EXAMINATION OF DNA REPAIR BY QUANTITATIVE PCR

AN EXAMINATION OF NUCLEOTIDE EXCISION REPAIR IN HUMAN CELLS BY A NOVEL QUANTITATIVE POLYMERASE CHAIN REACTION TECHNIQUE

By

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ABSTRACT

Host cell reactivation (HCR) of viruses has been used in the past to assess the DNA repair capacities of various mammalian cell types. In this study, a PCR-based HCR technique was developed for determining DNA repair capacity of mammalian cells. Many DNA lesions, including UV photoproducts, block DNA amplification by *Taq* polymerase, and the exponential nature of PCR imparts a tremendous potential for quantifying the remaining non-adducted DNA templates from small samples. Ad5HCMVsp1*lacZ* is a recombinant non-replicating adenovirus (Ad) containing the *lacZ* reporter gene under the control of the human cytomegalovirus (HCMV) immediate early promoter inserted into the deleted E1 region of the viral genome. This virus is unable to replicate, but it can efficiently express the reporter gene in many types of mammalian cells, including human fibroblasts. Using quantitative PCR, the induction and repair of UV photoproducts was measured in a 2.6 kb region of the *lacZ* reporter gene inserted into the deleted E1 region of Ad5HCMVsp1*lacZ* and in a 2.8 kb region of the endogenous E4 region of the virus. Primers flanking the regions were added to equal amounts of DNA extracted from cells infected with unirradiated or UV-irradiated Ad5HCMVsp1*lacZ* and each sample was amplified by PCR using radiolabelled nucleotides as substrates. PCR products were separated by agarose gel electrophoresis and quantified using a phosphorimaging system. Results show a simple exponential decrease in PCR product with increasing UV fluence

to the virus. There was a significant removal of UV photoproducts by 24 hours after infection of normal human fibroblasts. A reduced capacity for lesion removal was detected after infection of nucleotide excision repair deficient fibroblasts derived from patients with xeroderma pigmentosum (XP) and Cockayne syndrome (CS). Previous work from our lab using a β-gal reporter gene assay has shown that both UV light and heat shock treatment of cells prior to infection with UV-damaged Ad5HCMVsp1*lacZ* enhances HCR. Application of the quantitative PCR technique to the study of inducible repair shows there is an enhancement in the rate of lesion removal from both regions of the vector in UV-irradiated normal lung fibroblast cells, compared to unirradiated cells. This demonstrates that previous reports of enhanced host cell reactivation are indicative of a genuine enhancement of DNA repair. Also, the β-gal reporter gene assay was used to investigate inducibility of UV lesion repair by ionising radiation; no significant increase in HCR of β-gal activity was found in cells treated with γ-rays compared to untreated cells.

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I would like to thank my supervisor Dr. Andrew J. Rainbow for allowing me to pursue my M.Sc. in his lab and to develop the QPCR project. This project both presented an interesting challenge and provided a sense of gratification when results began trickling in. Thanks to Dr. Brad N. White for being a part of my M.Sc. supervisory committee, and for his advice in times of stress with molecular biology and academia. I would also like to thank Dr. Jim S. Quinn for his contributions as a member of my defence committee. Thanks to Todd Bulmer for his critical reviews, lucid insights and helpful discussions. Thanks to Murray Francis, Jim Stavropoulos, and Bruce McKay for technical help and discussions. Additionally, I'd like to thank Zhimin Tong, Victoria Nethercot, and the previously mentioned members of our lab group for making LSB 406 a comfortable place to live for two and a half years. I would like to thank the laboratory group of Dr. F. L. Graham for the Ad5HCMVsp1*lacZ* vector used in these studies, for the 293 cells used for propagation of the virus, as well as for helpful tips and instruction with respect to virus work. I would like to especially thank my parents, my siblings Orest and Marta, and my friends for all their support, as well as for the reality checks they occasionally provided.

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PREFACE

This thesis comprises three chapters and two appendices. Chapter 1 is an introduction to the material in the thesis, which presents relevant background information from the current literature. Chapter 2 is a co-authored manuscript to be submitted for publication, which presents a new quantitative polymerase chain reaction (PCR) technique for DNA repair, and its application to the study of nucleotide excision repair in human cells. I carried out all the research that is presented in this chapter. The information in Chapter 3 is a summary of my research into the inducibility of host-cell reactivation (HCR) of UV-damaged adenovirus by ionising radiation in human cells, using a β-gal reporter gene assay. Appendix A is an extension of Chapter 2, and it summarises results of two experiments examining the inducibility of lesion removal in xeroderma pigmentosum complementation group C cells by UV light. Appendix B outlines the construction of an internal standard for competitive PCR, which may be used to enhance the PCR assay described in Chapter 2.

LIST OF ABBREVIATIONS

6-4PP – 6-pyrimidine-4-pyrimidone photoproduct

α-MEM – α minimal essential medium

γRER – gamma ray-enhanced reactivation

Ad – adenovirus

Ad5HCMVsp1*lacZ* – adenovirus 5 with *lacZ* inserted into the deleted E1 region under the control of the human cytomegalovirus promoter

AP – apurinic/apyrimidinic

AT – ataxia telangiectasia

ATM – ataxia telangiectasia mutated

BER – base excision repair

CAK – CDK-activating kinase

CAT – chloramphenicol acetyltransferase

CDK – cyclin-dependent kinase

CHO – Chinese hamster ovary

CPD – cyclobutane pyrimidine dimer

CS – Cockayne syndrome

CSA and *CSB* – Cockayne syndrome genes

CS-A and CS-B – Cockayne syndrome complementation groups

DHFR – dihydrofolate reductase

DNA – deoxyribonucleic acid

DSB – double strand break

FBS – foetal bovine serum

GGR – global genome repair

Gy - Gray

HCR – host cell reactivation

HSV – herpes simplex virus

IR – ionising radiation

J - Joule

MMR – mismatch repair

(continued)

MOI – multiplicity of infection

NER – nucleotide excision repair

NHEJ – non-homologous end-joining

PIBIDS – photosensitivity, ichthyosis, brittle hair and nails, intellectual impairment, decreased fertility and short stature

PIC – preincision complex

PBS – phosphate buffered saline

PBS^{2+} – PBS supplemented with 1% calcium chloride and 1% magnesium chloride

PCNA – proliferating cell nuclear antigen

PCR – polymerase chain reaction

pfu – plaque forming units

Pol - polymerase

pTpT – thymine dinucleotide

QPCR – quantitative polymerase chain reaction

RNAPII – RNA polymerase II

RPA – replication protein A

SCC – squamous cell carcinoma

scid – severe combined immunodeficiency

SF – surviving fraction

TBE – Tris-borate/EDTA electrophoresis buffer

TCR – transcription-coupled repair

TFIIH – transcription factor IIH

TRCF – transcription-repair-coupling factor

TTD – trichothiodystrophy

UV - ultraviolet

UV-DDB – UV DNA damage binding (protein)

UVER – UV-enhanced reactivation

V(D)J – variable, diversity and joining gene segments

XP – xeroderma pigmentosum

XPA through *XPG* – xeroderma pigmentosum genes

XP-A through XP-G – xeroderma pigmentosum complementation groups

XP-V – xeroderma pigmentosum variants

Chapter 1

Introduction

A. Ultraviolet Radiation and DNA Repair

The genome of every organism is continuously being damaged by hydrolysis, oxidation and methylation reactions of endogenous metabolic processes, as well as by exogenous agents such as ultraviolet light (UV), ionising radiation and chemical mutagens. These sources cause distortions of the normal double-helical structure of the DNA. There are many complex pathways involving a multitude of proteins that recognise and repair DNA damage, and the broad range of cellular DNA repair mechanisms underscores the importance of maintaining an unmodified and intact DNA structure. With the constant introduction of DNA damage, and its reversal by DNA repair mechanisms, the genome is in a continuous state of flux. The balance between DNA damage and repair efficiency has implications in cell survival, mutagenesis, and genomic instability. In mammalian cells, these are preliminary steps in carcinogenesis.

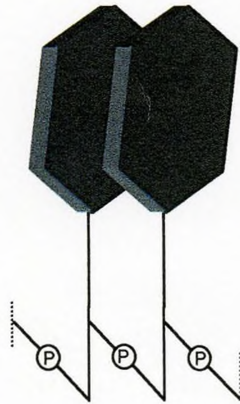
The most common types of human skin cancers are squamous cell carcinoma and basal cell carcinoma, which are caused by exposure to sunlight (Kraemer, 1997). In particular, it is the ultraviolet (UV) component of sunlight that induces DNA damage. If this damage is not properly repaired or eliminated, it can lead to mutagenesis and carcinogenesis. The UV spectrum is arbitrarily divided into three types of UV light, based on wavelength: UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (200-280 nm). Since the UV-C component

is blocked by the atmospheric ozone layer, the primary source of DNA damage from sunlight exposure is thought to be the UV-B component. However, for historical reasons, UV-C is the classic DNA-damaging agent that is best-characterised in DNA repair studies.

The two major DNA lesions introduced by UV-C and UV-B are the cyclobutane pyrimidine dimer (CPD) and the 6-pyrimidine-4-pyrimidone photoproduct (6-4PP). CPDs are the most common type of lesion in UV-C damaged DNA; they are formed when two adjacent pyrimidines undergo a 5-6 double-bond opening, and form a stable ring structure (see Figure 1-1). 6-4PPs are the second-most prevalent type of UV-induced lesion, formed when the C6 (position 6 carbon) of an open 5-6 pyrimidine double bond reacts with the C4 of an adjacent pyrimidine (McGregor, 1999). The ratio of CPDs to 6-4PPs depends on the sequence context and chromatin environment, but generally occurs at approximately 3 or 4 to 1. Other infrequent lesions induced include: pyrimidine monoadducts, purine dimers and a photoproduct between adjacent A and T bases (Sage, 1993). All of these lesions must be repaired for normal cell function to continue.

UV light not only causes DNA damage, but it affects cell membrane and cytoplasmic targets, which are involved in the cellular response to UV irradiation, that appear to be independent of nuclear events (Adler, et al., 1995). It is thought that UV light (and other cell-stressing agents) activates pathways

Figure 1-1. Major UV-induced DNA lesions. Upon absorption of UV light, the two major DNA lesions formed between adjacent pyrimidines are cyclobutane pyrimidine dimers (CPDs) and 6-pyrimidine-4-pyrimidone photoproducts (6-4PPs). (Adapted from van Steeg and Kraemer, 1999).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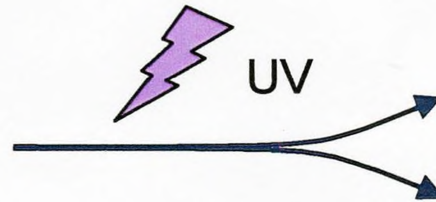
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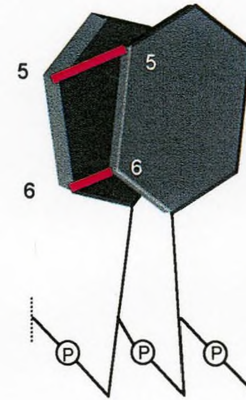
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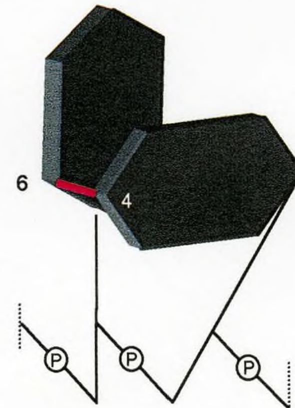
6
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6-pyrimidine-
4-pyrimidone
product [(6-4)PP]

originating at the cell membrane and/or cytoplasm, causing phosphorylation and de-phosphorylation signalling cascades. As a result, several kinases translocate into the nucleus and alter gene regulation by selectively activating transcription factors (Canman and Kastan, 1996). The tumour-suppressor TP53 (formerly known as p53) is induced by UV light and other DNA-damaging agents, and this cellular response is important for cell cycle arrest (allowing time for DNA repair) and apoptosis (cell death). Although it has not been shown directly, TP53 is thought to play a role in inducible DNA repair pathways (Hwang, et al., 1999; McKay, et al., 1999).

"DNA repair" is a term that encompasses a broad range of metabolic processes that allow a cell to deal with damaged DNA. The damage can be reversed directly (e.g. by photolyases or by alkyltransferases) or indirectly by removing damaged single bases (base excision repair) or a stretch of bases (nucleotide excision repair), and then resynthesising the removed patch (Jiricny, 1998). The repair pathways also include mechanisms that repair strand breaks and cross-links, or in some cases allow initial bypass of non-repairable or unrepaired DNA damage (post-replication repair). The overall goal of these highly developed mechanisms is to protect the fidelity of the DNA for efficient transcription and replication.

The nucleotide excision repair (NER) pathway removes UV-induced DNA damage, i.e. CPDs and 6-4PPs. In human cells, 6-4PPs are repaired very

efficiently in the entire genome (McGregor, 1999; van Steeg and Kraemer, 1999). Removal of CPDs, however, appears to be slower in the bulk of the genome (Bohr, et al., 1985), but more rapid in the transcribed strand of active genes (Hanawalt, 1991; McGregor, 1999; Mitchell and Nairn, 1989). Defective DNA repair (NER and other types) leads to mutagenesis, and possibly to carcinogenesis.

The importance of NER in the prevention of carcinogenesis is demonstrated in patients, whose cells are defective in NER. Sunlight-sensitive xeroderma pigmentosum (XP) patients experience a very high rate of skin cancer. NER is also important in the prevention of cancers induced by genotoxic agents other than UV. Smoking, which is causative in approximately 30% of cancer mortalities, produces bulky DNA adducts formed by benzo(a)pyrene or other polyaromatic hydrocarbons and arylamines found in cigarette smoke (Asami, et al., 1997), which are removed by NER. The NER pathway also impinges upon cellular response to chemotherapy, as many of the drugs used in this type of cancer treatment damage DNA, producing adducts that are substrates for NER. Clearly, selective inhibition of NER in tumour cells could provide a useful approach to increasing tumour cell sensitivity to the drugs. Thus, elucidation of NER pathways and their regulation is of great importance in both the prevention and treatment of cancer.

B. Human DNA Repair Mechanisms

The DNA repair mechanisms that have been identified in human cells can be divided into five major repair pathways: 1/ mismatch repair, 2/ homologous recombination, 3/ non-homologous end-joining, 4/ base excision repair, and 5/ nucleotide excision repair. Homologous recombination and non-homologous end-joining can be grouped as double-strand break repair mechanisms, and the term "excision repair" includes both base and nucleotide excision repair. Much has been learned about human DNA repair pathways through studying repair systems in bacteria and yeast, which are remarkably well conserved.

