Effect of hRev3 antisense expression on UV-induced cytotoxicity and mutagenicity-- In our 2002 study, cell strains expressing high levels of *hRev3* antisense were also compared to the 9N.58 for their sensitivity to the cytotoxic and mutagenic effects of UV. Because 9N is the more appropriate control for such studies, they too were repeated using 9N as the parental strain. Consistent with our results from 2002, the data presented in Figure 6A show that the *hRev3* antisense expressing strains (6I and 12C) did not differ from their parental strain

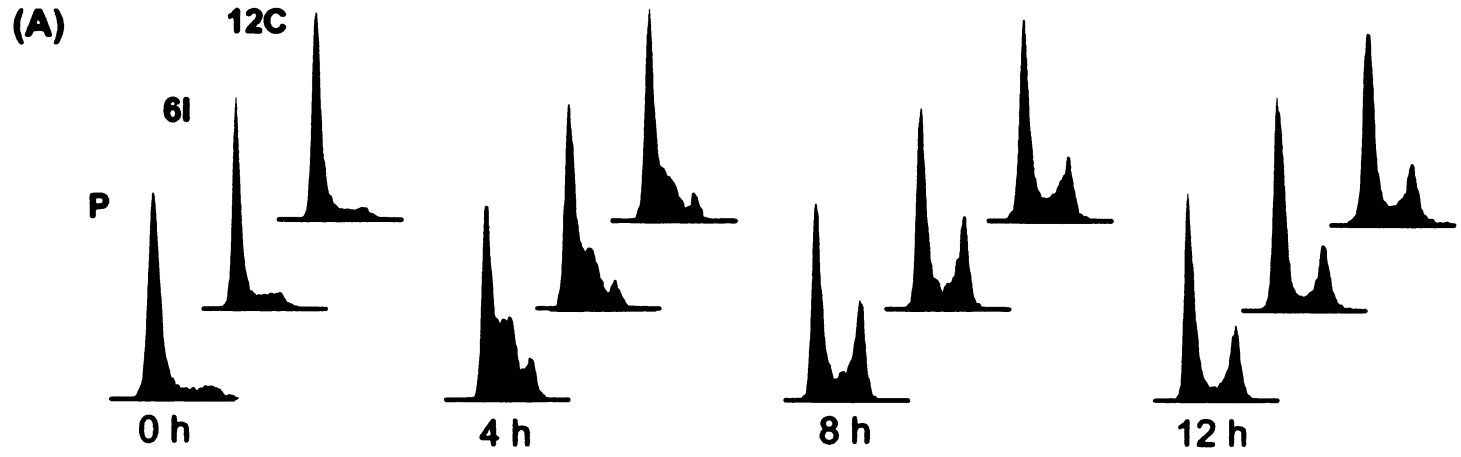
Figure 6 - Expression of high levels of *hRev3* antisense does not affect the survival of human fibroblasts exposed to UV radiation, but does cause a significant reduction in UV-induced mutations. Cell strains expressing *hRev3* antisense, designated clones 6I and 12C (closed symbols), and their parental strain, 9N, (open circles) were treated with BPDE and assayed for **(A)** cell survival and **(B)** induced mutation frequency. The solid lines represent the least squares regression for the data.

