Recognition and Repair of *trans-syn* II Cyclobutane Thymine Dimer by Mammalian Excision Nuclease Indicate that the Affinities of Damage Sensors do not Dictate the Repair Efficiency

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ABSTRACT

Stephanie Quinn Hutsell: Recognition and Repair of *trans-syn* II Cyclobutane Thymine Dimer by Mammalian Excision Nuclease Indicate that the Affinities of Damage Sensors do not Dictate the Repair Efficiency
(Under the direction of Dr. Aziz Sancar)

The *trans-syn* cyclobutane pyrimidine dimer is a minor, but biologically significant ultraviolet photoproduct that is produced primarily in single-strand DNA. The only known repair system for this lesion is nucleotide excision repair. In this study I investigated the recognition and repair of the *trans-syn* cyclobutane thymine dimer by mammalian excision nuclease. I find that the *trans-syn* cyclobutane thymine dimer is recognized by RPA, XPA, and XPC damage sensor proteins with high specificity comparable to that of the [6-4] photoproduct; however, this lesion is excised by the mammalian excision nuclease with efficiency comparable to that of the poorly recognized *cis-syn* cyclobutane pyrimidine dimer. These data suggest that kinetic factors, after the initial damage recognition step, play a major role in the overall catalytic proficiency of the mammalian excision nuclease.

ACKNOWLEDGEMENTS

I would like to thank all those in my circle of trust; I could not have come this far and discovered who I am without you.

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ATP	- adenosine 5'-triphosphate
bp	- base pair
º C	- Celsius
C	- cytosine
CHO	- Chinese Hamster Ovary
CPD	- cyclobutane pyrimidine dimer
DEAE	- diethylaminoethyl
DNA	- deoxyribose nucleic acid
E. coli	- *Escherichia coli*
EMSA	- electrophoretic mobility shift assay
ERCC1	- excision repair cross-complementing rodent repair deficiency, complementation group 1
fmol	- femtomole
HR23B	- human homologue RAD23B
KCl	- potassium chloride
K_D	- dissociation constant
µg	- microgram
µL	- microliter
mA	- milliamp
$MgCl_2$	- magnesium chloride
min	- minute
mL	- milliliter

mM - millimolar concentration

ng - nanogram

Ni - nickel

nm - nanometer

nM - nanomolar concentration

^{32}P - radioactive phosphorus isotope

PCNA - proliferating cell nuclear antigen

pH - parts hydrogen

PIC1 - pre-incision complex 1

PIC2 - pre-incision complex 2

PIC3 - pre-incision complex 3

Pyr<>Pyr - cyclobutane pyrimidine dimer

Pyr[6-4]Pyr - pyrimidine pyrimidone 6-4 photoproduct

Pyr<*c,s*>Pyr - *cis-syn* cyclobutane pyrimidine dimer

Pyr<*t,s*>Pyr - *trans-syn* cyclobutane pyrimidine dimer

RAD23 - RADiation sensitivity abnormal 23

RFC - replication factor C

RPA - replication protein A

SAD - seasonal affective disorder

S. cerevisiae - *Saccharomyces cerevisiae*

T - thymine

T[6-4]T - thymine-thymine 6-4 photoproduct

T<*c,s*>T - thymine-thymine *cis-syn* cyclobutane pyrimidine dimer

T<*t*,*s*>T - thymine-thymine *trans-syn* cyclobutane pyrimidine dimer

UM - unmodified

UV - ultraviolet

UVA - ultraviolet A

UVB - ultraviolet B

UVC - ultraviolet C

v - volume

XP - xeroderma pigmentosum

XPA - xeroderma pigmentosum complementation group A

XPB - xeroderma pigmentosum complementation group B

XPC - xeroderma pigmentosum complementation group C

XPD - xeroderma pigmentosum complementation group D

XPF - xeroderma pigmentosum complementation group F

XPG - xeroderma pigmentosum complementation group G

Chapter One

Nucleotide Excision Repair

1.1. Introduction

Genetic material is under constant assault from endogenous and exogenous agents. Ultraviolet rays from sunlight are a major source of such insult. All organisms have developed a biochemical pathway for the removal of this genetic insult before it progresses to a mutagenic event, furthermore some organisms have evolved multiple mechanisms for the removal of UV-induced DNA damage. These mechanisms include nucleotide excision repair and damage specific photolyase enzymes.

Photolyases are a class of enzymes capable of harnessing blue-light (350-450 nm) energy to split apart UV-induced cyclobutane pyrimidine dimers (Rupert *et al.*, 1958; Sancar, 1994) or pyrimidine-pyrimidone [6-4] photoproducts (Todo *et al.*, 1993; Kim *et al.*, 1994) in a process called photoreactivation. Each of these UV-lesions requires a unique photolyase, cyclobutane pyrimidine dimer photolyase and (6-4) photolyase, respectively.

Due to the absence of photolyases, nucleotide excision repair is the only known mechanism in placental mammals for the removal of bulky DNA adducts produced by sunlight (Sancar, 1996; Sancar *et al.*, 2004; Wood, 1997; Araujo and Wood, 1999; Reardon and Sancar, 2005; Sancar and Reardon, 2004). In addition, the excision nuclease recognizes and excises a broad spectrum of DNA lesions beyond the ultraviolet-light

induced [6-4] photoproduct and cyclobutane pyrimidine dimer (CPD), including benzo(a)pyrene, acetylaminofluorene, cisplatin, and psoralen DNA adducts (Sancar *et al.*, 2004). Failure to remove such lesions leads to mutation, and eventually carcinogenesis, because replication polymerases are unable to bypass DNA damage with high fidelity often mis-incorporating nucleotides opposite the lesion site.

Skin cancer is the most commonly diagnosed neoplasm in the United States (Christenson *et al.*, 2005). Sunlight exposure is the major environmental factor leading to UV-induced DNA damage. Failure of the excision nuclease to detect and remove these lesions leads to an accumulation of damage, which over time increases the likelihood of mutagenesis and skin cancer development. Additionally, mutations in any of the components of the excision repair pathway manifest as the disease xeroderma pigmentosum (XP) (Kraemer, 1997). Xeroderma pigmentosum is an autosomal recessive photosensitive disorder with over 1,000-fold higher incidence of UV-related skin cancers (Kraemer et al., 1984; Kraemer et al., 1994) characterized by an impaired ability to repair UV-induced DNA damage. Because of the direct link between sunlight-induced DNA damage, nucleotide excision repair, and cancer development, it is essential to understand the repair factors and mechanism involved in nucleotide excision repair, the only mammalian repair pathway for the removal of UV-photoproducts.

1.2. Nucleotide Excision Repair

The nucleotide excision repair pathway is present in all species from bacteria to humans (Petit and Sancar, 1999). This process is characterized by three steps: damage recognition, damage excision and release, and repair synthesis and ligation (Sancar *et al.*, 2004; Sancar, 1996; Wood, 1997).

In bacteria the products of three genes are responsible for damage recognition and excision, UvrA, UvrB, and UvrC. These three factors form the (A)BC excision nuclease. Two molecules of UvrA and one molecule of UvrB constitute the damage recognition complex (Orren and Sancar, 1989). Once a lesion is detected, UvrA dissociates and UvrC binds to the UvrB-DNA complex. UvrC then sequentially makes the 3' and 5' incisions, which bracket the lesion. The excision product ranges in size from 12-13 nucleotides in prokaryotes and is produced by the (A)BC excinuclease (Huang *et al.*, 1994). UvrD helicase facilitates the removal of the damaged oligomer from DNA and the resultant gap is filled by DNA polymerase I (Sibghat-Ullah *et al.*, 1990).