Mismatch Repair

Mismatch repair (MMR) corrects misincorporations in DNA that have escaped the cellular proof-reading exonuclease activity (Jiricny, 1998). MMR takes place immediately after replicative DNA synthesis (during the S phase of the cell cycle), by using the genetic information on the parental strand to correct errors in the nascent strand. Restoration of the original DNA sequence requires both recognition of mismatches, and the ability to distinguish between parental and daughter strands. Human MMR genes were identified by sequence similarity to the well characterised *Escherichia coli* (*E. coli*) mismatch repair system (Fishel and Kolodner, 1995). Mutations in human MMR genes are associated with the development of hereditary non-polyposis colon cancer

(Bronner, et al., 1994; Nyström-Lahti, et al., 1994). A germline mutation in one allele of a DNA mismatch repair gene may predispose an individual to early-onset colorectal cancer, and since this discovery much research has focussed on MMR.

Double Strand Break Repair

Homologous recombination (HR) and non-homologous end joining (NHEJ) are two pathways utilised for double-strand break (DSB) repair. HR is the only DSB repair mechanism present in *E. coli,* and also the pathway predominantly used by yeast, but in mammalian cells it is thought to play only a minor role in DSB repair (Taylor and Lehmann, 1998). In eukaryotic cells, HR is used to repair a DSB on one chromosome by using the information on the homologous chromosome as a template for alignment and repair. NHEJ (which is sometimes referred to as "illegitimate recombination") is a process that reconnects two ends of broken DNA without the aid of a template. NHEJ is the dominant mechanism of DSB repair in mammalian cells (Chu, 1997), and recent research in the fields of radiation biology and immunology has clarified the basic mechanism involved in this type of repair. NHEJ is a common repair mechanism for DSB repair after exposure to ionising radiation, and for the site-specific V(D)J recombination of genes used to generate diversity in the MHC that is necessary for immune system function (Chu, 1997; Grawunder, et al., 1996). The first piece of evidence that provided a link between the repair of radiation-induced DSBs

and V(D)J recombination in mammalian cells came from research on *scid* mice (reviewed by Zdzienicka, 1995). The autosomal recessive *scid* mutation impairs both DSB repair and V(D)J recombination, leading to radiosensitivity and immune defects in these mice. The human autosomal recessive disorder ataxia telangiectasia (AT) is associated with defective DSB repair (Foray, et al., 1997), but despite having cloned *ATM* (Savitsky, et al., 1995), the exact molecular deficiency and the connection to the DSB repair process has not yet been determined. Individuals with this disorder display sensitivity to ionising radiation and cancer-proneness.

Excision Repair

Excision repair processes utilise the information encoded on the complementary strand of DNA as a template for the repair of DNA damage. There are two types of excision repair: a/ base excision repair (BER) and b/ nucleotide excision repair (NER). In BER, non-bulky lesions (including uracil, thymine glycols and hydrates, N3-MeAde and 8-oxoguanine) are removed in a two-step process (Sancar, 1996). The first step is cleavage of the glycosidic bond between the modified base and the sugar-phosphate backbone by DNA glycosylase, which releases the damaged base, creating an apurinic/apyrimidinic (AP) site (Taylor and Lehmann, 1998). In human cells, there is one major AP endonuclease encoded by the *HAP1/APE1* gene (Taylor and Lehmann, 1998), and several DNA glycosylases, each of which recognises very specific base

damage (Sancar, 1996). In the second step, the AP site is excised and released by AP endonucleases and AP lyase, creating a one-nucleotide gap, which is then filled and ligated by a DNA polymerase and DNA ligase, respectively. There is a high degree of evolutionary conservation in the genes required for BER in *E. coli,* yeast and mammalian cells.

Nucleotide excision repair (NER) is a versatile repair system found in all organisms examined thus far, which repairs a wide range of DNA alterations, including those induced by UV radiation. Compared to BER, it is a more complex mechanism (requiring the concerted participation of many proteins), which recognises the lesion, opens up the DNA strands to make the lesion accessible for repair, and hydrolyses two phosphodiester bonds (one on each side of the damage) to release a damage-containing DNA fragment. The resulting gap is filled in using the opposite strand of DNA as a template, and then ligated. This sequence of events (functional homology) and the protein complex structural homology appear to be identical in prokaryotes and eukaryotes, but the genes for the subunits involved in the two systems do not share any sequence homology (Sancar, 1994; Taylor and Lehmann, 1998). NER has a wide substrate range, but it is most effective in repairing bulky lesions, such as UV-induced photoproducts, which distort the DNA double-helical structure. Two subpathways of NER have been characterised in both *E. coli* and mammalian cells: a/ transcription-coupled repair (TCR), which preferentially

repairs DNA damage in actively transcribed genes, and b/ global genome repair (GGR), which repairs damage homogeneously throughout the genome.

C. Human Nucleotide Excision Repair (NER)

Although the general principles and basic steps of prokaryotic and human NER are the same, there is little sequence and protein *interaction* (biochemical) homology in these two systems. In *E. coli* only six proteins are required for NER, whereas human NER requires approximately 30 polypeptides (reviewed in Tornaletti and Pfeifer, 1996). However, in the human NER system the polypeptides involved are not usually found in free form, but as components of six fractions, each containing 2-7 polypeptides in tight assemblies (Sancar, 1996), which may represent a functional homology with *E. coli*. The two systems are thought to reflect a case of convergent evolution (Sancar, 1996). Genetic and biochemical complementation between mammals and other studied species is rare or absent, and this has made research of human NER mechanisms a more formidable task.

Human NER-Deficiency Diseases (the genetics)

The first report of a DNA repair defect being associated with a human hereditary disease was James Cleaver's (1968) report that skin fibroblasts from a patient with the disease xeroderma pigmentosum (XP) were unable to carry out

NER following UV irradiation. Since then, the XP clinical and cellular phenotypes have been extensively characterised. Two other diseases have also been identified that are associated with defective excision repair: Cockayne syndrome (CS) and a photosensitive form of trichothiodystrophy (TTD). The study of mutant cell lines derived from patients with these three diseases, as well as mutant rodent cell lines with similar phenotypes, have allowed the elucidation of the normal human NER pathway.

i. Xeroderma Pigmentosum (XP)

XP is an autosomal recessive disorder, which affects approximately 1 in 250,000 people in the US and Europe, and 1 in 40,000 in Japan. Clinical symptoms of this disease include severe freckling, extreme sensitivity of the skin to sunlight and neurological abnormalities. Affected individuals have a 1000-fold greater frequency of skin tumours compared to normal individuals (van Steeg and Kraemer, 1999), which is predominantly manifested as basal cell and squamous cell carcinoma, as well as malignant melanoma (Sancar, 1996). Approximately 90% of the basal cell and squamous cell carcinomas in XP patients occur in areas of the body with greatest sun exposure - the face, head and neck. The median age of onset of symptoms is between 1 and 2 years (Cleaver and Kraemer, 1995), with onset of skin cancer at approximately 8 years (Friedberg, 1995). Cancer of internal organs is also elevated to a rate 10-20 fold higher than in the general population (Kraemer, et al., 1984). The neurological

abnormalities associated with XP include mental retardation, progressive ataxia and deafness. Approximately 20-30% of XP patients develop such neurological abnormalities; this generally occurs in those with mutations in the *XPA*, *XPB*, *XPD* or *XPG* genes. Age of onset and the severity of neurological symptoms vary, but are characterised by progressive deterioration (Robbins, et al., 1991). It has been reported (Andrews, et al., 1978) that there is a correlation between the severity of neurological abnormalities and the degree of UV sensitivity of cultured skin fibroblasts, as tested by colony forming ability after exposure to UV radiation.

"Classic" XP cells are sensitive to cell killing by UV light and other DNA damaging agents because of their deficiency in NER. Approximately 80% of identified XP patients have defective cellular DNA repair, and are of the "classical" type of XP (van Steeg and Kraemer, 1999). The remaining 20% are designated as XP variants (XP-V) and are thought to have a defect in post-replication repair, with no obvious defects in NER, and only a slightly elevated sensitivity to UV radiation (Friedberg, 1995). Seven "classic" XP complementation groups have been identified and labelled XP-A through XP-G. Cells from each complementation group have a characteristic range of UV hypersensitivity, as assessed by colony forming assays. Host cell reactivation (HCR) studies of adenovirus (discussed in "E. Use of Adenovirus Vectors for DNA Repair Studies") showed that XP fibroblasts have a reduced HCR capacity, and this is attributable to the known repair deficiency in these cells (Day, 1974a;

Day, 1974b). Also, studies of reporter gene activity from UV-irradiated adenovirus showed reduced HCR in XP cells compared to normal cells (Francis and Rainbow, 1999). XP cells also exhibit chromosomal abnormalities following UV irradiation, including increased levels of sister chromatid exchange and formation of micronuclei (Friedberg, 1995), as well as an increased rate of mutagenesis following DNA damage. XP cells from complementation group C are deficient in GGR, but retain proficient TCR (Evans, et al., 1993; van Hoffen, et al., 1995; Venema, et al., 1991; Venema, et al., 1990b), which makes them informative for TCR studies. Cells from all other XP complementation groups appear to be deficient in both TCR and GGR (Evans, et al., 1993; van Steeg and Kraemer, 1999).

ii. Cockayne Syndrome (CS)

CS is an autosomal recessive disease, which is much more rare than XP (Cleaver and Kraemer, 1995). CS patients are also sun sensitive, but unlike XP patients, they do not have severe freckling or an increased rate of skin cancer. Other CS symptoms include short stature, progressive deafness, optic atrophy, mental deficiency, body malformations and progeria (the appearance of premature ageing) (Cleaver and Kraemer, 1995; Lambert and Lambert, 1991). It is thought that the clinical features of CS cannot simply be accounted for by a deficiency in NER, but can be better understood if CS is considered a

"transcription syndrome" (van Gool, et al., 1997b; Vermeulen, et al., 1994) (see "Role of TFIIH in cellular response to UV").

As in XP cells, cultured CS cells show a hypersensitivity to killing by UV radiation (Friedberg, 1995). CS cells fall into two complementation groups -- CS-A and CS-B. Mutations in the *XPB*, *XPD* and *XPG* genes may also give rise to a CS phenotype in a mixed syndrome with both CS and XP clinical features (Frit, et al., 1999). Some CS cell strains show an increased number of sister chromatid exchanges (SCE) after treatment with UV radiation, but there does not appear to be a correlation between levels of SCE and sensitivity to UV (Friedberg, 1995). CS cells differ from XP in NER assays in that repair of bulk genomic DNA is normal in CS cells. HCR study of UV irradiated adenovirus shows a decreased capacity of CS fibroblasts to reactivate the virus (Day, et al., 1981). In HCR studies of reporter gene activity from UV-irradiated adenovirus, CS cells show decreased HCR compared to normal cells, but they are not as deficient in HCR ability as some XP cells (Francis and Rainbow, 1999). CS cells have the converse defect to that in XP-C cells, i.e. normal GGR, but deficient TCR (van Hoffen, et al., 1993; Venema, et al., 1990a).

iii. Trichothiodystrophy (TTD)

TTD is an autosomal recessive disorder with a very low prevalence in the general population. The general clinical features of TTD include brittle hair,

ichthyosis (fish-like scaly skin), mental and physical retardation (reviewed in Friedberg, 1995). There is a subset of TTD patients that displays sun sensitivity. This syndrome is referred to as PIBIDS, which has the clinical features of *p*hotosensitivity, *i*chthyosis, *b*rittle hair and nails, *i*ntellectual impairment, *d*ecreased fertility and *s*hort stature (van Steeg and Kraemer, 1999). TTD is not associated with cancer.

Fibroblast cells from PIBIDS patients have similar abnormalities to cells from XP patients -- hypersensitivity to killing by UV radiation and reduced NER as evident in reduced unscheduled DNA synthesis (Cleaver and Kraemer, 1995). Patients with this syndrome appear to have mutations in the *XPB* or the *XPD* gene, with the possible involvement of a putative third gene (*TTDA*). XPB and XPD are both helicase components of the basal transcription factor TFIIH, and they are therefore essential for gene transcription and TCR. Fusion studies of cells from TTD families showed a defect in the *XPD* gene in 18 out of 20 families (Stefanini, et al., 1993a; Stefanini, et al., 1993b; Vermeulen, et al., 1994), which demonstrates that mutations in one gene can be associated with three distinct clinical phenotypes (XP, TTD and XP with CS). Mutation site analysis of the mutated *XPD* gene was done by Taylor and co-workers (1997), and showed that the clinical phenotypes of XP and TTD can be attributed to the mutation sites within the gene.

Mechanism for NER (the biochemistry)

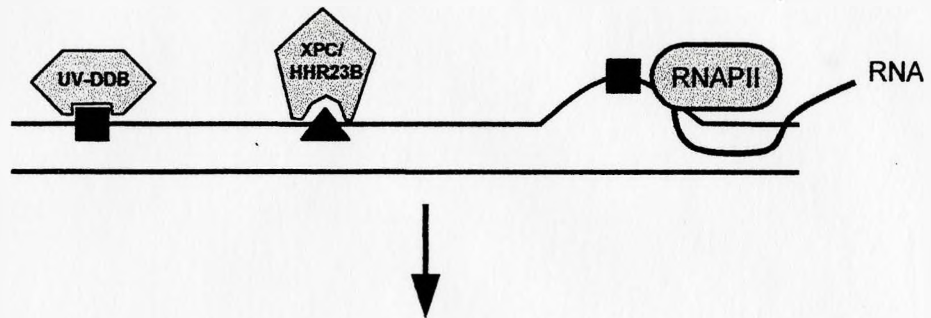
The function of NER is to remove bulky DNA adducts that impair transcription and replication. This is achieved by the orchestrated activity of a group of polypeptides in an ATP-dependent process that recognises lesions, locally unwinds the DNA at the damage site, excises the lesion by dual incisions, and re-synthesises the removed oligonucleotide.