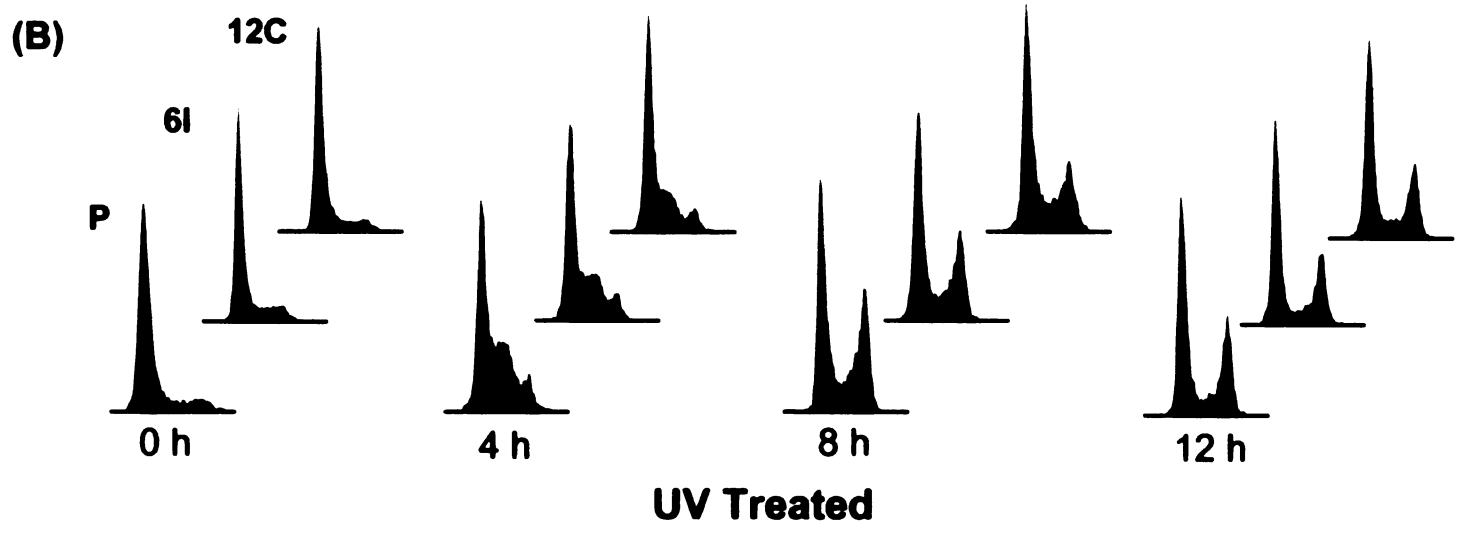

A
Percent Survival
100
90
70
50
30
20
0
2
4
6
8
10
12
14
Parent
CL 6I
CL 12C
B
Induced TG' Mutants per 10^6 Clonable Cells
200
160
120
80
40
0
UV (J/m^2)

(9N) in sensitivity to the cytotoxic effect of UV, indicating that hRev3 is not required to protect cells from UV-induced killing. When these same cell strains were assayed for their frequency of UV-induced mutations (Figure 6B), cell strains 6I and 12C exhibited considerably lower UV-induced mutation frequencies than their parental strain. These results are consistent with the results obtained in our previous study and suggest that, unlike with BPDE, hRev3 is required for mutagenesis induced by UV in human fibroblast cells.

Effect of hRev3 antisense expression on cell cycle progression following UV or BPDE treatment-- Cell strains with reduced hRev7 progress through the cell cycle at a considerably slower rate than control cells following treatment with either BPDE or UV. Consequently, we were interested to determine how treatment with these agents would affect the cell cycle progression of strains expressing *hRev3* antisense. Therefore, cell strains 6I and 12C, which express high levels of hRev3 antisense, as well as their parental strain, 9N, were synchronized at the G_1/S border, released from synchrony, and exposed to either BPDE or UV. To determine the distribution of cells in each stage of the cell cycle, populations of each of the three cell strains were harvested every 4 h for 12 h following BPDE or UV exposure, and analyzed by flow cytometry. As shown in Figure 7, expression of *hRev3* antisense had no effect on the progression of human fibroblasts through the cell cycle after either BPDE treatment (Figure 7A) or UV treatment (Figure 7B). These results indicate that expressing high *hRev3*

Figure 7 - Expression of high levels of *hRev3* antisense has no affect on the progression of human fibroblasts through the cell cycle after BPDE or UV treatment. Cell strains expressing high levels of hRev3 antisense (6I and 12C) and their parental cell strain (P) were synchronized at the G_1/S border and treated with BPDE or UV immediately after release from synchrony. Cells were harvested, fixed, and stained with propidium iodide for analysis of DNA content by flow cytometry. The distribution of cells in each phase in the cell cycle 0 h, 4 h, 8 h, and 12 h post-BPDE treatment are depicted.


(A)
12C
6I
P
0 h
4 h
8 h
12 h
BPDE Treated


(B)
12C
6I
P
0 h
4 h
8 h
12 h
UV Treated

antisense has no affect on the progression of human fibroblasts through the cell cycle following BPDE or UV treatment.

DISCUSSION:

The results presented in Figure 2A show that cells with reduced expression of hRev7 protein are more sensitive than the control cell strains to the cytotoxic effect of BPDE, indicating that the hRev7 protein protects human cells from BPDE-induced killing. This finding is especially interesting because our mutagenesis data demonstrate that reducing the level of hRev7 in human fibroblasts has no affect on the frequency of BPDE-induced mutations in such strains (Figure 2B), indicating hRev7 is not required for TLS past BPDE-induced DNA lesions. Taken together, these results indicate that although hRev7 acts to protect cells from BPDE-induced cell killing, this function is independent of the role of hRev7 in TLS.