The necessary and sufficient mammalian excision repair machinery consists of six factors, RPA, XPA, XPC, TFIIH, which includes XPB and XPD helicases, XPG, and XPF-ERCC1 (Figure 1.1), the six factors include XP proteins named for xeroderma pigmentosum complementation groups A through G. RPA, XPA, and XPC recognize the damage, TFIIH unwinds the helix, then XPG and XPF-ERCC1 make the respective 3' and 5' incisions, releasing the damaged strand. The excised product ranges between 24-32 nucleotides among eukaryotes (Huang and Sancar, 1994). The resultant gap is filled by replication polymerases and is sealed by DNA ligase 1. Additionally, but beyond the scope of this discussion, repair is enhanced by transcription repair-coupling factors in transcribed regions of the genome (Venema *et al.*, 1990).

1.2.1 Damage Recognition

Locating damage in a sea of undamaged DNA is the least well understood of the three steps describing excision repair. Damage recognition by the mammalian excision nuclease is an energy requiring multi-step process (Petit and Sancar, 1999). RPA, XPA,

and XPC are the mammalian repair factors implicated in damage recognition because of their moderately higher affinity for damaged over undamaged duplex DNA (Reardon and Sancar 2003; Wakasugi and Sancar, 1998; Clugston *et al.*, 1992; Burns *et al.*, 1996; Jones and Wood, 1993; Saijo *et al.*, 1996; Reardon *et al.*, 1996). The three repair factors may assemble in any order at the damage site (Reardon and Sancar, 2003; Reardon and Sancar, 2004; Reardon and Sancar, 2005), and complex assembly is encouraged not only by the affinity of the repair factors for DNA, but for each other as well. Assembly of the first detectable pre-incision complex (PIC1) includes TFIIH which is usually complexed with XPC. TFIIH transcription factor/repair factor is a multi-subunit complex containing helicases XPB and XPD. Once TFIIH is recruited to the lesion, DNA is unwound around the damage site.

1.2.2 Damage Excision and Release

XPC serves as a molecular matchmaker (Wakasugi and Sancar 1999; Wakasugi and Sancar 1998) to recruit XPG to the excision complex while displacing itself. This intermediate is termed pre-incision complex 2 (PIC2). XPF-ERCC1 binds specifically to PIC2 to form pre-incision complex 3 (PIC3). In PIC3, XPG makes the first incision 3′ to the DNA damage followed by the 5′ incision made by XPF-ERCC1 to release the damaged strand (Huang *et al.*, 1992). In mammals, the 3′ incision occurs first at the 6^{th} ± 3 phosphodiester bond from the damage site and the second incision is made at the 20^{th} ± 5 phosphodiester bond 5′ to the damage site, releasing an oligomer 24-32 nucleotides in length (Reardon and Sancar, 2005; Huang *et al.*, 1992).

1.2.3 Repair Synthesis and Ligation

The gap left by excising the damaged oligomer is filled by repair synthesis proteins RPA, RFC, PCNA, and PCNA-dependent DNA polymerases δ and ε. The repair patch is sealed by DNA ligase I in the last step of nucleotide excision repair (Mu *et al.*, 1996; Reardon and Sancar, 2003; Hutsell and Sancar, 2005). The repair patch corresponds exactly with the number of the removed nucleotides (Reardon Thompson Sancar 1997) suggesting the region is well protected from further nuclease insult.

1.2. Damage Recognition Factors

Of the necessary and sufficient proteins required for excision, RPA, XPA, and XPC are implicated as the damage-identifying factors in nucleotide excision repair (Wakasugi and Sancar, 1999; Sugasawa *et al.*, 1998; Missura *et al.*, 2001; He *et al.*, 1995).

1.3.1 RPA

Replication protein A (RPA) is a heterotrimeric single-strand DNA binding protein required for replication, recombination, and nucleotide excision repair (Wold and Kelly, 1988; Reardon and Sancar, 2005). RPA was later demonstrated to bind duplex DNA as well as damage containing single-strand and double-strand DNA (Clugston *et al.*, 1992; Patrick and Turchi, 1999; Lao *et al.*, 1999). The helix destabilizing ability of RPA (Lao *et al.*, 1999) coupled with its ability to selectively bind damage (Reardon and Sancar, 2003; Wang *et al.*, 2000) suggest RPA is directly involved in damage recognition. Additionally, RPA and XPA bind cooperatively to DNA damaged sites (Wang *et al.*, 2000; Wakasugi and Sancar, 1999; Hey *et al.*, 2001; Patrick and Turchi,

2002), effectively enhancing the discriminatory power of the two damage-sensing repair factors.

1.3.2 XPA

Xeroderma pigmentosum A complementing protein (XPA) is 273 amino acids in humans and is essential for nucleotide excision repair (Mu *et al.*, 1995). It has been shown by electrophoretic mobility shift assay (EMSA) to independently bind damaged DNA and is implicated in the damage recognition step of excision repair (Wakasugi and Sancar, 1999; Buschta-Hedayat *et al.*, 1999). There are still divided camps in the excision repair battle field over the damage sensing ability of XPA. One side suggests there are three damage-identifying repair factors, RPA, XPA, and XPC, each capable of recognizing damage independently and cooperatively (Reardon and Sancar, 2003; Reardon and Sancar, 2004). The model proposed by Sugasawa and colleagues maintains XPC is the damage recognition molecule and it is responsible for the initial DNA binding and recruitment of all subsequent repair factors and XPA is the 'verifier' of DNA damage incapable of independently recognizing damage (Sugasawa *et al.*, 1998; Sugasawa *et al.*, 2002). However, XPC is incapable of discriminating between cyclobutane pyrimidine dimers (CPDs) and undamaged DNA, thus implicitly favoring the model for random assembly of three repair factors at the damage site.

1.3.3 XPC

Xeroderma pigmentosum C complementing protein demonstrates the highest affinity for duplex DNA of the three damage-identifying repair factors (Reardon and Sancar 2003). In humans, XPC frequently forms a heterodimeric complex with HR23B, a

homologue of *S. cerevisiae* RAD23. It is thought that HR23B stabilizes XPC (Ng *et al.*, 2003), as it is not required for *in vitro* reconstitution of excision repair (Reardon *et al.*, 1996). XPC has been termed a molecular matchmaker (Sancar and Hearst, 1993) because it aids in the assembly of the final enzymatic complex PIC3, but is not present in it (Wakasugi and Sancar 1999; Wakasugi and Sancar 1998).

1.3. Ultraviolet Photoproducts Formed in DNA

Irradiation of DNA with UV light leads to the formation of a number of photoproducts including: the [6-4] photoproduct, its Dewar isomer, and the cyclobutane pyrimidine dimer (Pyr< >Pyr)(Figure 1.2) (Patrick and Rahn, 1976). Two stereoisomers of (Pyr< >Pyr) form in DNA by UV irradiation, the *cis-syn* cyclobutane pyrimidine dimer (Pyr<*c*,*s*>Pyr), which is by far the major photoproduct formed, and *trans-syn* cyclobutane pyrimidine dimer (Pyr<*t*,*s*>Pyr), which is formed at about 2 % the frequency of the Pyr<*c*,*s*>Pyr in duplex DNA (Patrick and Rahn, 1976). The pyrimidine UV-photoproducts exist in thymine, thymine (TT), cytosine, cytosine (CC), thymine, cytosine (TC), or cytosine, thymine (CT) nucleotide sequence contexts, but for the following study the pyrimidine sequence TT was used, as it is the most commonly detected di-pyrimidine adduct.

1.4.1 [6-4] Photoproduct

Previous studies estimated the [6-4] photoproduct to comprise 30 % of total DNA damage after UVC-irradiation (Patrick and Rahn, 1976). Recently it was discovered, using only UVA and UVB wavelengths which simulate sunlight and omitting UVC wavelengths, that [6-4] photoproduct forms at an almost undetectable level (Yoon *et al.*,

2000). Nevertheless, the [6-4] photoproduct is considered the best recognized of the UV-photolesions, K_D values in molar concentrations are: 2.2×10^{-7} for RPA, 1.5×10^{-7} for XPA, and 2.6×10^{-8} for XPC, and best excised substrate, 10 % of total DNA is excised in the repair assay, of the excision nuclease (Reardon and Sancar, 2003). The lesion bends DNA 44˚ and unwinds the helix 30˚ (Wang and Taylor, 1993). The structural deformation of the helix is posited as the reason why the [6-4] photoproduct is the best recognized substrate by the excision nuclease.