The initial step in NER is the recognition of DNA damage. Research has shown that substrates that have a high affinity in recruiting the NER machinery have both a helical distortion and a modification in the DNA chemistry (reviewed in Wood, 1999). Damage recognition is thought to be the rate-limiting step of NER, since rare lesions must be located in the genome, and since the lesion recognition is carried out by a small subset of proteins (Sancar, 1996). There is no consensus in the literature as to which proteins function as the initial DNA damage recognition proteins, which may in part be due to a difference in specific affinities of the involved proteins for different types of lesions. XPA, XPC and replication protein A (RPA) have each been shown to have higher affinities for damaged DNA than for undamaged DNA (Petit and Sancar, 1999), but the relatively modest affinity of each of these proteins individually does not seem to account for the high specificity of the NER system. XPA has been shown to form a tight complex with RPA (He, et al., 1995; Li, et al., 1995; Matsuda, et al., 1995), and the binding of this complex to damaged DNA is greater than the binding of each protein individually. XPA binds mainly to (6-4) photoproducts,

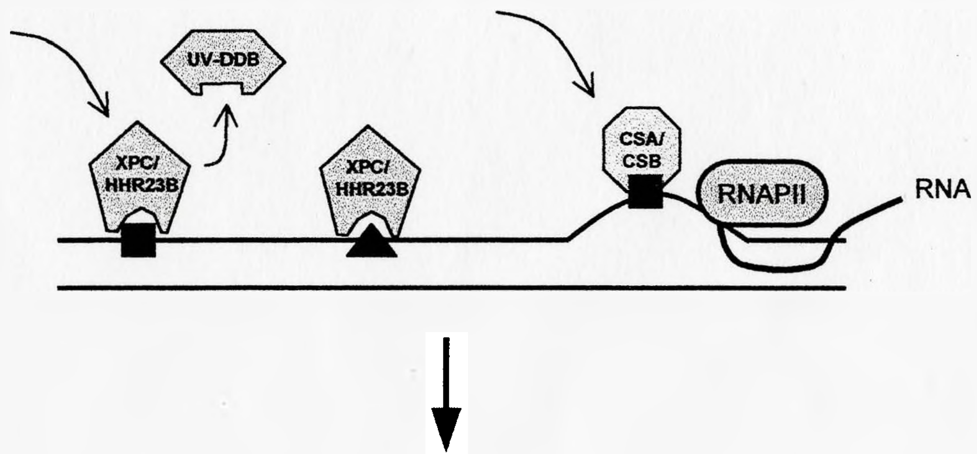
rather than pyrimidine dimers in UV-irradiated DNA (Friedberg, 1995). XPC coupled with HHR23B (as shown by biochemical fractionation) binds DNA with a relatively high affinity, with a slightly higher affinity for damaged DNA. Hwang and co-workers (1999) have proposed a GGR model that may clarify the roles of most of these molecules in lesion recognition and repair initiation (see Figure 1-2). This model suggests that an UV-damage DNA binding (UV-DDB) protein (mutated in NER-deficient DDB^- XPE cells) recognises CPDs with a 5×10^5 higher affinity for damaged DNA over undamaged DNA. In preparation for repair coupling, it is thought that XPC/HHR23B recognises UV-DDB bound to DNA, and replaces it at the lesion site. Although UV-DDB proteins can also recognise 6-4PPs, Hwang and co-workers (1999) propose that this type of damage may distort the DNA sufficiently to be directly recognised by the XPC/HHR23B complex, without the intermediate action of UV-DDB. Regardless of the initial lesion-recognising proteins, XPC/HHR23B is thought to help stabilise pre-incision subassemblies on nucleosomal DNA, and therefore to ensure proper assembly of the pre-initiation complex and recruitment of the nuclease subunits XPG and XPF/ERCC1 (Sancar, 1996). In the case of TCR, it is proposed that at the damage site the stalled RNA polymerase II is recognised and displaced by the CSA/CSB complex, which then recruits the core NER apparatus (see Figure 1-2).

Figure 1-2. Model for UV photoproduct recognition in nucleotide excision repair. (A) CPDs (square) are recognised by UV-DDB. Although 6-4PPs (triangle) can be recognised by UV-DDB, they may sufficiently distort the DNA to be directly recognised by XPC/HHR23B. Both photoproducts are recognised by arrest of RNAPII. (B) XPC/HHR23B recognises UV-DDB bound to DNA, possibly replacing it at the lesion and then partially opening the DNA. The CSA/CSB complex recognises and displaces the arrested RNAPII, opening the DNA at that site. XPC/HHR23B recruits the core NER apparatus for GGR, whereas CSA/CSB recruits the core apparatus for TCR. (Adapted from Hwang et al, 1999).

A. Damage Recognition



B. Preparation for repair coupling



Recruitment of core repair apparatus

Other researchers (Mu, et al., 1997; Wakasugi and Sancar, 1998) have shown that the first complex that forms with high specificity for DNA damage consists of XPC/ HHR23B, XPA, RPA (bound to factor HSSB), and TFIIH (containing the helicases XPB and XPD). This complex (see Figure 1-3) is referred to as Pre-Incision Complex 1 (PIC1[1]). Formation of this complex requires ATP, and the only two ATPases present are the XPB and XPD helicase components of TFIIH (Wood, 1999). This complex is relatively unstable, but is stabilised by the binding of XPG. It appears that at this point, upon recruiting XPG, the XPC protein dissociates from the pre-incision complex, since XPC is not detected in either PIC2 or PIC3 (Wakasugi and Sancar, 1998). This altered structure (PIC2) has high affinity and specificity for DNA damage, even though XPG does not play a direct role in damage recognition. The following step is the binding of XPF/ERCC1 to PIC2, which gives rise to the PIC3 structure. The order of assembly is specific, as XPF/ERCC1 cannot bind to PIC1 (Wakasugi and Sancar, 1998). Thus, XPG, which functions as the 3' nuclease in a later step, appears to recruit XPF/ERCC1 (the 5' exonuclease) to the complex, allowing for the dual incisions.

An incision is made on either side of the DNA damage releasing an oligomer 24-32 nucleotides in length. XPG makes the 3' incision at the 6th ± 3 phosphodiester bond 3' to the damage site, immediately followed by the 5'

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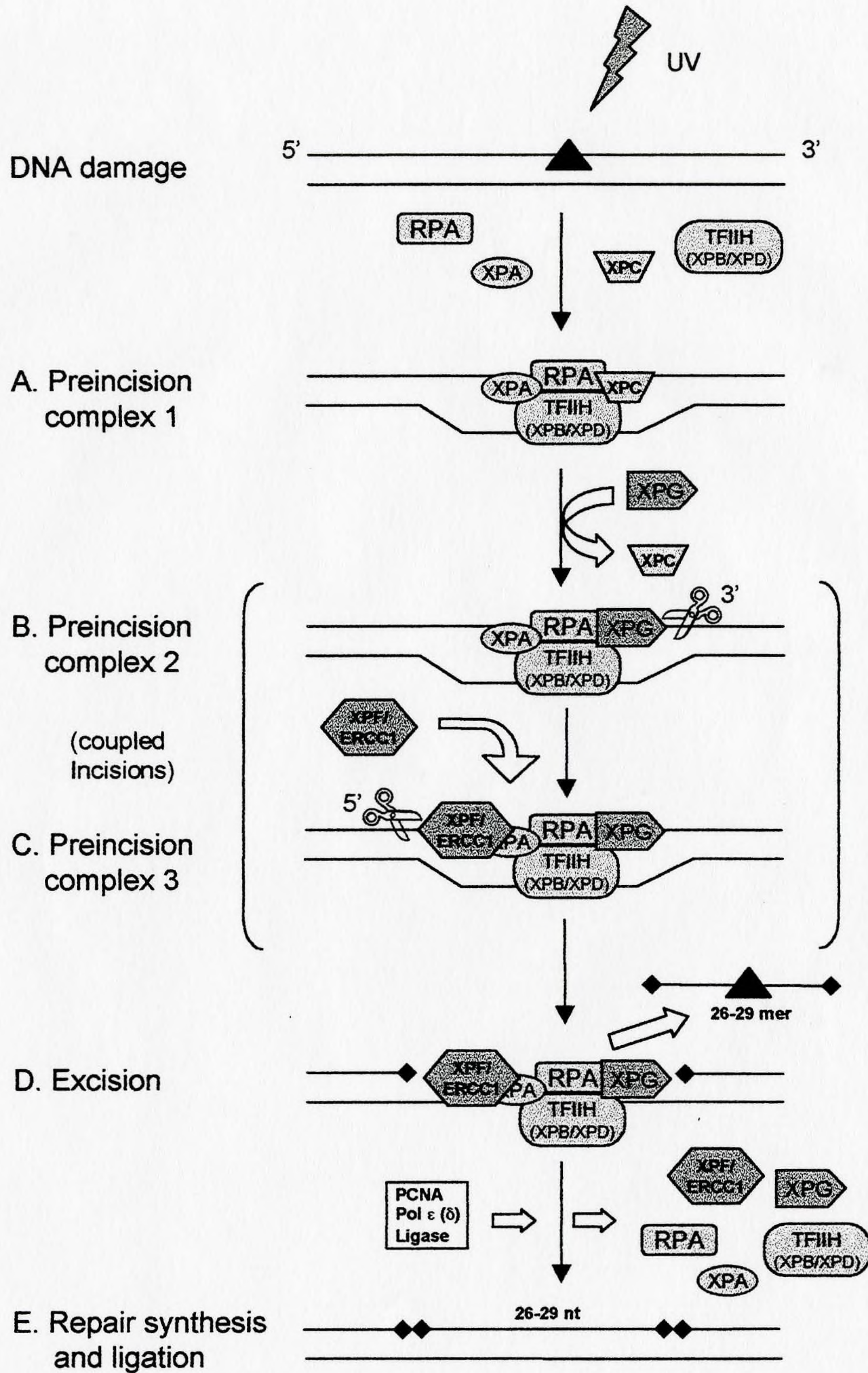
[1] In this thesis (and in the literature) PIC refers to pre-incision complex, and should not be confused with the use of the same acronym in the literature in reference to the pre-initiation complex of transcription.

Figure 1-3. Model for GGR in human cells. (**A**) After the initial lesion recognition step (see Figure 1-2) the recognition complex bound to the DNA recruits TFIIH (possibly bound to XPC) to form Pre-Incision Complex 1 (PIC1). TFIIH components XPB (3′-5′) and XPD (5′-3′) have helicase activities that unwind the DNA by ~20 base pairs around the damage. (**B**) XPG binds to PIC1, while XPC dissociates, leading to a more stable PIC2 complex. (**C**) PIC2 recruits XPF/ERCC1 to form PIC3. XPG makes the 3′ incision and then XPF/ERCC1 makes the 5′ incision. (**D**) Excised fragment containing DNA damage is released by exinuclease complex, leaving in place a "post-incision" complex. PCNA is loaded on DNA as Pol ε/δ clamp, replacing the post-incision complex with repair synthesis proteins. (**E**) the gap is filled and repair patch is ligated. (Adapted from Petit and Sancar, 1999).


UV
DNA damage
5'
3'
RPA
XPA
XPC
TFIIH (XPB/XPD)
A. Preincision complex 1
XPG
XPC
B. Preincision complex 2
3'
(coupled Incisions)
XPF/ ERCC1
5'
C. Preincision complex 3
26-29 mer
D. Excision
PCNA
Pol ε (δ)
Ligase
26-29 nt
E. Repair synthesis and ligation

incision catalysed by XPF/ERCC1 at the 20th ± 5 phosphodiester bond (reviewed in Petit and Sancar, 1999). Precise incision location appears to be influenced by the type of lesion and its sequence context. The release of the excised oligomer does not require any additional factors.

Completion of the NER process requires re-synthesis of the excised fragment and ligation of the newly synthesised patch. It has been shown that re-synthesis requires PCNA (Nichols and Sancar, 1992; Shivji, et al., 1992), and it is thought that DNA polymerases δ and/or ε are involved in re-synthesis, since PCNA functions as their polymerase clamp. Current data is consistent with both polymerases being involved in repair synthesis (Sancar, 1996). It is not known which of the three human DNA ligases is/are involved in the last step of NER - ligation.

Transcription-Coupled Repair (TCR)

Human cells have two general pathways for initiating NER. The global genome repair pathway (GGR) recognises and repairs damage throughout the genome, regardless of transcriptional activity. Transcription-coupled repair (TCR) specifically recognises and repairs lesions in the transcribed (template) strand of genes transcribed by RNA polymerase II (Hanawalt, 1994), at a rate faster than the GGR pathway (Bohr, 1987). It is believed that several features of transcription contribute to the increased rate of repair by TCR: 1/ open

chromatin structure of actively transcribed genes, making DNA more easily accessible to DNA repair factors, 2/ the transcribed strand may be more accessible to DNA repair proteins than the coding (non-transcribed) strand, and 3/ bulky lesions in the template strand can block RNA polymerase (reviewed in Sancar, 1996; Tornaletti and Pfeifer, 1996). The preferential repair of actively transcribed genes and the strand bias may be mechanistically related. This reflects NER activity that is coupled to transcription elongation by RNA polymerase II (RNAP II) at DNA lesions (Friedberg, 1996). The difference in the rates of repair by TCR versus GGR depends on both the cell strain and the type of damage. Chinese hamster ovary (CHO) cells were the first mammalian cells that were demonstrated to possess TCR, with a five-fold faster rate of repair of CPDs in the transcriptionally active DHFR gene than in the genome overall (Bohr, et al., 1985). It has since been shown that hamster cells are defective in the GGR of CPDs (Hwang, et al., 1998; Nichols, et al., 1996), and thus the marked difference initially observed may be due to poor repair of CPDs in the non-transcribed strand. Human cells also exhibit a faster rate of repair in an active gene than in the bulk genome (Bohr, 1987). A higher rate of repair of active genes versus bulk genomic DNA is seen in XP-C cells as well, but not in CS or XP-A cells. It is important to note that in human cells, although the **rate** of repair of the active *DHFR* gene is twice as fast as the bulk of the genome (4 and 8 hours after damage), after 24 hours no difference in the amount of damage repaired (85% repaired) is seen (Bohr, 1987). Within these preferentially repaired genes, there is also a bias for repairing the transcribed strand over the untranscribed

strand. Bohr and co-workers reported (1985) an 80% removal of lesions from the transcribed strand after four hours, with few lesions removed in the coding strand. In contrast, a study of human cells (Mellon, et al., 1987) showed significant repair of CPDs in both the transcribed and coding strands of the *DHFR* gene. Strand selectivity appears to be more prominent for repair of CPDs than for 6-4PPs (Bohr, 1991). This may be because 6-4PPs are efficiently removed by GGR. In general, for mammalian cells, the strand bias for NER observed in the transcribed strand is 2-5 fold higher than the rate in the non-transcribed strand (Friedberg, 1996).

Individuals with CS have cells defective in TCR (van Hoffen, et al., 1993; Venema, et al., 1991). Two genetic complementation groups have been defined for CS: CS-A and CS-B, and both genes have been cloned (Henning, et al., 1995; Troelstra, et al., 1990). CS cells show a moderate sensitivity to UV light, and have an increased rate of UV-induced mutations, particularly in the template strand (reviewed in Sancar, 1996). These cells are also slow to resume RNA synthesis after DNA damage (Mayne and Lehmann, 1982) and show phenotypic similarities to *E. coli* mfd$^-$ mutants (Selby and Sancar, 1993). MFD was shown to be the transcription-repair-coupling factor (TRCF) in *E. coli* (Selby and Sancar, 1993) and to function by direct interaction with RNA polymerase (Selby and Sancar, 1995). It is suspected that in human cells CSA and CSB proteins (at least in part) fulfil a function similar to that carried out by TCRF, since: the genetic evidence from CS mutant cells shows a necessity of CS for TCR, biochemical

evidence shows CSB associates with RNAP II (van Gool, et al., 1997a), and there is similarity between CS and *E. coli* TRCF (Vermeulen, et al., 1997). This has yet to be supported by direct evidence. It has recently been shown (Tu, et al., 1998) that CS-A cells are deficient in repair only during the elongation stages of RNAPII transcription, further implicating CSA as the mediator between transcription and repair. CSA does not directly bind RNAPII (Tantin, et al., 1997), but interacts with CSB and a subunit of TFIIH (Henning, et al., 1995).