We also found that cell strains with reduced hRev7 are more sensitive to the cytotoxic effects of cisplatin, ionizing radiation and MNU (Figure 4A-C). What is more, our laboratory previously demonstrated that reducing the level of hRev7 renders cells more sensitive to the cytotoxic effect of UV radiation [21]. These results suggest that hRev7 is generally required to protect human fibroblasts cells from cell killing induced by exposure to DNA damaging agents. Similar results were reported by Cheung et al. [25], who showed that downregulation of hRev7 (also referred to as MAD2B) in nasopharyngeal carcinoma cells rendered such cells generally more sensitive to DNA damaging agents, but not to cytotoxic agents that have different mechanisms of action, such as anti-metabolite or

microtubule-disrupting agents. These results indicate that hRev7 plays a role in preventing cell death caused by DNA damage in both normal and cancer cells.

Analysis of cell cycle progression by flow cytometry revealed that following exposure to BPDE, cell strains with reduced hRev7 progress through S-phase at a slower rate than control strains (Figure 3). A similar effect was demonstrated by previous studies in our laboratory, which showed that human fibroblast cells with reduced hRev7 also progressed through S-phase more slowly than control strains following UV-irradiation [21]. Interestingly, expressing high levels of *hRev3* antisense did not alter the rate of cell cycle progression of such strains following treatment by either UV or BPDE (Figure 7A and 7B), indicating that the requirement for hRev7 in completing S-phase following exposure to DNA damage is independent of its role as an accessory subunit of hPol ζ. Moreover, because hRev7 was not required for TLS past BPDE-induced DNA lesions, we conclude that it is unlikely that the delay in S-phase we observe in the human cells with decreased expression of hRev7 resulted from impaired ability to carry out TLS.

Interestingly, a similar effect has been demonstrated in mouse embryonic fibroblasts (MEFs) lacking Pol κ [26]. Such cells are more sensitive to UV-induced cytotoxicity than the wild-type cells. In addition, MEFs lacking Pol κ undergo a sustained replication block following treatment with UV [27]. Because Pol κ plays a very small role, if any, in TLS past UV-induced DNA lesions it was

unclear why cells lacking this polymerase demonstrate such phenotypes in response to UV radiation. However, more recent experiments by Ogi and Lehmann [28], demonstrate that the UV-induced sensitivity of MEFs lacking Pol κ can be attributed to a substantial reduction of nucleotide excision repair activity in such cells, indicating an unexpected additional role for Pol κ in nucleotide excision repair. Perhaps, similar to what has been demonstrated with Pol κ, the fact that hRev7 is dispensable for TLS past BPDE-induced DNA lesions, but is required both for efficient progression through S-phase and for survival of BPDE treated cells, indicates of an additional, as yet uncharacterized, function of hRev7.

The fact that we found that the accessory subunit of hPol ζ (hRev7) is dispensable for BPDE-induced mutagenesis in human fibroblasts was initially surprising, given that previous studies conducted in our laboratory [20] indicated a requirement for the catalytic subunit of hPol ζ (hRev3) in such mutagenesis. However, as explained above, for that study the control strain used was not the direct parent of the *hRev3* antisense expressing strains. Instead 9N.58 was a subclone of their actual parental strain, 9N. For reasons yet unknown to us, cell strain 9N.58 exhibits a much higher frequency of BPDE-induced mutations than cell strain 9N does (Figure 6B). As a result, when the *hRev3* antisense expressing strains were compared to 9N.58 in the previous experiments [20], the results were misinterpreted as showing that the frequency of BPDE-induced

mutations in cell strains expressing *hRev3* antisense were lower than in the control cell strain.

In our current study, we compared cell strains expressing high levels of *hRev3* antisense, 6I and 12C, to the more appropriate control strain, 9N, for the frequency of BPDE-induced mutations, and found that expressing *hRev3* antisense had no affect on the frequency of mutations induced by BPDE in these cell strains (Figure 5B). Although it is still not possible to demonstrate directly that our *hRev3* antisense expressing strains have reduced levels of hRev3 protein (because of a lack of a suitable antibody), these two cell strains demonstrate significantly lower UV-induced mutation frequencies than their parental strain (Figure 7B). Such a result is consistent with having reduced levels of Rev3 [18, 29, 30]. Taken together, these data provide evidence that hRev3 is not involved in TLS past BPDE-induced DNA lesions. Combined with the fact that in the present study, we also found that hRev7 is not required for TLS past BPDE-induced DNA lesions, we conclude that hPol ζ is not required for TLS past BPDE-induced DNA lesions in human fibroblasts.

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