1.4.2 Cis-syn Cyclobutane Pyrimidine Dimer

The most abundant UV-photoproduct is the cis-syn pyrimidine dimer accounting for almost 70 % of total DNA damage after irradiation (Patrick and Rahn, 1976). The *cis-syn* pyrimidine dimer is a poorly excised substrate by the excision nuclease, about 2 % of total damaged DNA is excised in the excision repair assay (Reardon and Sancar, 2003). This damage product bends DNA 30˚ and unwinds the duplex about 10˚ (Park *et al.*, 2002). Structural studies (Taylor *et al.*, 1990; Bdour *et al.*, 2006) suggest that the helix structure is perturbed on the 5'-side of the *cis-syn* dimer, and the distortion to the helix resembles a distended helix turn. The *cis-syn* thymine dimer is recognized slightly better than undamaged DNA, K_D values in molar concentrations are: 6.3×10^{-7} for RPA, 2.1×10^{-7} for XPA, and 3.8×10^{-8} for XPC, compared with undamaged DNA K_D values in molar concentrations are: 5.2×10^{-7} for RPA, 2.2×10^{-7} for XPA, and 3.9×10^{-8} for XPC (Reardon and Sancar, 2003).

1.4.3 Trans-syn I and Trans-syn II Pyrimidine Dimer

The *trans-syn* isomer exists as two structural isomers, *trans-syn*-I and *trans-syn*-II and it is not known which is formed *in vivo* (Patrick and Rahn, 1976; Wang and Taylor, 1993; Kao *et al.*, 1993; McCullough *et al.*, 1998; Smith and Taylor, 1993). The *trans-syn* thymine dimer bends DNA 22˚ and unwinds it 15˚ (Wang and Taylor, 1993). Structural studies (Taylor *et al.*, 1990; Bdour *et al.*, 2006) suggest that the helix structure is perturbed on the 3'-side of the *trans-syn* dimer, and acutely kinks the helix, which may contribute to damage recognition. It has been shown that *E. coli* (A)BC excinuclease excises T<*c,s*>T, T<*t,s*>T, and T[6-4]T from DNA with the [6-4] photoproduct being the most efficiently repaired substrate (Svoboda *et al.*, 1993). A similar study completed in the mammalian system, is described in the following chapter.

It is possible that the [6-4] photoproduct is formed at the same frequency as the *trans-syn* thymine dimer. Therefore, it is of biologic relevance to characterize this photoproduct because it may be a more significant or mutagenic lesion than previously thought and analyses of its recognition and removal may provide insight into the excision repair mechanism.

1.4. Conclusion and Model

The following study will further our understanding of how DNA damage, specifically UV-photoproducts, is recognized in mammals by the damage-identifying subunits RPA, XPA, and XPC. Understanding the fundamental mechanism of nucleotide excision repair may have direct relevance to the treatment of cancer. If drugs can selectively inhibit or reduce excision repair in cancer cells by modulating a component of damage recognition, clinicians could increase the therapeutic index of DNA damaging

agents commonly used to treat cancer. Finding DNA damage among the roughly 4 x 10^9 base pairs in the human genome is finding the proverbial needle in the haystack, blindfolded. The random assembly model first proposed by Reardon and Sancar (Reardon and Sancar, 2003; Reardon and Sancar, 2004) suggests that cooperative interactions among the damage-identifying repair factors enhance damage recognition not accounted for thermodynamically in the DNA binding constants of single repair factors, and subsequently provides a scaffold for the remaining repair factors to assemble and to complete repair. This study provides support for their model and elucidates properties of a little studied, but potentially important UV-photoproduct.

Figure 1.1. Model for nucleotide excision repair. Damage is recognized by RPA, XPA, and XPC in a cooperative way. TFIIH is recruited to form pre-incision complex 1 (PIC1). XPG displaces XPC from PIC1 to form PIC2. Finally, XPF-ERCC1 is recruited to form PIC3 in which XPG makes the 3' incision 6 ± 3 nucleotides 3' from the damage site and XPF-ERCC1 makes the 5' incision 20 ± 5 nucleotides 5' to the damaged bases releasing the damage in the form of a 27-nucleotide-long oligomer. Repair synthesis proteins replication factor C, proliferating cell nuclear antigen, and DNA polymerases δ and ε fill the gap. Repair patch is sealed by DNA ligase. Figure adapted from Hutsell and Sancar *Clin Cancer Res* 2005 Feb 15;11(4):1355-7.

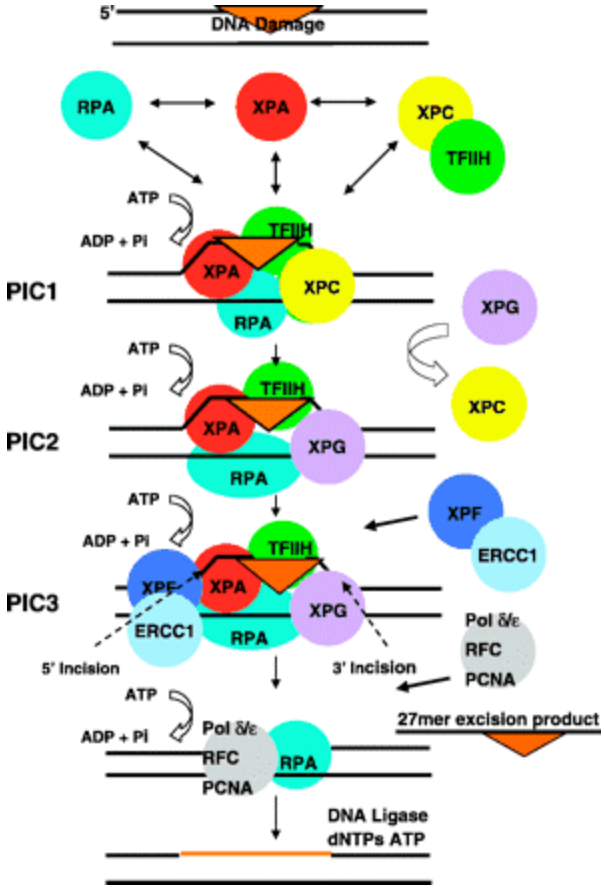
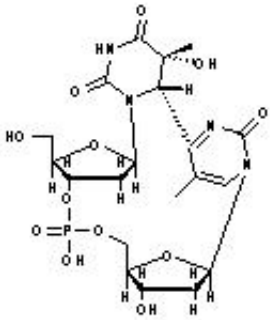
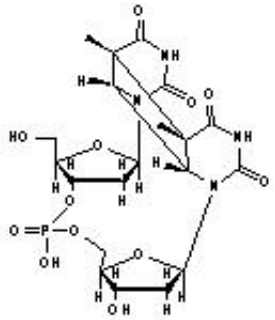

5'
DNA Damage
RPA
XPA
XPC
TFIIH
ATP
ADP + Pi
PIC1
XPG
PIC2
PIC3
XPF
ERCC1
5' Incision
3' Incision
Pol δ/ε
RFC
PCNA
27mer excision product
DNA Ligase
dNTPs ATP

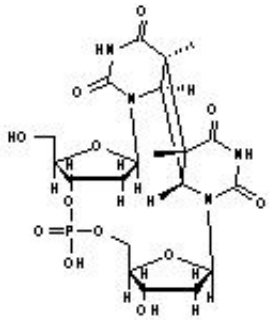
Figure 1.2 Structure of major UV-induced DNA photoproducts: (6-4) photoproduct, cis-syn dimer, and trans-syn dimers I and II. (6-4) photoproduct, *cis-syn* cyclobutane thymine dimer, and *trans-syn* I and II cyclobutane thymine dimers were incorporated into synthetic oligonucleotides and used in this study.