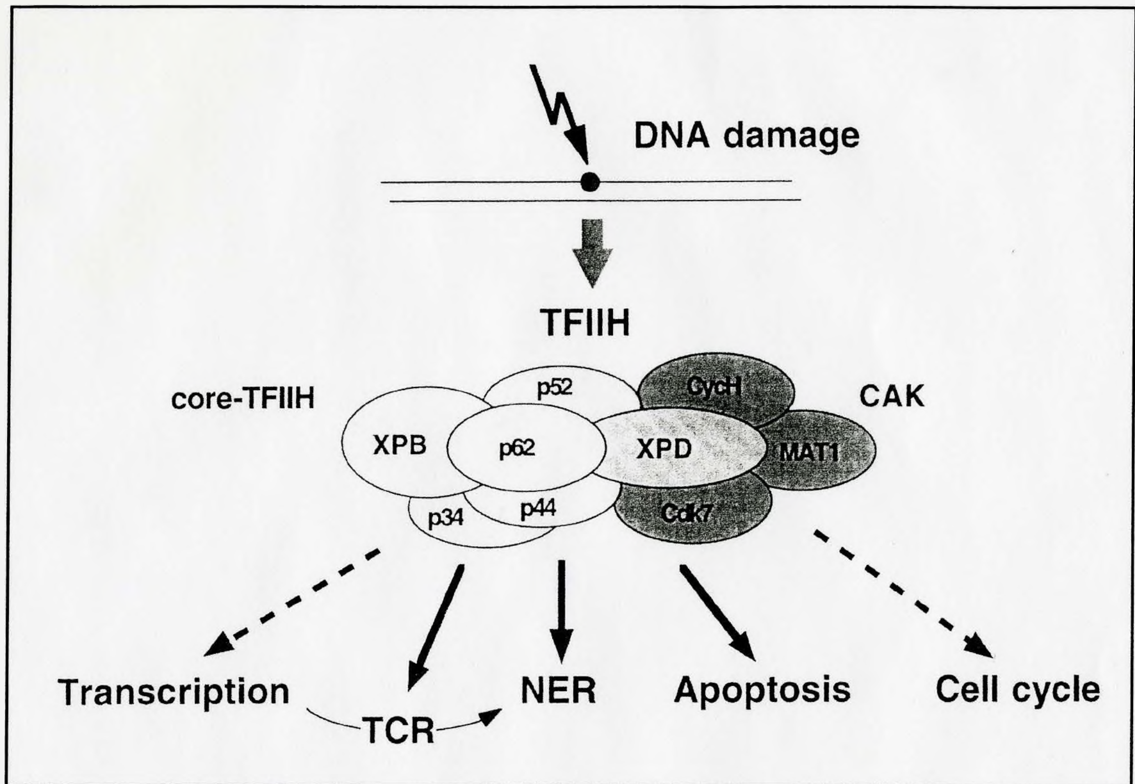
Role of TFIIH in Cellular Response to UV

TFIIH is a large multiprotein assembly, consisting of nine subunits, which is necessary for RNAP II transcription initiation (see Figure 1-4). It has several catalytic activities, including: DNA-dependent ATP hydrolysis, ATP-dependent DNA helicase activity, serine/threonine kinase activity (phosphorylating the C-terminal domain of the large subunit of RNAPII), and it is in turn regulated by the cyclin H subunit (Nikolov and Burley, 1997). In addition to the enzymatic activity provided by TFIIH, it is also an integral part of the protein scaffold necessary for NER (reviewed in Frit, et al., 1999).

i. The Connection Between NER and Transcription

There are three experimental observations that link nucleotide excision repair and transcription: 1/ actively transcribed genes are repaired faster than

Figure 1-4. TFIIH plays a key role in cellular response to DNA damage. Following DNA damage, the functions of TFIIH in transcription and cell cycle control would be downregulated, leading to transcription inhibition and cell cycle arrest. The function of TFIIH in DNA repair (particularly TCR) would be upregulated by DNA damage. Depending on the extent of damage, TFIIH could also play a role in apoptosis. (From Frit et al, 1999).


DNA damage
TFIIH
core-TFIIH
CAK
p52
CycH
XPB
p62
XPD
MAT1
p34
p44
Cdk7
Transcription
TCR
NER
Apoptosis
Cell cycle

inactive parts of the genome, 2/ the basal transcription factor TFIIH that is required for initiation of transcription by RNAPII is also involved in NER, and 3/ upon the induction of DNA damage, transcription is transiently repressed, suggesting co-ordination between the regulation of repair and transcription (Karmarkar, et al., 1998). TFIIH is not only involved in TCR, but also the GGR pathway, as certain viable TFIIH mutants show deficient NER activity (Araujo, et al., 2000; Gözükara, et al., 1994; Winkler, et al., 2000) or a complete abolition of NER activity (Karmarkar, et al., 1998). Thus, TFIIH is required for NER, and it may be important in co-ordinating repair with cell cycle progression, and signalling the cellular apoptotic response due to DNA damage.

Two subunits of TFIIH were initially identified as proteins necessary for NER: XPB (p89) and XPD (p80). These two proteins are helicases with opposite polarity that unwind the DNA helix locally to allow transcription by RNAP II and DNA repair. Mutations in *XPB* and *XPD* are associated with the diseases XP, XP/CS and TTD. The relative expression of *XPB* has been shown to be important in determining the XP/CS or TTD cellular phenotype (Riou, et al., 1999). Coin and co-workers (1999) showed that the *XPB* mutations in two XP-B/CS patients decrease the transcriptional activity of TFIIH by preventing promoter opening. They also showed that mutations in *XPD* affect the stoichiometric composition of TFIIH, which leads to partial reduction in transcription due to a weakened interaction between the XPD-CAK complex (discussed later) and the core TFIIH. This evidence supports the idea that XP-B

and XP-D patients are more likely to be affected by compound transcription-repair syndromes rather than general DNA repair disorders.

The deficiency in CS can be better understood if the clinical features are considered a result of a transcription syndrome, rather than just a DNA repair defect. Consistent with this model are the findings that CSB interacts with RNAPII (Tantin, et al., 1997; van Gool, et al., 1997a), that CSB stimulates transcript elongation (Selby and Sancar, 1997) and that transcriptional activity is reduced in CS cells (Balajee, et al., 1997; Dianov, et al., 1997). It has also been shown that CSA and CSB are not required for repair at transcription initiation sites, but are required for repair at elongation sites (Tu, et al., 1998). These investigators proposed a model where recruitment of TFIIH to the pre-initiation complex for transcription may obviate the need for CSA/CSB in repair. However, in the elongation stage (where TFIIH is no longer associated with RNAPII), CSA and/or CSB may promote the return of TFIIH to the downstream lesion within the transcribed strand of an active gene. A role for CSA and CSB has also been suggested in the ubiquitination of RNAPII, leading to RNAPII degradation, as a result of transcriptional arrest due to DNA damage (Bregman, et al., 1996; Ratner, et al., 1998). The RNAPII degradation may reflect a role of the CS proteins in a transcriptional arrest response, or it may be a transcription-coupled repair response that recruits the repair machinery to the site of the damage.

The transient suppression of transcription after exposure of cells to UV, as evident in a decrease in mRNA synthesis, is primarily due to direct blockage of RNAPII by UV-induced DNA lesions. However, there is evidence (reviewed in Mullenders, 1998) which suggests that a direct block of RNAPII is not the only mechanism in transcription inhibition. It is possible that the recruitment of TFIIH (necessary for initiation of transcription by RNAPII) to DNA repair sites throughout the genome, may limit the amount of TFIIH available for transcription initiation. This model is supported by evidence that shows p62 and p89 (TFIIH components), which are normally located at transcription sites, disperse throughout the nucleus upon treatment of the cells with UV (Karmarkar, et al., 1998). After some time, p62 and p89 return to their normal foci of activity in the nucleus. This restoration of localisation to the original sites is not seen in NER deficient XP-A cells, suggesting that in normal cells a limited amount of TFIIH is recruited for DNA repair and is not freed up for transcription until repair is complete.

ii. Cell Cycle Progression

The sequential activation and inactivation of a family of serine-threonine kinases - the cyclin-dependent kinases (CDKs) - regulates mammalian cell cycle progression. Upon exposure to DNA damaging agents, there is a cell cycle arrest, which allows DNA damage to be repaired prior to replication and mitosis. TFIIH has CDK-activating kinase (CAK) activity in a trimer (see Figure 1-4)

composed of the catalytic subunit Cdk7, its regulator Cyclin H, and Mat1 (which promotes a stable association between Cdk7 and Cyclin H). XPD is thought to anchor the CAK trimer to the core TFIIH (Rossignol, et al., 1997). The CAK trimer appears to have different substrate specificity depending on whether it is bound to TFIIH, or in free form (reviewed in Frit, et al., 1999). When bound to TFIIH, the CAK can phosphorylate the CTD domain of RNAPII, TFIIEa and TFIIFa, which promotes the transcription reaction. Unbound CAK does not perform these roles, but rather phosphorylates Cdk2, playing a role in cell cycle progression. Regulation of TFIIH function may depend on an equilibrium state between unbound CAK and that bound to TFIIH (Rossignol, et al., 1997; Yankulov and Bentley, 1997).

The tumour suppressor TP53 (formerly known as p53) is a multifunctional protein, with activity in regulation of gene expression, that accumulates in response to UV irradiation, and is associated with cell cycle arrest (thought to occur by transcriptional activation of *CDKN1A* [formerly known as *p21, WAF1*], which encodes a CDK inhibitor protein). The TP53 protein directly interacts with three subunits of TFIIH: p62 (Léveillard, et al., 1996; Xiao, et al., 1994), XPD (Léveillard, et al., 1996) and XPB (Wang, et al., 1995). The result of these interactions is inhibition of TFIIH helicase activity. Also, *in vitro* studies have shown that Cdk7/Cyclin H can phosphorylate TP53, which enhances its specific DNA-binding activity. A model (reviewed in Frit, et al., 1999) suggests TFIIH could be the link between transcriptional arrest at a DNA damage site and cell

cycle arrest via TP53 phosphorylation (activation). Regulation of TP53 activity is complex and occurs at many levels, including phosphorylation, nuclear localisation, transcription and translation. The functions of TP53 are many and, as yet, poorly understood, despite much evidence of its involvement in many cellular processes.

iii. Apoptosis

TFIIH has also been implicated in the apoptotic response of cells exposed to DNA damaging agents. In a study by Wang and co-workers (1996), it was shown that exogenous TP53 expression induced apoptosis in normal human fibroblasts, but not in XP-B or XP-D cells. The apoptotic response could be restored in these mutant cells by transferring the wild-type *XPB* or *XPD* gene back into the cells. Since XPB and XPD are components of TFIIH, this directly implicates TFIIH in the TP53 pro-apoptotic response after DNA damage.

Thus, the transcription factor TFIIH appears to be a key player in cellular response to UV light. Not only is it directly involved in transcription and DNA repair, it is involved in cell cycle regulation and apoptosis in response to UV light exposure. Although it is obviously not the only molecule involved in cellular stress responses, using TFIIH in all these capacities appears to be an efficient use of limited cellular resources.

D. Inducibility of Nucleotide Excision Repair

The best-characterised radiation-induced damage-response system is the "SOS repair" system in *Escherichia coli* (*E. coli*). This bacterial damage-response mechanism involves more than 20 genes that control DNA replication, excision repair, recombination, post-replication repair and mutagenesis. The "SOS repair" model has served as a basis for investigation of radiation-induced responses in mammalian cells. Both UV light and ionising radiation initiate a variety of responses in mammalian cells. There are sensors in the cell membranes that rapidly initiate a series of signal transduction cascades of protein kinases (Bender, et al., 1997). The immediate early response genes are turned on within minutes, and these include genes coding for transcription factors such as c-jun, c-fos and NF-κB. These transcription factors alter gene expression of the intermediate response genes (activated within several hours), with "sensors" that may recognise DNA damage such as UV photoproducts and double-strand breaks. DNA repair proteins may also fall into this category of response genes (Eckardt-Schupp and Klaus, 1999). The overall goal of these response systems is to improve cellular survival by affecting cell cycle arrest, DNA repair, DNA recombination (reviewed in Eckardt-Schupp and Klaus, 1999). In cases of extreme DNA damage that cannot be sufficiently repaired, these signalling pathways could also modulate a late response -- apoptosis.

There are several lines of evidence that suggest mammalian NER is an inducible process. Historically, the major approaches used in studying inducible DNA responses include: split-dose experiments using UV light, enhanced survival of UV-damaged virus, and enhanced reactivation of UV-damaged reporter gene activity. In split dose experiments, cells are treated with a low dose of "priming treatment" prior to a later challenging dose. Tyrell (1984) showed that exposure of non-dividing primary human fibroblast cells to an initial dose of UV-C light enhanced their ability to repair potentially lethal damage from the subsequent challenging UV stress. This enhancement develops over 2-3 days, and then decays over the following 2-3 days. However, this effect (observed by Tyrell, 1984) is thought to be at least partially independent of excision repair, since UV-induced response was also seen in repair-deficient XP-A cells (although at lower doses).

Enhanced survival of UV-damaged virus has been used widely to examine inducible NER. The survival of UV-irradiated nuclear replicating viruses is increased in mammalian cells treated (prior to infection) with UV light (Bockstahler and Lytle, 1970; Jeeves and Rainbow, 1983b), carcinogens (Lytle, et al., 1978; Sarasin and Hanawalt, 1978) and also with ionising radiation (Jeeves and Rainbow, 1979). The enhancement seen with carcinogen treatment (Lytle, et al., 1978) increases with delayed infection for 24-36 hours, and then decreases. The enhanced survival of herpes simplex virus (HSV) due to UV treatment of CV-1 monkey kidney cells is highest after 5 days (Bockstahler, et al., 1976) at

which time plaque formation increases exponentially with increasing UV fluence to the cells.

A third approach used to investigate inducible NER is reactivation of reporter gene expression. Using a plasmid expressing the *E. coli* chloramphenicol acetyltransferase (CAT) gene, Protic and co-workers (1988) showed that treatment of normal human fibroblast cells with UV radiation or mitomycin C (a DNA-damaging carcinogen) 24-48 hours before transfection, increased reactivation of damaged reporter in normal cells, but not in NER-deficient XP-A cells. In a follow-up study (Protic, et al., 1989) this group identified a UV damage-specific binding (DDB) protein induced by UV light, mitomycin C and aphidicolin (inhibitor of DNA polymerase α) in normal cells. This was not observed in XP-C cells, transformed XP-A cells, transformed XP-D cells, virus transformed repair-proficient cells, or certain XP-E cells. UV-DDB activity has been correlated with a cell's capacity to repair 6-4PPs in the whole genome (Otrin, et al., 1997), and it has recently been suggested that UV-DDB plays an important role in identifying CPDs for GGR (Hwang, et al., 1999). Viruses have also been used in enhanced reporter gene HCR studies, and this is discussed in the following section.

Further evidence of a mammalian SOS-type of response comes from work with treatment of cells with thymine dinucleotides (pTpT), which resemble sequences excised during repair of UV-induced photoproducts. It has been

shown that pTpT can mimic the effects of UV irradiation on pigmentation by increasing melanogenesis in normal human melanocytes (Eller, et al., 1996) and it can also enhance repair of UV-induced DNA damage in a TP53-mediated response (Eller, et al., 1997). Treatment of human keratinocytes with pTpT also enhances repair of damage induced by the chemical carcinogen benzo(a)pyrene in a response that upregulates TP53, PCNA and XPA proteins within 48 hours (Maeda, et al., 1999).

Another recent study (Chang, et al., 1999) showed that the drug emodin (a constituent of an herb used in Chinese medicine as cancer therapy) increases the repair of UV and cisplatin-induced DNA damage in human cells. An elevation in ERCC1 gene expression (but not XPF, XPA, RPA or XPG) and an increase in cellular uptake of Ca^{2+} accompanied this enhanced repair. Quinacrine mustard treatment of human fibroblast cells (Ye, et al., 1999) also induces an increased rate of repair of CPDs by GGR, with only a marginal increase in the repair of CPDs in the transcribed strand, compared to untreated cells.

E. Use of Adenovirus Vectors for DNA Repair Studies

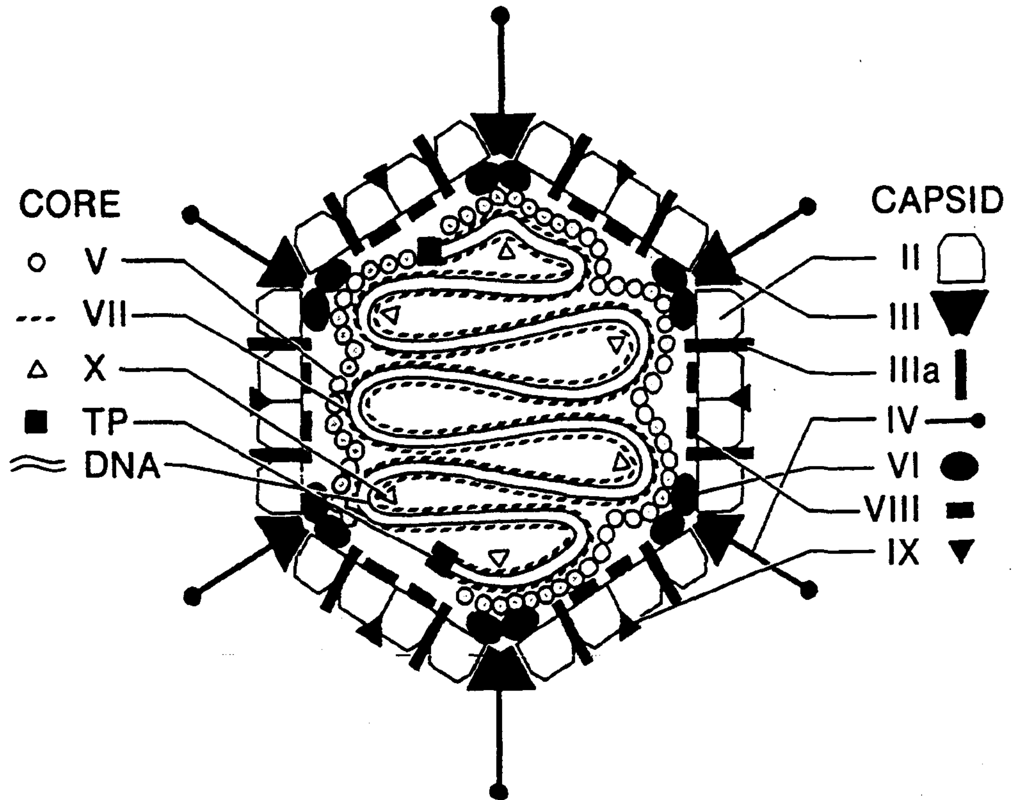
Host-Cell Reactivation Studies

Reactivation of UV-irradiated viruses has been used as a measure of excision repair capacity in both bacterial and mammalian cells (reviewed in

Defais, et al., 1983; Rainbow, 1981). "Host cell reactivation" (HCR) is a term used to describe the capacity of a given cell line to reactivate damaged virus. Usually, a dose-response curve is generated comparing some endpoint of viral infection from cells infected with damaged virus to cells infected with undamaged virus. Such relative survival curves are then used to compare the HCR capacities of various cells lines. In order for such studies to reflect host cellular DNA repair, viruses that depend entirely on host cell enzymes for DNA repair are most useful as probes. For UV-damaged virus, HCR-proficient cells are capable of eliminating the pyrimidine dimers from the viral genome (Boyle and Setlow, 1970) **as well as** from their own genome (Boyce and Howard-Flanders, 1964; Setlow and Carrier, 1964). Most studies of HCR in cultured mammalian cells utilise herpes simplex virus (HSV), adenovirus (Ad), or simian virus 40 (SV40) – all double-stranded DNA viruses that replicate in the nucleus of the cell (reviewed in Defais, et al., 1983).

HCR of UV-damaged adenovirus has been used to assess DNA repair in mammalian cells for many years. Adenoviruses are non-enveloped viruses that have a linear double-stranded DNA genome, which is contained in an icosahedral protein shell (Shenk, 1996; Stewart, et al., 1991) (see Figure 1-5). These viruses are convenient for study because they are easily propagated to a high titre, and they readily infect non-replicating cell strains in a highly synchronous manner. The capacity of cells to reactivate UV-damaged Ad can be assessed by a variety of endpoints, including: plaque-forming ability

Figure 1-5. Model of adenovirus virion. This cross-section shows the polypeptide components (designated by numerals) and the DNA component. TP represents the terminal protein. (From Stewart et al, 1991).


CORE
V
VII
X
TP
DNA
CAPSID
II
III
IIIa
IV
VI
VIII
IX

(Shenk, 1996; Stewart, et al., 1991), viral structural antigen formation (Jeeves and Rainbow, 1983b) and viral DNA synthesis (Arnold and Rainbow, 1996; Davis, et al., 1996). Such studies have shown that for XP cell strains, the relative amount of HCR correlates with the reduced excision repair capacity of these cells (reviewed in Defais, et al., 1983). HCR is also reduced in the repair deficient CS cells (Rainbow and Howes, 1982). More recently, a HCR technique utilising a recombinant non-replicating Ad5 vector has been applied in DNA repair studies, which assesses the expression of a reporter gene as an endpoint (Francis and Rainbow, 1999; McKay, et al., 1997a; McKay, et al., (in press); McKay and Rainbow, 1996). In this technique, the UV-damaged Ad5HCMVsp1*lacZ* vector, which expresses the bacterial *lacZ* gene efficiently in mammalian cells (Morsy, et al., 1993), is used to infect cultured cells and *lacZ* gene expression is quantified by a colourimetric reaction. Expression of *lacZ* from UV-damaged virus is compared to that from undamaged virus at 44 hours post-infection, indicating HCR capacity. It has been shown that several XP-C cell strains, XP-D, XP-F, XP-G and several CS cell strains exhibit a significant deficiency in HCR of UV-damaged reporter gene following infection of unirradiated fibroblast cells (Francis and Rainbow, 1999). Levels of HCR in CS cells are higher than in some XP-C cells, suggesting that the GGR pathway plays an important role in the repair of the transcriptionally active *lacZ* gene of Ad5HCMVsp1*lacZ* in untreated cells (Francis and Rainbow, 1999).

Enhanced Reactivation of UV-irradiated Virus

Several studies have looked at the inducibility of HCR, and shown that treatment of cells with UV light, ionising radiation, chemical carcinogens or heat shock prior to infection with an UV-irradiated virus enhances the survival of the virus. This effect is called "enhanced reactivation", and it has been shown for SV40 (Bockstahler and Lytle, 1977; Sarasin, 1978; Sarasin and Hanawalt, 1978), adenovirus (Bockstahler and Lytle, 1977; Francis and Rainbow, 1999; Jeeves and Rainbow, 1979; McKay, et al., 1997a; McKay and Rainbow, 1996) and several other viruses (reviewed in Abrahams, et al., 1984; Abrahams, et al., 1988; Defais, et al., 1983) in a variety of host cells, including human fibroblasts. Ionising radiation (X-rays and γ-rays) has been shown to enhance survival of UV-irradiated viruses (Bockstahler and Lytle, 1971; Bockstahler and Lytle, 1977). In particular, Jeeves and Rainbow (1979) showed that γ-irradiation of normal human fibroblasts lead to the enhanced survival of UV-irradiated adenovirus. These results were partially the basis for the work described in Chapter 2 of this thesis.

Recently, the HCR technique for UV-damaged adenovirus reporter gene expression has been applied in the study of enhanced reactivation. Francis and Rainbow (1999) showed that irradiation of cells with low UV fluences prior to infection with the Ad5HCMVsp1*lacZ* vector resulted in UV-enhanced reactivation (UVER) in normal and XP-C cell strains, but not in CS or other XP cells strains. In a similar study, McKay and Rainbow (1996) showed that heat

shock treatment to the cells prior to infection with this vector also enhanced HCR in normal and XP-C cell strains, but not in other XP or CS cell strains. This enhanced reactivation effect appears to be TP53-dependent (McKay, et al., 1997a). These studies suggest that UV light and heat shock enhance the repair of UV-damaged DNA through a mechanism that involves the TCR pathway. Chapter 2 of this thesis outlines a study of DNA repair using a quantitative PCR technique for HCR of Ad that provides additional evidence of inducible DNA repair mechanisms in normal fibroblast cells.

F. Polymerase Chain Reaction-Based DNA Repair Assays

The polymerase chain reaction (PCR) has recently been applied to the study of DNA damage and repair due to UV light (Chakalova and Russev, 1998a; Chakalova and Russev, 1998b; Chandrasekhar and Van Houten, 1994; Drouin and Therrien, 1997; Gao, et al., 1994; McCarthy, et al., 1996; McCarthy, et al., 1997; Tornaletti and Pfeifer, 1994), cisplatin treatment (Jennerwein and Eastman, 1991; Kalinowski, et al., 1992; Murata, et al., 1990) and exposure to ionising radiation (Ploskonosova, et al., 1999a; Ploskonosova, et al., 1999b). This type of assay is based on the fact that many types of DNA lesions can block amplification by *Taq* polymerase, and that the exponential nature of PCR amplification imparts a tremendous potential to quantify the remaining undamaged template from small samples. By applying Poisson analysis, lesion frequency can be determined for a quantitative comparison. This type of assay

has the advantage of being reproducible and precise in the investigation of highly localised genomic regions, even to the point of nucleotide resolution (Drouin and Therrien, 1997; Gao, et al., 1994; Tornaletti and Pfeifer, 1994) without the use of specific enzymes that recognise and cleave DNA adducts. The power of studies using QPCR to look at DNA repair in UV-irradiated human fibroblast cells is demonstrated in the specificity of results, such as: 1/ DNA repair within certain investigated genes is biphasic (McCarthy, et al., 1996) and 2/ the repair rate at individual nucleotides is highly variable and sequence dependent (Tornaletti and Pfeifer, 1994). We have taken advantage of the precision, reproducibility and ease of QPCR to investigate HCR of very specific genomic regions of the vector Ad5HCMVsp1*lacZ*. This is described in Chapter 3 of this thesis.

G. Current Work

This thesis summarises two research avenues: 1/ the development and application of a novel QPCR-based HCR assay for defined genomic regions of Ad5HCMVsp1*lacZ*, and 2/ the inducibility of HCR of the UV-irradiated *lacZ* reporter gene in Ad5HCMVsp1*lacZ* by γ-rays.

Chapter 2 of this thesis outlines the development and application of a novel QPCR technique for the investigation of HCR in human cells. This technique was developed in order to more directly assess HCR capacity of cells

by studying lesion frequency directly by PCR amplification, rather than expression of a reporter gene as was done previously (Francis and Rainbow, 1999; McKay, et al., 1997a; McKay and Rainbow, 1996). In this work, it was determined that HCR of Ad5HCMVsp1*lacZ* in untreated human fibroblast cells relies to a large extent on the GGR pathway, which is in accordance with previously published data (Francis and Rainbow, 1999; McKay, et al., 1997a; McKay and Rainbow, 1996). Also, it was determined that TCR plays a larger role in UV lesions removal in actively transcribed genes in Ad5HCMVsp1*lacZ*, than in less transcribed regions of the genome. This QPCR technique is particularly useful for investigating inducible HCR, since the endpoint examined is a direct representation of lesion frequency in the DNA. Thus, any variables related to reporter gene expression, e.g. RNAPII bypass of lesions, are not factors. Using this technique, we have shown that UV irradiation of normal human cells prior to infection with UV-damaged Ad5HCMVsp1*lacZ* leads to an enhanced rate of photoproduct removal in both the actively transcribed *lacZ* gene, and the less transcribed E4 region.

It has previously been shown (Jeeves and Rainbow, 1979) that irradiation of cells with γ-rays prior to infection with an UV-irradiated Ad virus enhances HCR of the virus in normal cells. In the study outlined in Chapter 3, the HCR technique for a reporter gene in the UV-damaged non-replicating Ad5HCMVsp1*lacZ* vector was applied to investigate the inducibility of HCR by

γ-rays. No significant enhancement of HCR by γ-rays was found in any cell strains/lines tested.

Chapter 2

A novel quantitative PCR assay for host-cell reactivation studies shows inducible removal of UV lesions in human cells.

A novel quantitative PCR assay for host-cell reactivation studies shows inducible removal of UV lesions in human cells.

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Abstract

We have developed a sensitive new PCR-based assay for determining DNA repair capacity in a broad range of mammalian cells. Many DNA lesions, including UV photoproducts, block DNA amplification by *Taq* polymerase, and the exponential nature of PCR imparts a tremendous potential for quantifying the remaining non-adducted DNA templates from small samples. Ad5HCMVsp1*lacZ* is a recombinant non-replicating adenovirus (Ad) containing the *lacZ* reporter gene under the control of the human cytomegalovirus (HCMV) immediate early promoter, inserted into the deleted E1 region of the viral genome. This virus is unable to replicate, but it can efficiently express the reporter gene in many types of mammalian cells, including human fibroblasts. Using quantitative PCR we have measured the induction and repair of UV photoproducts in a 2.6 kb region of the actively transcribed *lacZ* reporter gene encoded by Ad5HCMVsp1*lacZ* and in a 2.8 kb region of the endogenous E4 region of the virus, which is believed to have a decreased level of transcription. Primers flanking the regions were added to equal amounts of DNA extracted from cells infected with unirradiated or UV-irradiated Ad5HCMVsp1*lacZ* and each sample was amplified by PCR using radiolabelled nucleotides as substrates. PCR products were separated by agarose gel electrophoresis and quantified using a phosphorimaging system. Results show a simple exponential decrease in PCR product with increasing UV fluence to the virus, which corresponds to approximately 1 cyclobutane pyrimidine dimer per PCR-terminating lesion.

There was a significant removal of UV photoproducts after infection of normal human fibroblasts, but a reduced removal of lesions, compared to normal fibroblasts, after infection of nucleotide excision repair deficient fibroblasts derived from patients with xeroderma pigmentosum (XP) and Cockayne syndrome (CS). Results indicate that both the global genome repair (GGR) pathway and the transcription-coupled repair (TCR) pathway are important in lesion removal from the vector. Also, we have shown that UV-irradiation of normal human fibroblasts prior to infection with the vector results in an enhanced rate of removal of photoproducts from Ad5HCMVsp1*lacZ*, demonstrating that previous reports of enhanced host cell reactivation of vector regions are indicative of a genuine inducible DNA repair response in human cells.