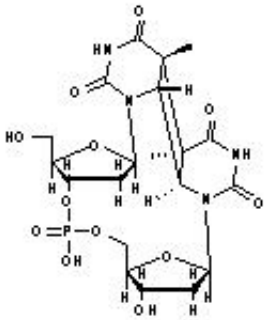
(6-4) photoproduct



Cis-syn thymine dimer



Trans-syn I thymine dimer



Trans-syn II thymine dimer

Chapter Two

Recognition and Repair of the *trans-syn* II Cyclobutane Thymine Dimer

2.1 Introduction

Exposure of DNA to ultraviolet (UV) light leads to the formation of a number of photoproducts including the [6-4] photoproduct, and the cyclobutane pyrimidine dimer (Pyr<>Pyr) (Taylor, 2002). Two geometric isomers of Pyr<>Pyr form in DNA by UV irradiation, the *cis-syn* cyclobutane pyrimidine dimer which by far is the major photoproduct and the *trans-syn* isomer, which forms at about 2 % the frequency of Pyr<*c,s*>Pyr in duplex DNA (Patrick and Rahn, 1976; Kao *et al.*, 1993; Smith and Taylor, 1993). The *trans-syn* isomer exists as two stereoisomers, *trans-syn* I and *trans-syn* II and at present it is not known which is formed *in vivo* (Kao *et al.*, 1993; Smith and Taylor, 1993). It has been shown that *E. coli* (A)BC excinuclease excises T<*c,s*>T, T<*t,s*>T, and T[6-4]T photoproducts from DNA with the [6-4] photoproduct being the most efficiently repaired substrate (Svoboda *et al.*, 1993). Similarly, it has been extensively documented that both T<*c,s*>T (Huang *et al.*, 1992; Reardon *et al.*, 1993) and T[6-4]T (Mu *et al.*, 1997; Reardon and Sancar, 2003) are repaired by the human excision nuclease with the [6-4] photoproduct being excised at 5-10 fold faster rates than the T<*c,s*>T (Reardon and Sancar, 2006). The mechanistic work on the excision of [6-4] photoproduct and T<*c,s*>T by the human excision nuclease revealed that the former was recognized by the damage sensors of the human excision nuclease, RPA, XPA, and XPC,

with reasonable specificity, but that T<*c,s*>T was not discriminated by these factors from undamaged DNA (Reardon and Sancar, 2003). These and related findings led to the formulation of a model proposing that efficiency of repair is due to a combination of damage recognition by thermodynamic cooperativity and kinetic proofreading of human excision nuclease (Reardon and Sancar, 2004; Sancar *et al.*, 2004). In this study I investigated the recognition of T<*t,s*>T II by RPA, XPA, and XPC and the excision of the photolesion by the excision nuclease ensemble. I found that T<*t,s*>T II is recognized like a [6-4] photoproduct, but it is excised like a T<c,s>T, revealing for the first time a lack of correlation between damage recognition by damage sensors and the efficiency of excision by the mammalian excision nuclease. The findings are consistent with the proposal that kinetic proofreading is the main determinant of specificity of mammalian excision nuclease.

2.2 Materials and Methods

2.2.1 Cell-Free Extract

Cell-free extract from the Chinese hamster ovary (CHO) cell line AA8 was prepared as described previously (Reardon and Sancar, 2006). The extract was stored at -80 ^{o}C in 25 mM HEPES-KOH, pH 7.9, 100mM KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 2mM dithiothreitol and 12.5 % glycerol (v/v) buffer. Repair factors RPA, XPA, and XPC-hR23B, were purified as recombinant proteins (Reardon and Sancar, 2006).

2.2.2 Repair Factor Expression and Purification

The three subunits of RPA are all contained on a single expression vector *p11d-tRPA* provided by Dr. Marc Wold at the University of Iowa (Henricksen *et al.*, 1994).

The vector was transformed into *E. coli* strain BL21 (DE3). Protein expression was induced by treatment with isopropyl-1-thio-β-D-galactopyranoside (IPTG), followed by Affi-Gel blue (Bio-Rad) chromatography (Henricksen *et al.*, 1994).

The $[His]_6$-XPA plasmid construct available in the laboratory (Park and Sancar, 1993) was transformed and expressed in *E. coli* strain DR153 (*recA*$^-$ *uvrB*$^-$). Protein expression was induced by treatment with isopropyl-1-thio-β-D-galactopyranoside (IPTG), followed by Ni-NTA affinity chromatography (Qiagen) with an imidazole elution step, and a heparin agarose (Sigma) chromatography step with a sodium chloride gradient elution (Hermanson and Turchi, 2000).

Recombinant XPC baculoviral stock was used to infect Sf21 insect cells. Cell free extract was applied to a phosphocellulose p11 (Whatman) column and bound protein was eluted with KCl. Fractions containing XPC were applied to a single stranded DNA-cellulose column (Sigma) and eluted with KCl, and finally applied to DEAE-agarose (Bio-Rad) column to remove contaminating DNA (Matsutani *et al.*, 1994; Reardon *et al.*, 1996).

Recombinant proteins were stored at -80 ^{o}C in 25 mM HEPES-KOH, pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 2mM dithiothreitol and 12.5 % glycerol (v/v) buffer.

2.2.3 Substrates

Internally radiolabeled 136 base pair (bp) or 138 bp DNA substrates with no damage or with one of three thymine photoproducts, T<*c,s*>T, T<*t,s*>T II, or T[6-4]T were prepared by ligating 6 partially overlapping oligonucleotides (Figure 2.1), one of which contained the photoproduct (Reardon and Sancar, 2006). The T<*c,s*>T and T[6-

4]T were in a 136 bp duplex and the T<*t,s*>T II was in a 138 bp duplex (Table 2.1). For electrophoretic mobility shift assays, internally radiolabeled 50 bp duplexes without damage (unmodified, UM), with T<*c,s*>T, and with T[6-4]T, or 52 bp duplex with T<*t,s*>T II were prepared in a similar manner using four oligonucleotides. The sequences of these 50 bp and 52 bp duplexes correspond to nucleotide positions 44–93 of the 136 bp substrate (Hara *et al.*, 2000) and nucleotide positions 45–94 of the 138 bp substrate.

2.2.4 Electrophoretic Mobility Shift Assay

Repair factors RPA, XPA, or XPC at the indicated concentrations and 2.5 fmol of 50 bp or 52 bp duplexes were incubated in 12.5 μL reaction mixtures containing 50 mM Tris-HCl, pH 8.0, 60 mM KCl, 5 mM $MgCl_2$, 100 μg/mL bovine serum albumin, and 10 % (v/v) glycerol at 30 °C for 30 min (Reardon *et al.*, 1993). Samples were resolved in 5 % nondenaturing polyacrylamide gels, with electrophoresis at room temperature and a constant current of 25 mA. The DNA-protein complexes were visualized by autoradiography and quantitative analysis of the percent of total DNA bound was carried out with the Storm 860 system and ImageQuant software (GE Healthcare).

2.2.5 Excision Assay

The assay measures the release of 24-32 nucleotide-long oligonucleotides from internally labeled substrates (Reardon and Sancar, 2006). The reaction mixture contained, in 25 μL, 0.6 fmol DNA substrate, 54 ng pBR322, and 50 μg cell-free extract in reaction buffer containing 17 mM HEPES-KOH, pH 7.9, 12 mM Tris-HCl, 35 mM KCl, 44 mM NaCl, 5.8 mM $MgCl_2$, 0.3 mM EDTA, 2 mM dithiothreitol, and 2.5 % (v/v) glycerol (Reardon and Sancar, 2006). The reactions were carried out at 30 ^{o}C for the indicated

time periods. At the end of each reaction the DNA was deproteinized, precipitated with ethanol, and resolved in 8 % polyacrylamide DNA sequencing gels. The excision products were located by autoradiography, and the percent excision of total substrate DNA was quantified by using the Storm 860 system and ImageQuant software (GE Healthcare).

2.3 Results and Discussion

2.3.1 Binding of Damage Recognition Factors to UV Photoproducts.

I determined the relative affinities of the three damage sensors, RPA, XPA, and XPC, to UV photoproducts T<*c,s*>T, T<*t,s*>T II, and T[6-4]T by electrophoretic mobility shift assays. The results are shown in Figures 2. 2 and 2.3. In agreement with earlier reports (Reardon and Sancar, 2003; Wakasugi and Sancar, 1999) all three factors bound to T[6-4]T with higher affinity than to T<*c,s*>T and undamaged DNA, which under our assay conditions have indistinguishable binding properties. Interestingly, T<*t,s*>T II is bound with an affinity similar to or greater than T[6-4]T by all three damage identifying repair factors (Figure 2.3, Table 2.2).

A possible explanation for these binding results may lie in the structures of the respective photoproducts. The [6-4] photoproduct bends DNA by 44 ° and unwinds the helix by 30 ° (Wang and Taylor, 1993). The T<*c,s*>T bends DNA by 30 ° and unwinds it by 10 ° (Husain *et al.*, 1998; Park *et al.*, 2002) and T<*t,s*>T bends DNA 22 ° and unwinds it by 15 ° (Wang and Taylor, 1993). However, it must be noted that even though the overall degrees of bending and unwinding caused by T<*c,s*>T and T<*t,s*>T are similar, the deformation associated with T<*c,s*>T is smooth compared to acute kinking associated with breaking of hydrogen bonds 5 ' to the T<*t,s*>T photoproduct (Wang and Taylor,

1993), and in that regard, T<*t,s*>T is closer to T[6-4]T in the degree, if not the exact geometry, of the structural perturbation inflicted upon the helix. These findings are in line with the general observation that severe helical deformities constitute high-affinity binding sites for the damage sensor factors of the human excision nuclease (Sancar, 1996; Wood, 1997; Reardon and Sancar, 2004).

2.3.2 Excision of UV Photoproducts by Mammalian Excision Nuclease.

As a general rule, the affinities of the damage sensors, RPA, XPA, and XPC, for a DNA lesion correlate with the efficiency with which that lesion is removed by the mammalian excision nuclease (Sancar, 1996; Reardon and Sancar, 2003; Reardon and Sancar, 2004). Thus I expected that T<*t,s*>T II would be excised at least as efficiently as the [6-4] photoproduct. Surprisingly, however, T<*t,s*>T II is repaired with a rate similar to that of T<*c,s*>T, which is repaired at about 20 % the rate of the [6-4] photoproduct (Figures 2.4 and 2.5).

This discrepancy between the binding affinity of the damage sensors and the catalytic step by the two nucleases XPG and XPF-ERCC1 to remove the damage is consistent with the notion that the initial binding affinity is not the ultimate determinant of the repair rate and that specificity is further modulated and amplified by the kinetic proofreading activity of the TFIIH ATPase/helicase (Reardon and Sancar, 2003; Reardon and Sancar, 2004).

2.4 Conclusion

In mammalian excision repair an ATP-independent damage recognition step is followed by ATP hydrolysis-dependent kinetic proofreading steps to achieve

physiologically relevant specificity at a biologically acceptable rate. Three levels of specificity have been recognized: 1) thermodynamic specificity whereby the damage sensors bind to undamaged and damaged DNAs with different affinities. 2) Cooperativity whereby protein-protein interactions among the binding proteins may preferentially increase the binding of the damage sensors to damaged DNA over undamaged DNA. 3) Finally, the presence of ATP-dependent kinetically irreversible steps on the pathway has led to the suggestion of kinetic proofreading before the final chemically irreversible steps of dual incisions. In the canonical kinetic proofreading model (Hopfield, 1974; Ninio, 1974), the kinetic steps enable the enzyme system to abort the enzyme-incorrect substrate complexes before catalysis. It is theoretically feasible that a system such as nucleotide excision repair, which has a wide substrate range, some of the substrates will be discarded at higher rates than others, even though the initial binding affinities of the two substrates may be identical. This is what I observe with the processing of T[6-4]T and T<*t,s*>T II, both of which are recognized with about the same affinities, but are excised at significantly different rates, indicating that the nature of the substrate affects the kinetic proofreading efficiency, and hence the overall repair rate. As has been observed in translation (Cochella and Green, 2005), it is likely that in excision repair, in addition to thermodynamic discrimination and kinetic proofreading, induced fit mechanisms play a role in the ultimate specificity as measured by the rate of excision of DNA damage. Further work with additional substrates is needed to define the contributions of thermodynamic, structural, and kinetic factors to specificity.

Figure 2.1. Schematic for preparation of linear DNA substrate. The damage-containing oligonucleotide is radiolabelled with ^{32}P and the six overlapping oligonucleotides are ligated and annealed to form substrate for the excision repair assay.

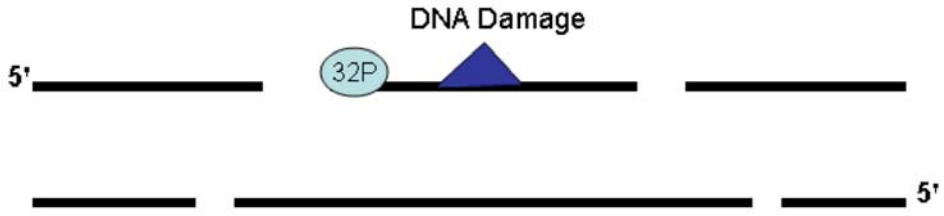

DNA Damage
5'
32P
5'

Figure 2.2. Binding of human damage recognition proteins RPA, XPA, and XPC to UV photoproducts. Electrophoretic mobility shift assays were done with the indicated proteins and 0.2 nM 50-bp or 52-bp duplexes with no lesion (UM), with a *trans-syn* II cyclobutane thymine dimer (T<t,s>T), with a (6–4) photoproduct (T[6-4]T) as indicated, or with a *cis-syn* cyclobutane thymine dimer (T<c,s>T, data not shown). The highest protein concentrations in the assays was 300 nM for RPA, 200 nM for XPA, and 15 nM for XPC, and the positions of free and bound DNA are indicated. Autoradiograms of electrophoretic mobility shift assays are presented here. The increasing protein concentrations in the binding reactions are indicated by triangles.