1. Introduction

UV radiation induces DNA lesions, including cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PP), which are primarily repaired by nucleotide excision repair (NER) in mammalian cells. Characterisation of NER has shown two interrelated, but separate pathways: (1) global genome repair (GGR) – which homogeneously repairs DNA damage in the entire genome, and (2) transcription-coupled repair (TCR) – which preferentially repairs damage in actively transcribed genes. Disruptions of these DNA repair processes have been shown to be associated with mutagenesis and carcinogenesis.

The genetic disorder xeroderma pigmentosum (XP) is an autosomal recessive disorder, resulting in hypersensitivity to sunlight (particularly the UV component), and in a predisposition towards the development of skin cancers (Friedberg, 1995). XP is characterised by deficiencies in NER (Cleaver, 1968), which may be caused by mutations in any one of at least seven distinct NER genes, giving rise to the complementation groups XP-A through XP-G. XP group C cells have been shown to be proficient in TCR (Venema, et al., 1991), but deficient in GGR, whereas the other XP complementation groups are deficient in both TCR and GGR. Cockayne syndrome (CS) is also an autosomal recessive disease, which results in hypersensitivity to UV light, but affected individuals do not have an increased rate of skin cancer (Cleaver and Kraemer, 1995). CS is divided into two complementation groups: CS group A and CS group B. Cells

from CS patients are also deficient in NER, but in contrast to XP cells, they are proficient in GGR and deficient in TCR (Venema, et al., 1990).

Host cell reactivation (HCR) of a damaged reporter gene introduced into cultured mammalian cells has been used to assess cellular DNA repair capacity. Ad5HCMVsp1*lacZ* is a recombinant non-replicating adenovirus vector that is unable to replicate in human fibroblasts in absence of exogenous E1 gene expression due to a deletion in the E1 region. This vector has the *lacZ* reporter gene under the control of the HCMV immediate early promoter inserted into the deleted E1 region, and it can efficiently infect and express the gene in human fibroblast cells (Morsy, et al., 1993). Since this virus is unable the replicate, reporter expression from an UV-damaged virus is dependent on repair of the transcribed strand of the gene. HCR of β–galactosidase (β–gal) activity for UV-irradiated Ad5HCMVsp1*lacZ* has been shown to be reduced in XP and CS cell strains, relative to normal diploid fibroblasts (Francis and Rainbow, 1999; McKay, et al., (in press)).

Treatment of cells with UV light, γ-radiation or heat shock enhances reactivation of DNA-damaged virus (Bockstahler, et al., 1976; Jeeves and Rainbow, 1979a; Jeeves and Rainbow, 1979b; McKay and Rainbow, 1996; Piperakis and McLennan, 1984). It has been suggested that UV-enhanced reactivation (UVER) of UV-damaged reporter genes results from induction of DNA repair mechanisms (Francis and Rainbow, 1999; Protic, et al., 1988; Smith,

et al., 1995). Brown and Cerutti (1989) showed that UV-enhanced reactivation of UV-damaged simian virus 40 (SV40) resulted from enhanced restoration of early viral gene function, suggesting enhanced repair or bypass of DNA lesions in these early expressed genes. UVER of β-gal activity from recombinant Ad5HCMVsp1*lacZ* is TP53-dependent (McKay, et al., 1997) and the induced response appears to involve the TCR pathway (Francis and Rainbow, 1999; McKay, et al., 1997). To help ascertain whether UVER of Ad5HCMVsp1*lacZ* truly reflects an increase in DNA repair (rather than enhanced RNA polymerase II bypass of lesions), we have developed a quantitative PCR assay to investigate HCR of this vector which does not rely on functional gene expression as an endpoint.

Recently, several quantitative PCR (QPCR)-based techniques have been used to study DNA damage and repair in mammalian cells (Chakalova and Russev, 1998; Drouin and Therrien, 1997; McCarthy, et al., 1996; McCarthy, et al., 1997). The basis for these assays is the blockage of DNA polymerase from *Thermus aquaticus* (*Taq* polymerase) by DNA lesions (both CPDs and 6-4PPs) generated by irradiation with UV light (Wellinger and Thoma, 1996). The exponential nature of PCR imparts a tremendous potential to quantify remaining non-damaged DNA templates from small samples. Previous studies using these techniques focused on DNA repair of endogenous mammalian genes after irradiation of cells with UV light. These techniques have the limitation of the inability to examine basal endogenous DNA repair mechanisms in cells, i.e.

without exposing cells to damaging agents such as UV light. A second limitation of these techniques is the inability to examine inducible repair of DNA damage from one type of agent in cells treated with another agent, e.g. examining UV damage repair in cells treated with ionising radiation.

This report details a novel QPCR technique for assessing UV photoproduct induction and repair using HCR of Ad5HCMVsp1*lacZ*. This E1-deleted adenovirus vector is able to efficiently infect many mammalian cell types (including human cells) and to express the *lacZ* reporter gene (Morsy, et al., 1993). It has been reported that in E1-deleted adenovirus vectors transcription of the E4 region is impaired (Berk, et al., 1979; Jones and Shenk, 1979; Nevins, 1981 ; Lusky, et al., 1998). We examined HCR of a 2.6 kb fragment of the actively transcribed *lacZ* gene (inserted into the deleted E1 region of Ad5), and a 2.8 kb fragment of the endogenous E4 region of the virus in unirradiated normal human fibroblast cells, and NER-deficient XP-C, XP-A, and CS-B cells. We show that UV irradiation of viral DNA results in a fluence-dependent uniform decrease in PCR product. There is significant removal of photoproducts from the two fragments observed in Ad5HCMVsp1*lacZ* after infection of normal human fibroblasts, whereas the removal of damage was significantly less than normal following infection of NER-deficient XP and CS cells. Results indicate that both GGR and TCR are important in NER of Ad5HCMVsp1*lacZ* in untreated cells. We also used this technique to assess repair of the two fragments after infection of UV pre-irradiated normal human fibroblasts cells. We found an increased rate

of lesion removal from the vector in irradiated normal human cells as compared to unirradiated cells for both regions of the vector examined.

2. Materials and Methods

2.1 Cells and Virus

The normal human fibroblast strains GM09503, GM08399A, GM00037F, IMR90 (lung fibroblast) and the NER-deficient XP-C GM00030A (XP3BE) and GM00510 (XP1PW), XP-A GM00082 and CS-B GM01428 (CS7SE) were obtained from the NIGMS Human Genetic Cell Repository (Camden, NJ). The Ad E1 region transformed human 293 cells (Graham, et al., 1977) for propagating virus were obtained from Dr. F. L. Graham (McMaster University). Cultures were maintained in α-minimal essential medium (Gibco BRL), supplemented with 10% foetal bovine serum (Sigma F-4135) and 1% antibiotic/antimycotic (100μg/ml penicillin G sodium, 100μg/ml streptomycin sulphate and 250 ng/ml amphotericin B (Gibco BRL)) in a humidified atmosphere of 5% CO_2 at 37°C.

The vector Ad5HCMVsp1*lacZ* is a non-replicating recombinant human adenovirus expressing the bacterial *lacZ* gene under the control of the HCMV immediate early promoter. This virus expresses β-gal in various human cell lines (Morsy, et al., 1993), and to ensure that it was not replicating a fresh stock was

grown up after plaque purification as described previously (Graham and Prevec, 1991).

2.2 UV Irradiation of Virus and Cells

UV irradiation of adenovirus has been described previously (Bennett and Rainbow, 1988). Briefly, viral suspensions in phosphate buffered saline (PBS) supplemented with 1% $MgCl_2$ and 1% $CaCl_2$ (PBS^{2+}) were irradiated on ice, with continuous stirring, using a General Electric germicidal lamp (G8T5) at an intensity of 2 J/m^2s at predominantly 254 nm. Aliquots of irradiated virus were removed sequentially during irradiation to vary the fluence, and diluted appropriately with PBS^{2+}. For UV-enhanced reactivation experiments, confluent cells were irradiated by the same UV source 24 hours after seeding, in 200 µl unsupplemented PBS, at a fluence rate of 1 J/m^2s, for a total fluence of 10 J/m^2.

2.3 Infection of Cells and DNA Isolation

Cells were seeded in 24-well plates (Falcon) at a density of 1×10^5 cells per well and left for 24 hours. Confluent cells were infected with either non-irradiated or UV-irradiated virus in a total volume of 200 µl (in PBS^{2+}) at a multiplicity of infection (MOI) of 50 plaque-forming units (pfu) per cell. Following viral adsorption for 60 minutes at 37°C, the viral suspension was

aspirated and replaced by 1 ml of supplemented growth medium until cell lysis. For UV-enhanced reactivation experiments, cells were infected with virus 24 hours after UV irradiation.

For the t=0 h time point samples, immediately after addition of virus to the cells, an equal volume of a pronase digestion solution (1mg/ml pronase (Gibco BRL) in 0.01M Tris-HCl pH 7.5, 0.01M EDTA, 1% SDS) was added, to lyse the cells. For all other time points, media was aspirated at the appropriate time after infection, 200 µl of PBS^{2+} was added, and then an equal volume of pronase solution was added. Pronase digestion was allowed to proceed for 3-4 hours at 37°C. Digested samples were stored at –20°C until DNA extraction.

Total DNA was isolated using a volume-controlled modified phenol/chloroform extraction procedure (Sambrook, et al., 1989). Briefly, one volume of phenol/chloroform/isoamyl alcohol (25:24:1 (Gibco BRL)) was added to the sample, and the aqueous layer removed. Then, a back extraction (Sambrook, et al., 1989) was performed by adding one volume of TE to the phenol-containing phase, again removing the aqueous layer and adding this to the first aqueous aliquot. This was followed by a chloroform/isoamyl alcohol (24:1) extraction of the combined aqueous solution, and ethanol precipitation. The DNA was resuspended in 50 µl TE, and an appropriate dilution of this stock was used for quantitative PCR.

2.4 Quantitative PCR

The sequence of the Ad5HCMVsp1*lacZ* vector (provided by Dr. F. L. Graham) was used for primer design. Four oligonucleotides (MOBIX Facility, McMaster University) were used as two pairs of PCR primers (see Figure 2-1) – one amplifying the *lacZ* gene inserted into the deleted E1 region, and one amplifying the adenoviral E4 region. The *lacZ* gene primers 5'-GGT GTG GGC CAT AAT TCA ATT C-3' (primer A, positions 782 to 803 in vector sequence) and 5'-ATA CTG TCG TCG TCC CCT CAA A-3' (primer B, 3385 to 3364) were used to amplify a 2604 bp fragment. The E4 region primers 5'-TAC TGG AGC CTT GGG TTT TGA T-3' (primer C, 32045 to 32066) and 5'-GGT TGA AGG TGC TGG AAT GTT T-3' (primer D, 34862 to 34841) were used to amplify a 2818 bp fragment. In initial experiments, the two regions were amplified independently, but in later work a more efficient multiplex PCR reaction with both pairs of primers was used. A typical 100 μl reaction in a 0.2 ml thin-walled PCR tube contained 10 μl of appropriately diluted template DNA, and 90 μl of PCR master mix (10 μl 10 x PCR buffer (Gibco BRL), 0.5 mM each primer, 200 μM each dNTP (Amersham Pharmacia Biotech), 1.5 mM $MgCl_2$, 2.5 U PlatinumTaq (Gibco BRL), 0.15 μl ^{33}P-dCTP and water (to a volume of 90 μl per reaction)). One master mix stock was used for all PCR reactions in each experiment. The samples were mixed and placed in the thermal cycler (M.J. Research PTC-100 with HotBonnet). The thermal cycler was programmed to operate with a 30 sec, 94°C denaturation step, followed by 24-26 reaction cycles of 1.5 min at 94°C, 1 min at 54°C and 2 min at

72°C, with an additional final 2 min, 72°C elongation step. These reactions were kept at 4°C until further analysis. PCR product was measured by quantitation of incorporated radiolabelled deoxyribonucleotide (^{33}P-dCTP). Specifically, 25 μl of PCR product and 5 μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) were separated by agarose gel electrophoresis (0.8% gel) in 0.5 x TBE (45 mM Tris-borate and 1mM EDTA). The gels were dried (BioRad Model 583 gel dryer) and then exposed to and quantitated using a phosphorimager system (Molecular Dynamics).

In order for PCR to be quantitative, PCR amplification must be measured in the exponential phase of the reaction (Morrison and Gannon, 1994). Since efficiency of viral uptake differs in the various cell types under various conditions (such as irradiated vs. unirradiated cells), and the amount of adsorbed virus is less that the total amount of virus used for infection, we needed to ensure that PCR amplification was indicative of initial amounts of undamaged DNA. Thus, a two-fold dilution series was carried out for aliquots of each unirradiated sample and was subjected to PCR in parallel with experimental samples (Figure 2-2A). The resulting calibration curve (Figure 2-2B) was used to determine the reduction in PCR product following UV exposure to the virus. For each of the experimental samples, duplicate PCR reactions were carried to determine the relative PCR product amount. A typical multiplex QPCR experiment with serial dilution is shown in Figure 2-3.

3. Results

3.1 The rationale

The basis of PCR assays for DNA repair is that UV photoproducts block DNA amplification by *Taq* polymerase (Wellinger and Thoma, 1996), and that the exponential nature of PCR imparts a tremendous sensitivity to the quantitation of remaining non-adducted DNA templates from small samples. Using a convenient multiplex PCR reaction to amplify several genomic regions simultaneously eliminates differences that may occur as a result of independent PCR amplifications, and also reduces total resources used. HCR techniques for DNA repair have the advantage of independent irradiation of DNA and host cells, which is advantageous when looking at inducible DNA responses. In this study, we have combined the attributes of multiplex QPCR and HCR by using a non-replicating Ad vector as a template for a PCR technique that measures repair of regions in the viral genome. Comparisons can be made with results that were previously obtained by examining HCR of reporter gene expression from the same viral vector.

3.2 UV damage to the virus

Unirradiated and UV-irradiated Ad5HCMVsp1*lacZ* virus was used to infect normal skin, normal lung, XP-C, XP-A and CS-B fibroblast strains. QPCR

was used to amplify a 2.6 kb region of the *lacZ* gene and a 2.8 kb region of the endogenous E4 region of this vector. For each experiment, a dose-response curve (t=0 hours samples) was obtained by plotting relative survival of PCR product amount after irradiation. A plot of the pooled survival curves of PCR product with increasing UV fluence (for all experiments) is shown in Figure 2-4. UV irradiation of Ad5HCMVsp1*lacZ* resulted in a simple exponential decrease in PCR product with increasing UV fluence for both the *lacZ* and E4 fragments. The fluence required to reduce the amount of PCR product to 37% of that for unirradiated virus (D_0) was calculated (using $SF = e^{-D/D_0}$, where SF is the surviving fraction of PCR product) to be 128.5 J/m^2 for the *lacZ* fragment (R^2>0.99) and 88.0 J/m^2 for the E4 fragment (R^2>0.99) from fitted simple exponential functions.