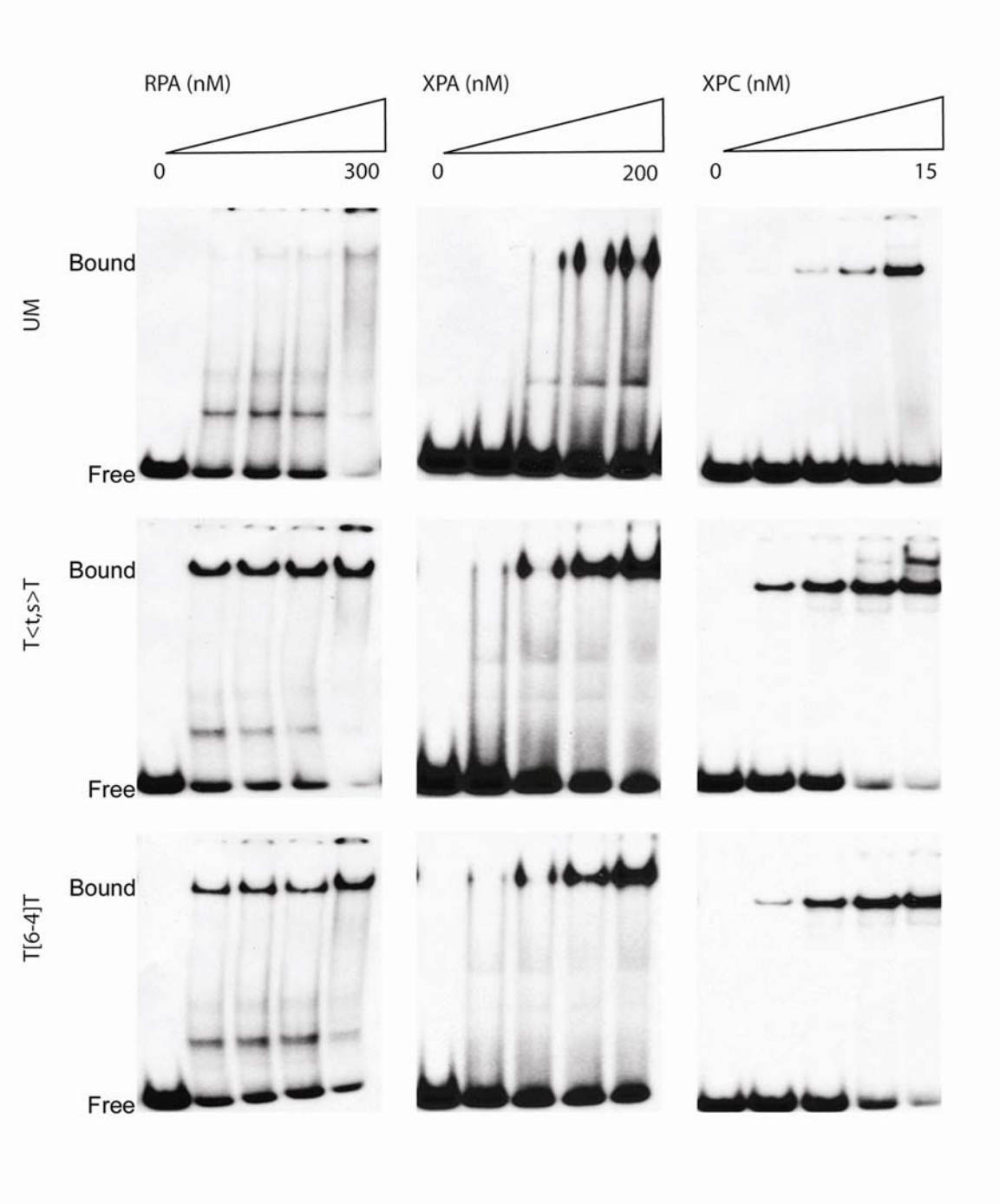
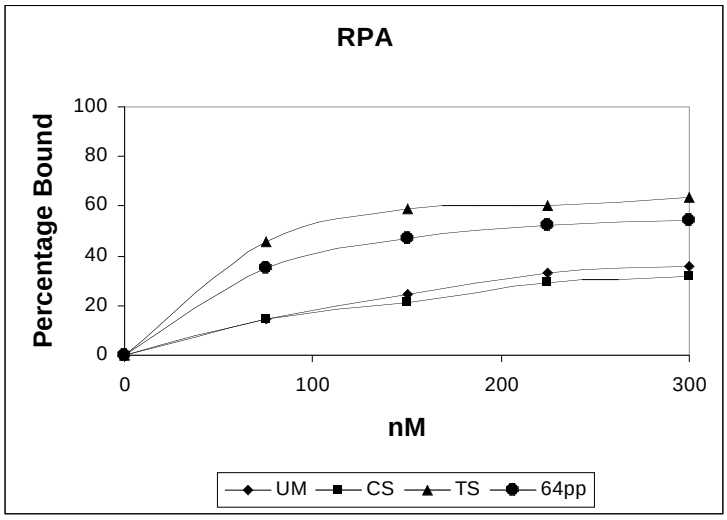
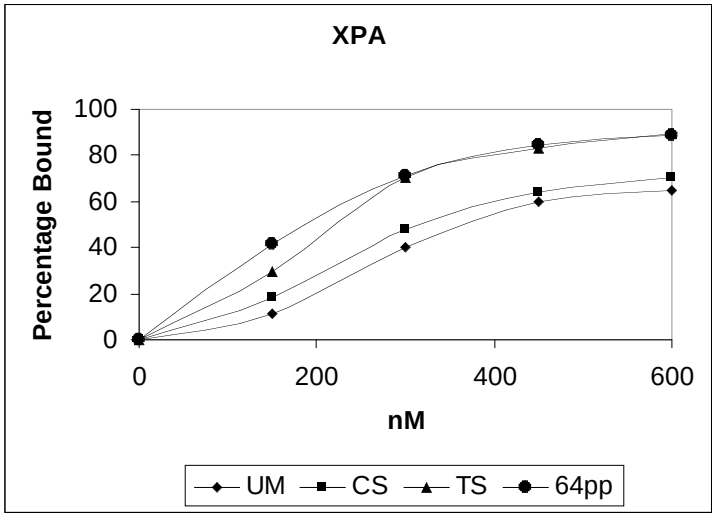

RPA (nM)
0
300
XPA (nM)
0
200
XPC (nM)
0
15
UM
Bound
Free
T<t,s>T
Bound
Free
T[6-4]T
Bound
Free

Figure 2.3. Binding isotherms generated from the average of four experiments including the ones shown in Figure 2.2. The RPA concentrations were 75, 150, 225, and 300 nM; XPA concentrations were 150, 300, and 450 or 300, 450, and 600 nM; XPC concentrations were 5, 7.5, 10, and 15 nM. (♦) UM DNA; (■) T<*c*,*s*>T; (▲) T<*t*,*s*>T II; (●) T[6-4]T.


RPA
Percentage Bound
100
80
60
40
20
0
0
100
200
300
nM
UM
CS
TS
64pp


XPA
Percentage Bound
100
80
60
40
20
0
0
200
400
600
nM
UM
CS
TS
64pp

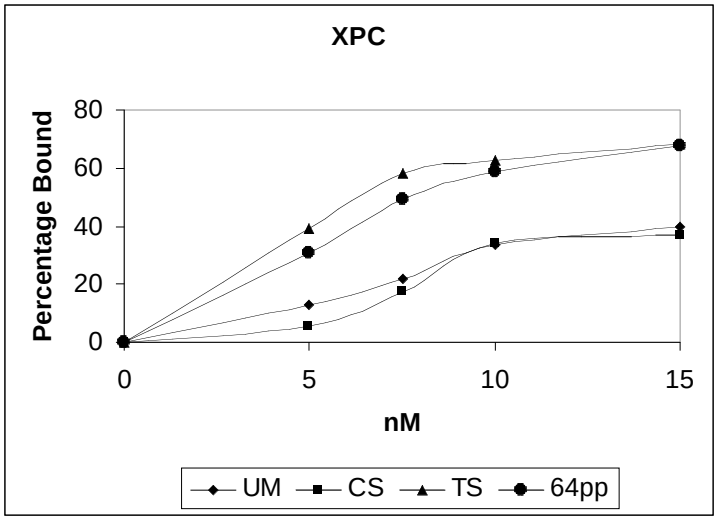

XPC
Percentage Bound
80
60
40
20
0
0
5
10
15
nM
UM
CS
TS
64pp

Figure 2.4. Excision activity of mammalian nucleotide excision repair. CHO cell free extract was incubated with DNA duplexes containing either *trans-syn* II cyclobutane thymine dimer (T<t,s>T II), *cis-syn* cyclobutane thymine dimer (T<c,s>T), or (6-4) photoproduct (T[6-4]T).

Excision products were separated in an 8 % denaturing polyacrylamide gel. Autoradiogram of a representative assay; original substrate and excision products are noted to the right and numbers to the left indicate positions of DNA size markers. Incubation time of each reaction is noted at the bottom and DNA substrate used is noted at the top.

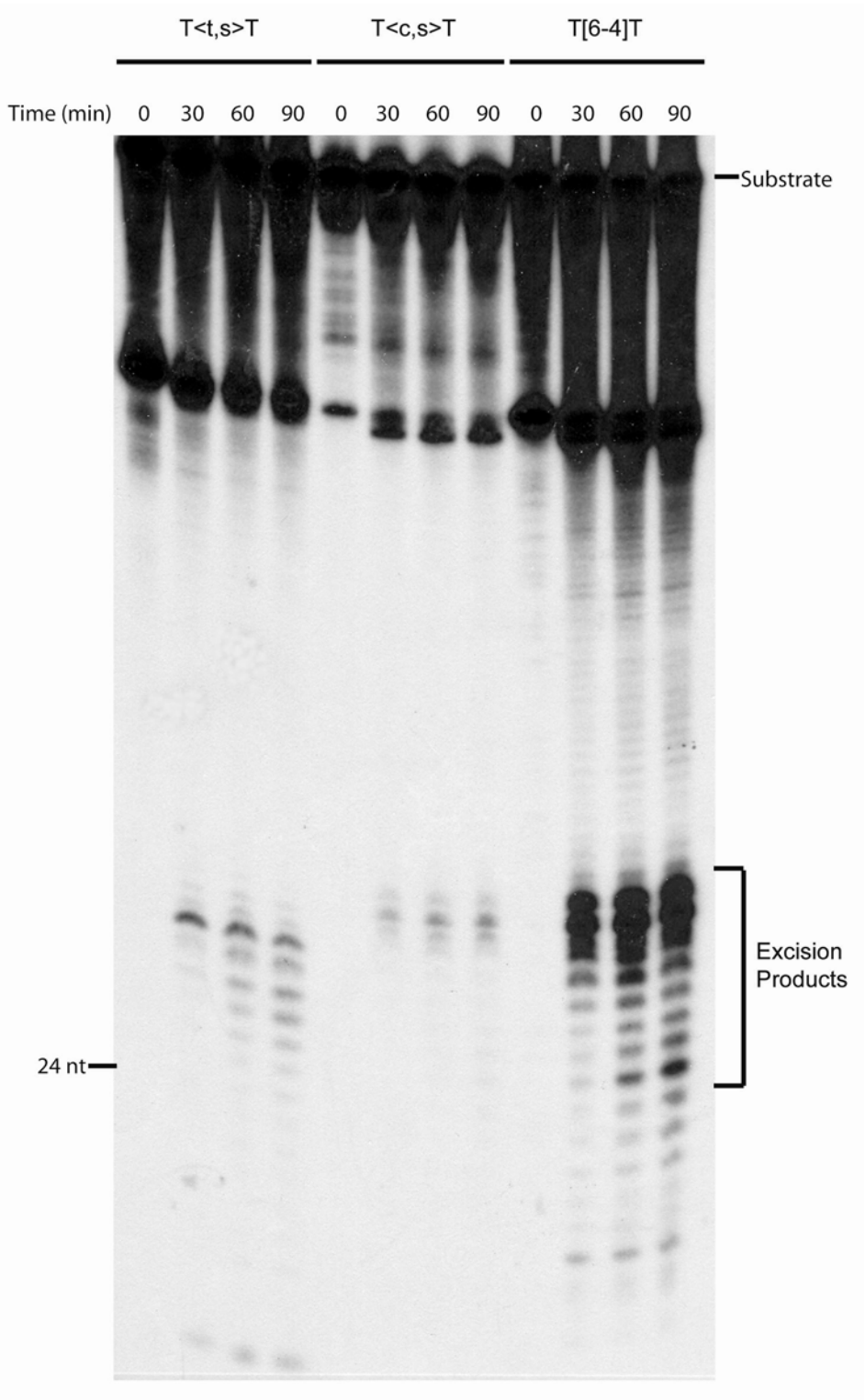

T<t,s>T
T<c,s>T
T[6-4]T
Time (min) 0 30 60 90 0 30 60 90 0 30 60 90
Substrate
Excision Products
24 nt

Figure 2.5. Excision kinetics. The statistical analysis of eighteen experiments from three substrate preparations are plotted. (●) T[6-4]T, (■) T<*c,s*>T, (▲) T<*t,s*>T II.

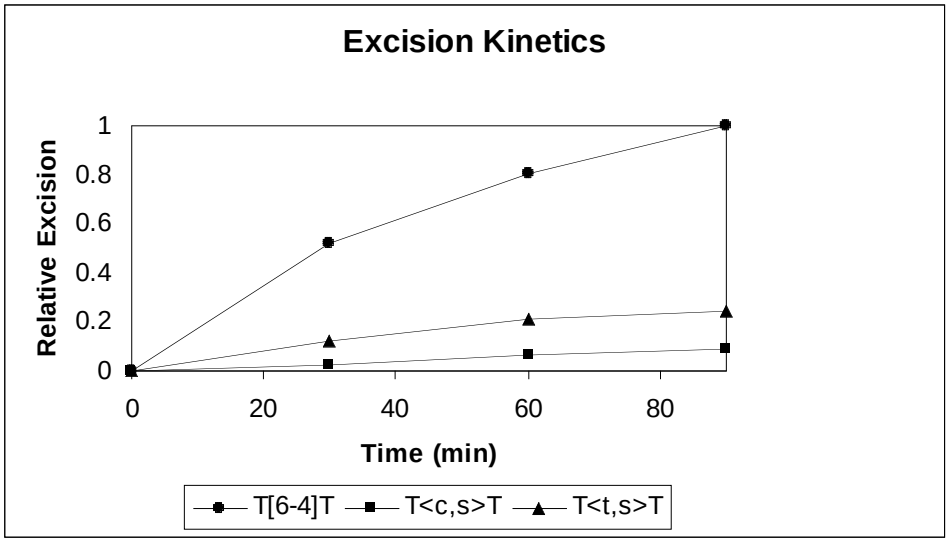

Excision Kinetics
Relative Excision
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
Time (min)
T[6-4]T
T<c,s>T
T<t,s>T

Table 2.1. Sequence of DNA substrate surrounding the lesion.

Damage	Sequence
UM	…CTGA_G**A**A**G** - **CTAC**_GAGC…
T[6-4]T	…CTGA_G**T**AT<>TA**T**G_GAGC…
T<c,s>T	…CTGA_G**T**AT<>TA**T**G_GAGC…
T<t,s>T II	…CTGACG**A**AT<>TA**A**GCGAGC…

Flanking sequence is identical for all substrates. Bold letters indicate a difference in substrate sequence. An underscore allows all sequences to be aligned with the trans-syn sequence which is 2 nucleotides longer than the other DNA substrates. The total guanine-cytosine content of these substrates does not differ significantly enough to consider the role of sequence differences in the binding experiments.

Table 2.2. Affinities of human DNA damage binding proteins to UV products.

	K_{UM}[a]	$K_{T<C,S>T}$[a]	$K_{T<T,S>T\ II}$[a]	$K_{T[6-4]T}$[a]
RPA	**5.0×10^{-7}**[b]	**5.5×10^{-7}**[b]	**1.2×10^{-7}**	**2.1×10^{-7}**
XPA	**3.8×10^{-7}**	**3.1×10^{-7}**	**1.8×10^{-7}**	**2.2×10^{-7}**
XPC	**3.5×10^{-8}**[b]	**3.8×10^{-8}**[b]	**5.5×10^{-9}**	**7.5×10^{-9}**

[a] K_{UM}, $K_{T<c,s>T}$, $K_{T<t,s>T\ II}$, $K_{T[6-4]T}$, are the estimated molar concentrations of repair factors at which 50 % of 0.2 nM unmodified, T<*c,s*>T, T<*t,s*>T II, or T[6-4]T-containing DNA were bound under excess repair factor conditions.

[b] Extrapolated from the binding isotherm to the amount required for 50 % binding.

Chapter Three

Final Discussion

'Here comes the sun, here comes the sun, and I say it's alright.' – George Harrison

While George Harrison may have been referring to the freedom he felt during his break from the Beatles, the physical and psychological benefits of the sun are well documented. Exposure to ultraviolet light regulates the melatonin-serotonin balance in humans, which if perturbed tends to manifest as depression, or in months with fewer daylight hours, as seasonal affective disorder (SAD) (Wehr and Rosenthal, 1989). Ultraviolet light has also been used to treat psoriasis and vitamin D deficiency (Lehmann *et al.*, 2004). So, as is the motto for my alma mater, fiat lux, let there be light. With one caveat, however, the ultraviolet component of sunlight is the greatest environmental factor contributing to the one million new cases of skin cancer diagnosed every year in the United States (Christenson *et al.*, 2005). Because of the cosmetic and mental benefits sun exposure affords, Americans tend to overindulge in self-irradiation activities on the beach, at the pool, and in tanning beds. In terms of sun exposure, as well as life's other pursuits, I find it is best to heed the advice of Aristotle; moderation of all things prevents a virtue from becoming a vice.