3.3 Host cell reactivation of PCR product in normal and deficient cell lines

We have used QPCR to examine HCR of an UV-damaged Ad5HCMVsp1*lacZ* vector in NER-proficient and NER-deficient cell strains. Typical survival curves for PCR product from irradiated Ad5HCMVsp1*lacZ* following infection of normal, XP-C, XP-A and CS-B human fibroblast strains are shown in Figure 2-5. It can be seen that relative survival increases significantly over time for normal fibroblast cell strains, whereas NER-deficient cell strains show reduced relative survival over time compared to normal cells. For the XP-C cell strain GM00030A (Figure 2-5, panel C) relative PCR product actually

decreased with time. Although we are not certain of the cause, it is possible that there is some degradation of UV-damaged vector over time, which would only be seen in extremely NER-deficient cells. Mean relative survival of PCR product was determined by pooling data from all experiments for each cell type, and reduction in PCR product was converted to number of lesions as follows.

By applying the principles of the Poisson distribution, the number of lesions induced or remaining can be calculated for each UV dose at each time point (average # lesions/fragment = - ln (SF)). By comparing the number of lesions at t=0 h and at a later time point, one can calculate the percentage of lesions removed up until that time point. By averaging the lesions removed for all the UV doses (100, 200, 300 J/m^2) at a time point, a single mean value can be determined that represents lesion removal. Such pooled lesion removal points were plotted for the E1 (Figure 2-6A) and E4 (Figure 2-6B) regions for all cell strains examined. The normal fibroblast cell strains were most proficient at lesion removal in both regions of the Ad5HCMVsp1*lacZ* genome, whereas the NER-deficient cell strains exhibited a significant deficiency in lesion removal from the UV-damaged Ad vector. The XP-C cell line, GM00030A, was the most deficient in lesion removal from both regions of the Ad5HCMVsp1*lacZ* vector compared to normal cells, with lesion removal being not significantly different from 0 % at all time points. Another XP-C cell strain (GM00510) and an XP-A strain were also deficient in reactivation of PCR product compared to that in the normal strains. The CS-B cell strain, GM01428, also showed decreased removal

of UV lesions from the Ad vector compared to normal cells. We observed a significant difference ($t(3) = -2.95$, $P=0.021$, one-tailed *t*-test) in lesion removal between the actively transcribed *lacZ* gene and the less-transcribed E4 fragment in this TCR-deficient cell strain. Repair was reduced in the *lacZ* fragment compared to repair in E4 at t=36 h by a factor of 1.49. At t=48 h repair was 1.51-fold lower in *lacZ* than in E4 (data not shown), but the difference was not statistically significant ($t(3)= -0.38$, $P=0.72$, one-tailed *t*-test).

3.4 *UV-enhanced reactivation in normal cells*

UV-irradiated and unirradiated Ad5HCMVsp1*lacZ* were also used to infect untreated and UV-treated (fluence of 10 J/m^2) normal lung fibroblast cells (IMR90). A typical experiment is shown in Figure 2-7. Lesion removal was calculated for the pooled data of the two experiments, and is shown in Figure 2-8. At t=24 h there was a significantly higher percentage of lesions removed from the vector in the UV-irradiated cells than in the unirradiated cells. At t=36 h there was no significant UVER over levels of unirradiated cells. Since UVER is present at 24 hours after infection, at a level similar to that of basal HCR at 36 hours, and there is no UVER at t=36 h, this suggests that there is a difference in the **rate** of repair between unirradiated and irradiated normal cells.

4. Discussion

UV irradiation of Ad5HCMVsp1*lacZ* resulted in single exponential dose-response curves for both the PCR amplified *lacZ* (inserted into the deleted E1 region) and the E4 region fragments. The fluence required to reduce the amount of PCR product to 37% of that for unirradiated virus (D_0 value) was calculated (using $SF = e^{-D/D_0}$, where SF is the surviving fraction of PCR product) to be 128.5 J/m^2 for the *lacZ* fragment ($R^2 > 0.99$) and 88.0 J/m^2 for the E4 region fragment ($R^2 > 0.99$). From these values, we calculated PCR-terminating lesion induction to be 2.99×10^{-6} lesions/nucleotide/J/m^2 in the *lacZ* fragment, and 4.04×10^{-6} lesions/nucleotide/J/m^2 in the E4 fragment. Previous reports (Rainbow and Mak, 1973) have calculated that the fraction of thymine in the adenoviral genome present as dimer after UV irradiation was 1.9×10^{-5} m^2J^{-1}. By correcting this value for the thymine content of the two fragments we studied (*lacZ* and E4) and taking into account formation of **all** CPDs induced by UV (as described in Setlow and Carrier, 1966), we calculate CPD induction in the *lacZ* fragment at a rate of 3.33×10^{-6} CPD/nucleotide/J/m^2, and in the E4 fragment at a rate of 3.79×10^{-6} CPD/nucleotide/J/m^2. This corresponds to 1.10 CPD per PCR-terminating lesion in the amplified *lacZ* fragment, and 0.94 CPD per PCR-terminating lesion in the E4 fragment (mean=1.02). Therefore, the frequency of induction of PCR-terminating lesions closely corresponds to CPD induction in adenovirus vectors.

Previous work from our lab group investigated HCR of UV-damaged Ad5HCMVsp1*lacZ* in normal and NER-deficient human fibroblast cells using expression of a UV-damaged reporter gene from this vector as the endpoint (Francis and Rainbow, 1999; McKay, et al., 1997). These studies showed that HCR in untreated cells was significantly reduced in NER-deficient cell strains compared to that in normal strains, and that both the GGR and TCR subpathways contribute to the repair of the actively transcribed strand of the *lacZ* gene. In the current work, the TCR-proficient but GGR-deficient (TCR^+GGR^-) XP-C cells, the TCR^-GGR^+ CS-B cells and the TCR^-GGR^- XP-A cells all showed a lower lesion removal capacity than normal cells, providing evidence that both GGR and TCR are used in HCR of both the actively transcribed *lacZ* gene and the less-transcribed E4 region. In normal skin fibroblasts, there was no difference in the repair between the actively transcribed *lacZ* gene and the less-transcribed E4 region, indicating no preferential repair in active genes of the Ad vector. In the XP-C and XP-A cells, repair of the E4 region was similar to that of E1 as well. In contrast, the CS-B cells showed significantly lower repair rate of the *lacZ* gene compared to the E4 region. Cockayne syndrome cells are deficient in TCR, which is driven by stalled transcripts that act as a signal to target repair enzymes to DNA lesion sites (Christians and Hanawalt, 1992; Leadon and Lawrence, 1991). It has been suggested that CSA and CSB play a role in re-recruiting TFIIH to the repair complex when RNAPII is arrested at DNA lesions (Tu, et al., 1998). Additionally, CSA and CSB are required for ubiquitination and targeting of RNAPII for proteosomal degradation (Bregman, et al., 1996; Ratner, et al., 1998).

In CS cells, both re-recruiting TFIIH and ubiquitination may be inhibited due to a mutation in one of the CS genes, thus promoting a persistence of RNAPII at the lesion site where it pauses. Since both TCR and GGR repair lesions in the actively transcribed strand (Mullenders, et al., 1998), a persistent RNAPII stall at a lesion site in CS cells may inhibit repair of lesions that could otherwise potentially be substrates for the GGR subpathway.

Other host-cell reactivation studies in human cells have provided varied results with respect to the involvement of TCR and GGR in repair. Many of these studies utilised reactivation of expression from an UV-damaged reporter gene as an endpoint, which is believed to represent the cellular capacity to remove transcription-blocking lesions from the vector. HCR studies of plasmid carrying the chloramphenicol acetyltransferase (CAT) reporter gene showed decreased HCR in NER-deficient XP cells (Barrett, et al., 1991; Lehmann and Oomen, 1985; Protic-Sabljic and Kraemer, 1985) and CS cells (Barrett, et al., 1991; Klocker, et al., 1985) compared to normal cells. Barrett and co-workers (1991) showed that CS cells are proficient in repairing non-CPD UV-induced lesions (6-4PP) in the actively transcribed strand of the gene, but deficient in CPD repair in this same region. In a study of CPD removal from plasmid which carried the *lacZ* gene (transfected into normal cells), CPD removal was not more rapid in the transcribed stand of the active gene than in the non-transcribed strand, nor faster from the active gene than from the rest of the (Ganesan and Hanawalt, 1994). In a follow-up study, Ganesan and co-workers (1999) compared CPD removal from

UV-damaged plasmid carrying the *lacZ* gene under the control of the CMV promoter in normal and NER-deficient cells. Observations of CPD removal in the transcribed strand of *lacZ* versus the non-transcribed strand were inconclusive. However, a comparison of lesion removal from full-length plasmid with that in the transcribed gene showed similar CPD removal in the two regions in transfected XP-C cells, but lower lesion removal in the active gene versus the rest of the plasmid after transfection of CS cells. Thus, the results of plasmid HCR studies, previous results from our lab with Ad5HCMVsp1*lacZ* (Francis and Rainbow, 1999), and the current results are consistent with a model for human cells in which both TCR and GGR play a role in the repair of actively transcribed genes in an exogenous vector, with no significantly enhanced repair of active genes over inactive areas of such vectors.

DNA repair studies in mammalian cells have revealed an inducible NER response similar to the bacterial "SOS response". Recently, Ye and co-workers (1999) showed that after quinacrine mustard treatment, human fibroblast cells repaired CPDs by GGR more rapidly than usually, with a smaller increase in repair of CPDs in the transcribed strand. Chang and co-workers (1999) showed that the drug emodin increases repair of UV- and cisplatin-induced DNA damage in normal human lung fibroblasts. Host-cell reactivation is particularly convenient for the study of inducible NER, since the DNA and the cells may be independently treated with DNA damaging agents. Research using a plasmid carrying the CAT reporter gene has shown that treatment of cells with UV light

or mitomycin C increases reactivation of UV-damaged reporter gene in normal, but not NER-deficient human cells (Protic, et al., 1988). Similarly, treatment of cells with thymidine dinucleotides (mimicking sequences excised during repair of UV photoproducts) also increases NER of damaged CAT reporter gene (Eller, et al., 1997; Maeda, et al., 1999).

The approach our laboratory group has used to study inducible mammalian NER mechanisms compares host cell reactivation of UV-irradiated virus in irradiated and unirradiated cells. The ability of UV light to enhance reactivation of UV-irradiated adenovirus and an UV-irradiated reporter gene have previously been described (Jeeves and Rainbow, 1983; Francis and Rainbow, 1999; McKay, et al., 1997). In particular, Francis and Rainbow (1999) showed that UV irradiation of normal and XP-C cells resulted in enhanced HCR of a UV-damaged reporter gene from Ad5HCMVsp1*lacZ*, whereas irradiation of other XP and CS cell strains deficient in GGR did not result in UV-enhanced reactivation (UVER). This suggests that there is an inducible DNA repair process, which requires efficient TCR for enhanced HCR. However, this does not completely rule out the possibility of UV-induced bypass of photolesions by RNAPII, which could also result in an enhancement of reporter gene expression. The QPCR assay we have developed and applied was used to amplify regions from the DNA directly, and thus any observed enhancement of HCR with this technique involves removal of the PCR-terminating lesions from the DNA. We have investigated UVER using the QPCR method in normal human lung

fibroblast cells (IMR90). In these cells we observed an enhancement in lesions removal from both the actively transcribed *lacZ* gene (inserted into the deleted E1 region) and the endogenous E4 region 24 hours after infection with the UV-damaged vector. The enhanced HCR levels at t=24 h are comparable to HCR levels in untreated cells at t=36 h (see Figure 2-8), and there is no further UVER above basal levels of repair at t=36 h. This suggests that irradiation of cells with UV increases the rate of repair, rather than increasing the total amount of lesion removal. These results demonstrate that previous reports of enhanced host cell reactivation of reporter gene activity are indicative of a genuine inducible NER response in human cells.

UV irradiation upregulates expression from the HCMV immediate early promoter, and it has been shown that β-gal expression from unirradiated Ad5HCMVsp1*lacZ* increases following UV irradiation of repair-proficient and repair-deficient cell strains (Francis and Rainbow, 1997). Francis and Rainbow (1999) showed that UV treatment of cells prior to infection with the vector results in an enhanced repair of the transcribed strand of the reporter gene in normal and XP-C cell strains by a TCR-dependent mechanism. In the current study we also investigated a region of the reporter gene in Ad5HCMVsp1*lacZ*, and our results show that UV treatment of cells prior to infection with the vector actually induces a higher rate of removal of DNA lesions. In addition to observing UVER in the actively transcribed *lacZ* gene, we also observed UVER of PCR product from the E4 fragment. Since the E4 region is believed to have no transcriptional

activity (Berk, et al., 1979; Jones and Shenk, 1979) or deficient transcription (Nevins, 1981; Lusky, et al., 1998) in E1 deleted adenovirus vectors, this suggests a role for GGR in the UV-inducible response. It is possible that enhanced HCR in E4 results from increased TCR due to a low level of transcription in this region. Results from this study together with previous work (Francis and Rainbow, 1999) are consistent with a model in which UV irradiation of cells upregulates GGR in both the *lacZ* gene and the E4 region, through a mechanism dependent on TCR. Recent results of Hwang and co-workers (1999) showed that transcription from the p48 gene, which is mutated in GGR-deficient DDB$^-$ XPE cells, is upregulated (in a TP53-dependent manner) in response to UV treatment of cells. This suggests that there is an UV-inducible GGR response, which is dependent on an increase in p48 transcription. This rapid increase in p48 transcription would require removal of UV-induced lesions from the p48 gene in UV-irradiated cells, which may implicate the participation of the TCR pathway in this inducible response.

In conclusion, we believe we have developed a sensitive and convenient DNA repair assay for a wide variety of mammalian cells, by combining the power of quantitative PCR with the advantages of host cell reactivation assays. The advantages of this assay include the precision, reproducibility and ease of PCR, the ability to specifically investigate several regions of the genome simultaneously and a short period for results (compared to plaque formation, or colony-forming assays). The improvements in this technique compared to other

HCR assays include the ability to directly assess initial lesion formation, and an endpoint that is directly based on lesion frequency in the DNA. We have used this assay to show a significant removal of UV photoproducts in Ad5HCMVsp1*lacZ* after infection of normal human fibroblasts, and significantly diminished lesion removal following infection NER-deficient XP-C, XP-A and CS-B cells. This supports a model in which basal levels of lesion removal from this vector involve both the GGR and TCR pathways. We also found an increased rate of lesion removal from the vector in irradiated normal cells as compared to unirradiated cells, providing evidence of a genuine UV-inducible DNA repair mechanism in human cells.

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Figure 2-1. PCR primer locations in Ad5HCMVsp1*lacZ* genome (not to scale). Primer pairs AB (for *lacZ* gene inserted into deleted E1 region) and CD (for endogenous E4 region) were used to amplify the two regions independently, or together in a multiplex PCR reaction.