Because life has evolved to require solar input, species must also be equipped to manage the consequences of UV exposure. To date, all studied species maintain at least one biochemical pathway for the removal of UV-induced DNA damage from their

genome. The study of UV-photoproducts and their removal from the genome is relevant to approaching Aristotle's golden mean, the balance between beneficial sun exposure and overindulgence.

The study herein pertains to the *trans-syn* thymine dimer; a UV-lesion that may not be as trivial as first considered. Biologically relevant spectra of solar irradiation include UVA and UVB wavelengths, as UVC does not penetrate the ozone layer, so long as it remains intact. Under UVC conditions, the *trans-syn* thymine dimer constitutes 2 % of total damage, the *cis-syn* thymine dimer accounts for over 60 % of damage, and the [6-4] photoproduct weighs in at approximately 20 % of total damage (Patrick and Rahn, 1976). A recent report using simulated sunlight suggests the [6-4] photoproduct may be formed at a lower frequency, 5 % of total damage, using the more biologically relevant UV fluence (Yoon *et al.*, 2000). Additionally, the *trans-syn* pyrimidine dimer forms at a higher frequency in single strand DNA, a condition that was not considered in the initial study (Douki, 2006; Douki and Cadet, 1992). Indirectly this suggests the *trans-syn* thymine dimer may be formed at a higher frequency under simulated sunlight conditions or may form in equivalent amounts to the [6-4] photoproduct. This UV-photoproduct merits further investigation because it may prove a more powerful mutagen than the [6-4] photoproduct and its unique chemistry may serve as an important biochemical tool in the study of nucleotide excision repair.

The work described here is only the beginning of such and investigation. My results show the *trans-syn* II thymine dimer is bound by the damage-identifying repair factors RPA, XPA, and XPC with similar affinity as the [6-4] photoproduct, the best recognized substrate. I also show the *trans-syn* II thymine dimer is a substrate for the

mammalian excision nuclease and its kinetics of repair are similar to the *cys-syn* thymine dimer. The results of my work are the first to elucidate the interaction of the dimer with the excision repair machinery; in addition, my work provides evidence of a secondary step in the pathway of excision repair which confers final specificity of the nuclease system.

This study inspired more questions than those it answered. Future experiments should focus on the *trans-syn* I thymine dimer. Because there is a structural difference between the two dimers, the binding affinity and excision kinetics could be markedly different. Those experiments would confirm that the *trans-syn* I thymine dimer is a substrate for the mammalian excision nuclease, that the chemical structures of these two lesions elicit unique binding affinities by each of the damage-identifying repair factors, and that the two lesions offer distinct repair profiles in the excision repair assay. Because it is not known which isomer, if any, is formed *in vivo*, it would be interesting to raise antibodies specific to either isomer I or II and detect the presence of the dimer in UV-irradiated cells. The laboratory of Dr. John-Stephen Taylor has investigated bypass of the *trans-syn* thymine dimer with a variety of polymerases (Smith *et al.*, 1998), but not with any of the human Y-family polymerases. Lesion bypass experiments could be done with polymerase η, κ, and ι to examine the error frequency of trans-lesion synthesis.

Another point of interest related to this study is the damage-sensing ability of XPA. The role of XPA in damage recognition could be demonstrated by obtaining a DNase I footprint of XPA protein on damaged DNA. XPA protein has been shown by EMSA to independently bind damaged DNA and is implicated in the initial damage recognition step of nucleotide excision repair (Reardon and Sancar, 2003; Missura *et al.*,

2001; Dip *et al.*, 2004; Camenisch *et al.*, 2006; this study). There are still widely accepted models of excision repair which suggest XPC is the single damage recognition factor and the initiator of nucleotide excision repair (Sugasawa *et al.*, 1998; Sugasawa *et al.*, 2002). DNase I footprint analysis of XPC has shown the repair factor is capable of producing a specific effect on a [6-4] photoproduct-containing duplex (Wakasugi and Sancar, 1999; Sugasawa *et al.*, 1998) and can also produce a footprint on three-base loops (Sugasawa *et al.*, 2002). The assembled human excision nuclease was also able to produce a DNase I footprint and it was demonstrated that the complex extends about twenty nucleotides 5' of the damage site and fifteen nucleotides 3' of the damage site (Mu *et al.*, 1997; Wakasugi and Sancar, 1998).Obtaining a DNase I footprint of XPA on damaged DNA will qualitatively demonstrate XPA is capable of recognizing damage on its own. Confirming the damage recognition property of XPA will provide insight into how the repair factor interacts with DNA damage to further elucidate mechanistic details of damage recognition.

A final point of interest tangentially related to this study is to more quantitatively determine the binding affinity of the damage-identifying repair factors RPA, XPA, and XPC for UV-photoproducts alone and in pairwise combinations. Such an investigation would elucidate the influence of cooperative interactions on damage recognition as proposed by the random assembly model of nucleotide excision repair (Reardon and Sancar 2003; Reardon and Sancar 2004). Increased site specific affinity of DNA binding proteins is unfortunately concomitant with increased affinity for nonspecific sites as well. One way to avoid the inevitable sequestration of high affinity binding proteins at nonspecific sites is to relinquish affinity in favor of cooperation. In this case a number of

factors work together to achieve specific binding of a substrate. Damage recognition by individual repair factors was demonstrated (Reardon and Sancar, 2003; this study). However, the affinity of single repair factors for damaged DNA is only moderately greater than that of undamaged DNA (Reardon and Sancar, 2003; this study), and therefore does not account for the overall selectivity of the pathway. There are reports discussing whether or not RPA increases the affinity of XPA for damaged DNA (Wakasugi and Sancar, 1999; Missura *et al.*, 2001; Wang *et al.*, 2000; Patrick and Turchi, 2002; He *et al.*, 1995; Stigger *et al.*, 1998; Hey *et al.*, 2001; Liu *et al.*, 2005; You *et al.*, 2003; Yang *et al.*, 2002). There is only one report demonstrating an interaction between XPA and XPC proteins (Wakasugi and Sancar, 1999). All of these reports suggest possible cooperative interactions increase substrate recognition by the repair factors RPA, XPA, and XPC, but none quantitatively confirm it. Surface plasmon resonance would be a technique capable of determining in real time the effect of these repair factors on each other (Wilson, 2002).

In brief, the outline of such an approach follows here. The biotinylated damaged and undamaged DNAs would be conjugated to the streptavidin-immobilized surface of individual BIAcore sensor chips. Damage recognition proteins would be injected into the flow-cell of the sensor chip and response units would be measured. Surface plasmon resonance studies have been done with RPA and XPA interacting alone with the *cis-syn* thymine dimer and [6-4] photoproduct and in combination using the [6-4] photoproduct (Wang *et al.*, 2000). Further studies should examine all pairwise combinations of damage-identifying repair factors and a broad spectrum of UV-induced lesions.

This technology is a more sensitive approach to investigating binding interactions and could provide insight into the molecular interactions which occur during damage recognition. The effect on binding affinity that cooperative interactions contribute to substrate specificity will be examined. Further studies could be done to investigate DNA independent interactions among the damage-identifying repair factors. In addition, this technology could be extended to look at complex formation of the excision nuclease, not just the damage recognition complex.

In summary, this study was the first to reveal that damage recognition does not necessarily correlate with efficiency of excision repair. This finding lends support to the model that another mechanism is responsible for conferring the overall specificity of the excision nuclease. One such model suggests this mechanism is kinetic proofreading (Reardon and Sancar, 2003). In any event, understanding the fundamental mechanism of damage recognition in nucleotide excision repair is relevant not only to understanding a basic biochemical mechanism, but to improving the efficacy of DNA damaging chemotherapeutic agents and developing technology for the repair of UV damage.

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