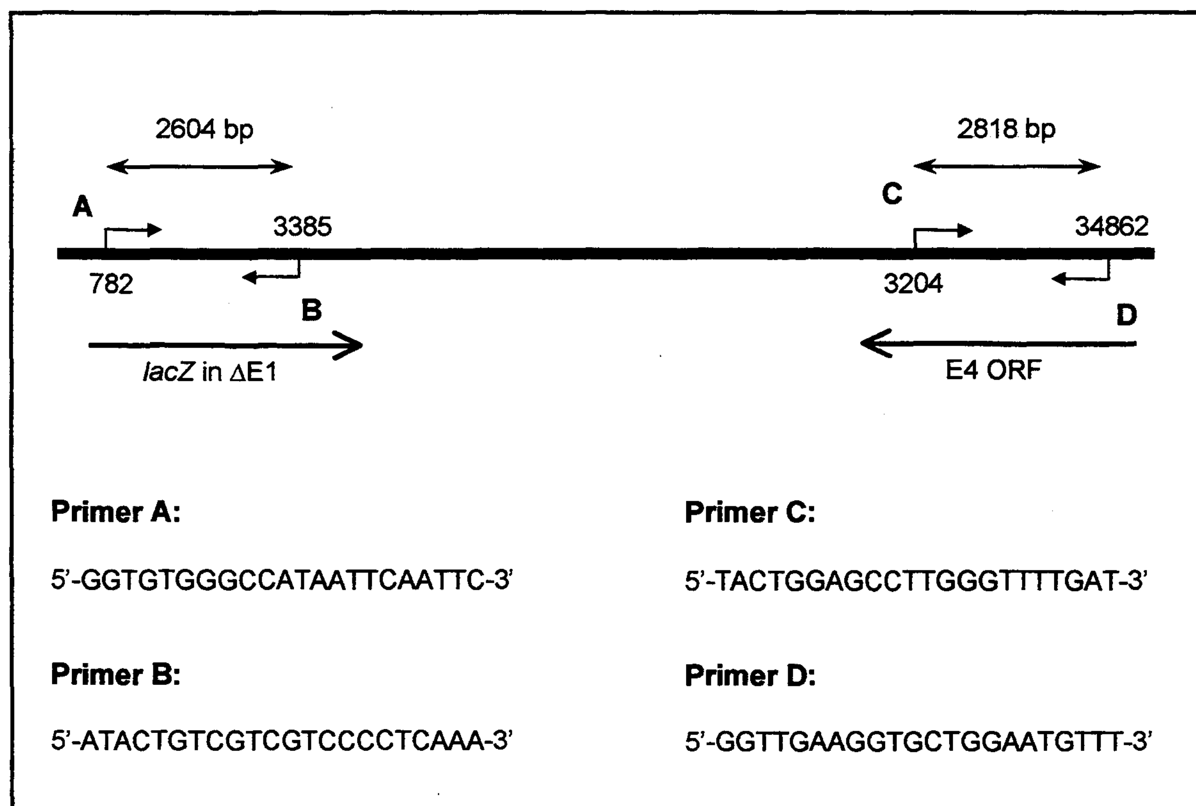

2604 bp
2818 bp
A
C
3385
34862
782
3204
B
D
lacZ in ΔE1
E4 ORF
Primer A:
5'-GGTGTGGGCCATAATTCAATTC-3'
Primer C:
5'-TACTGGAGCCTTGGGTTTTGAT-3'
Primer B:
5'-ATACTGTCGTCGTCCCCTCAAA-3'
Primer D:
5'-GGTTGAAGGTGCTGGAATGTTT-3'

Figure 2-2. Typical QPCR experiment using *lacZ* primers. (A) Image of PCR products amplified from DNA extracted from cells infected with Ad5HCMVsp1*lacZ* at an MOI of 50 (0.8% agarose gel). Lanes 1-6 show 2-fold serial dilutions, lanes 7 & 8 show duplicate PCR reactions from unirradiated samples, lanes 9-14 show duplicate reactions from UV irradiated Ad samples. (B) Plot of PCR amplified serial dilution of unirradiated Ad sample (lanes 7 & 8 in Fig. 2-2A). Line of best fit (minimised χ^2, Microcal Origin 6.0) was used to produce a calibration curve used to determine reduction in PCR product following UV irradiation.

A

B

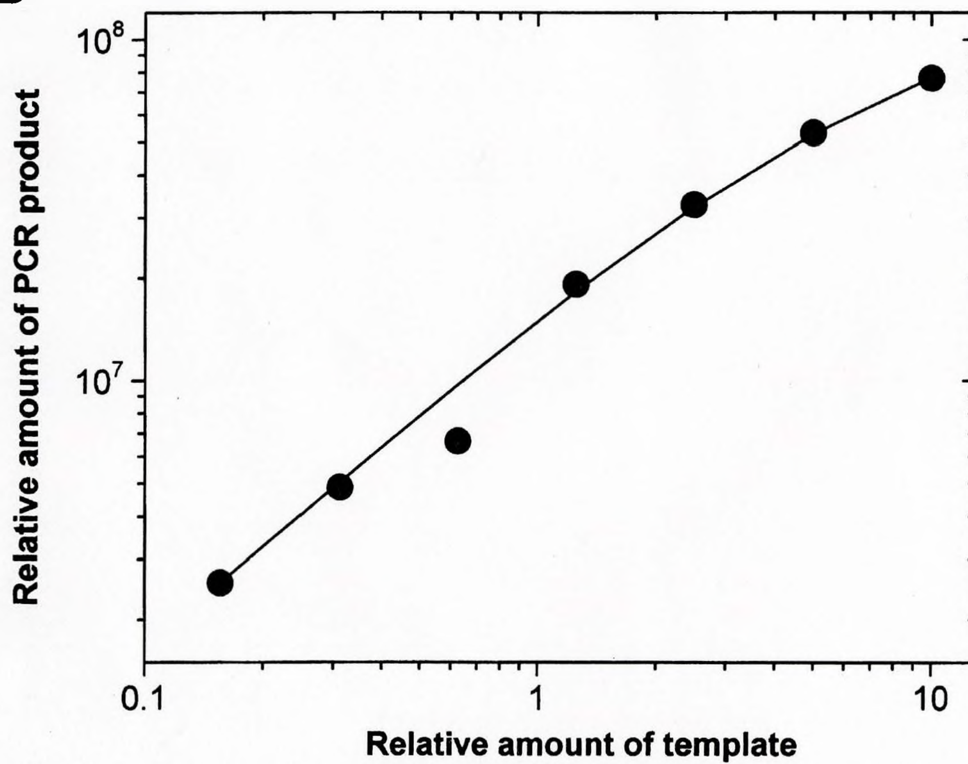
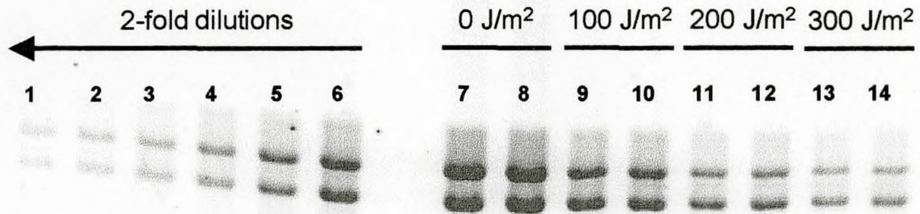


Figure 2-3. Typical multiplex PCR experiment using *lacZ* primers and E4 primers. (A) Image of PCR products amplified from DNA extracted from cells infected with Ad5HCMVsp1*lacZ* at an MOI of 50 (0.8% agarose gel). (B) Plot of PCR amplified serial dilution of unirradiated Ad sample (shown in Fig. 2-3A) for E1 (●) and E4 regions (■).

A



B

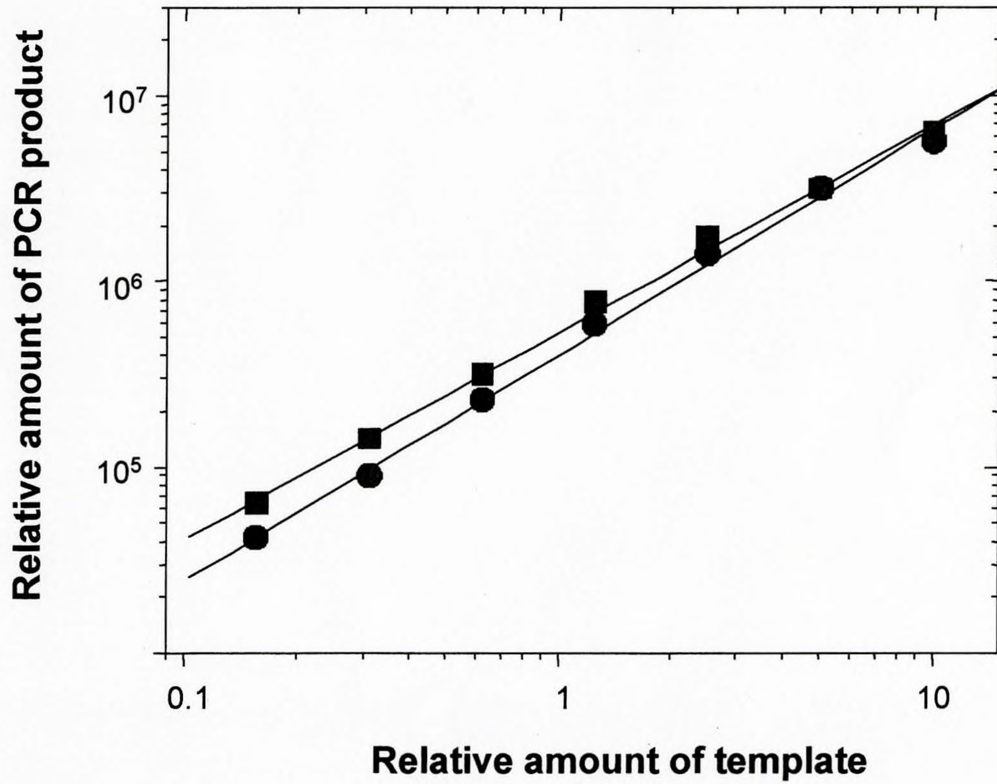


Figure 2-4. Dose-response curves for 2 regions of UV-irradiated Ad5HCMVsp1*lacZ*. A uniform single exponential decrease in PCR product is detected with increasing UV fluence. The points represent the mean (± SE) of 12 experiments (2 PCR reactions for each of 2 experimental samples per experiment) for the E1 (●) and E4 regions (■). E1 fragment D_0=128.5 J/m^2 and E4 fragment D_0=88.0 J/m^2. Based on the slope of the line of best fit (Microcal Origin 6.0), lesion frequency was 2.99×10^{-6} lesions/nucleotide/J/m^2 for the E1 fragment and 4.04×10^{-6} lesions/nucleotide/J/m^2 for the E4 fragment.

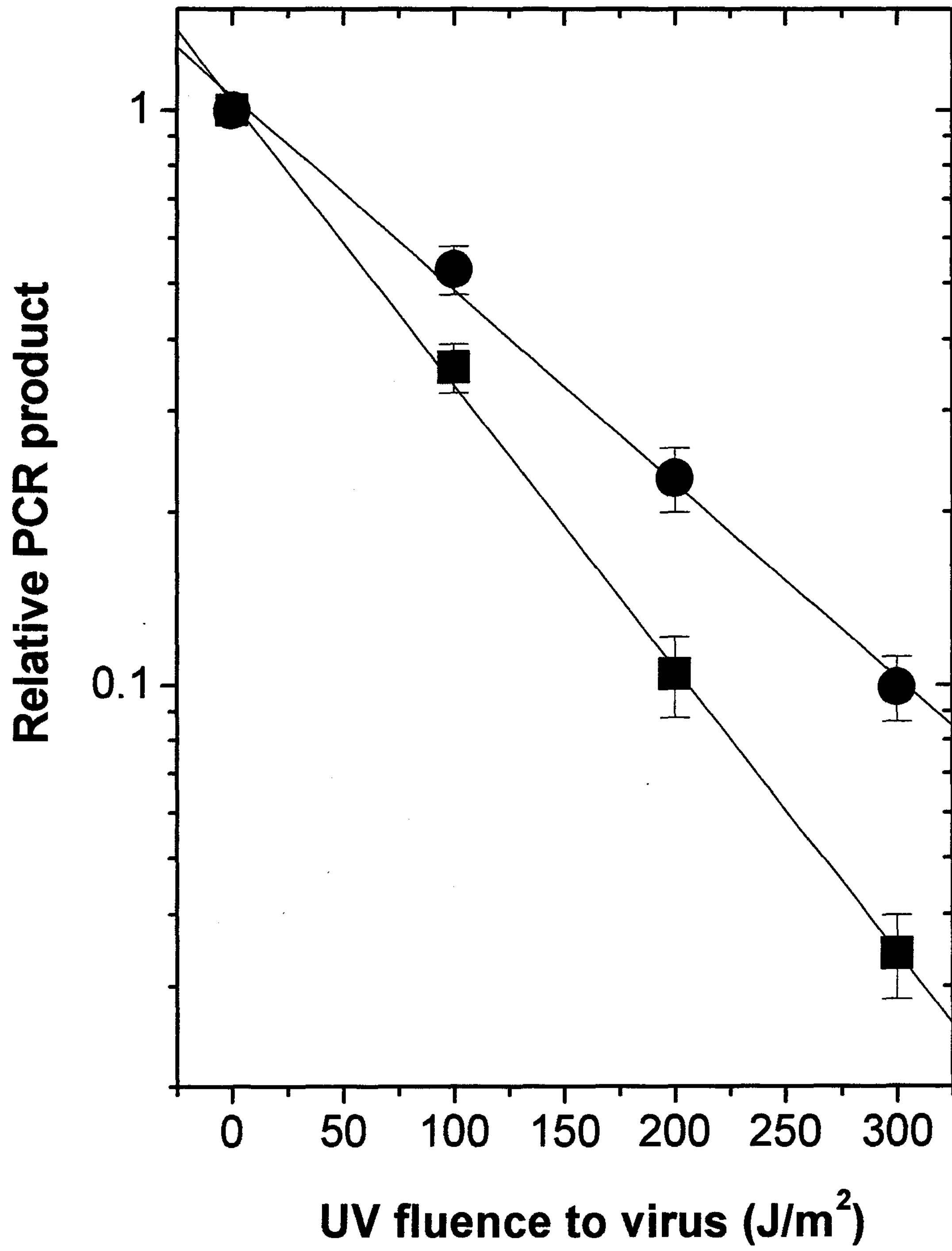

1
0.1
Relative PCR product
0
50
100
150
200
250
300
UV fluence to virus (J/m2)

Figure 2-5. Typical results of HCR experiments for normal and NER-deficient cells. Times shown are t=0 h (■), t=24 h (●), t=36 h (△) and t=48 h (◆). Cell strains: (A) GM09503 (normal), (B) GM08399 (normal), (C) GM00030A (XP-C), (D) GM00510 (XP-C), (E) GM01428 (CS-B) and (F) GM00082 (XP-A). Each data point represents duplicate PCR reactions (± SE) from one experimental sample.

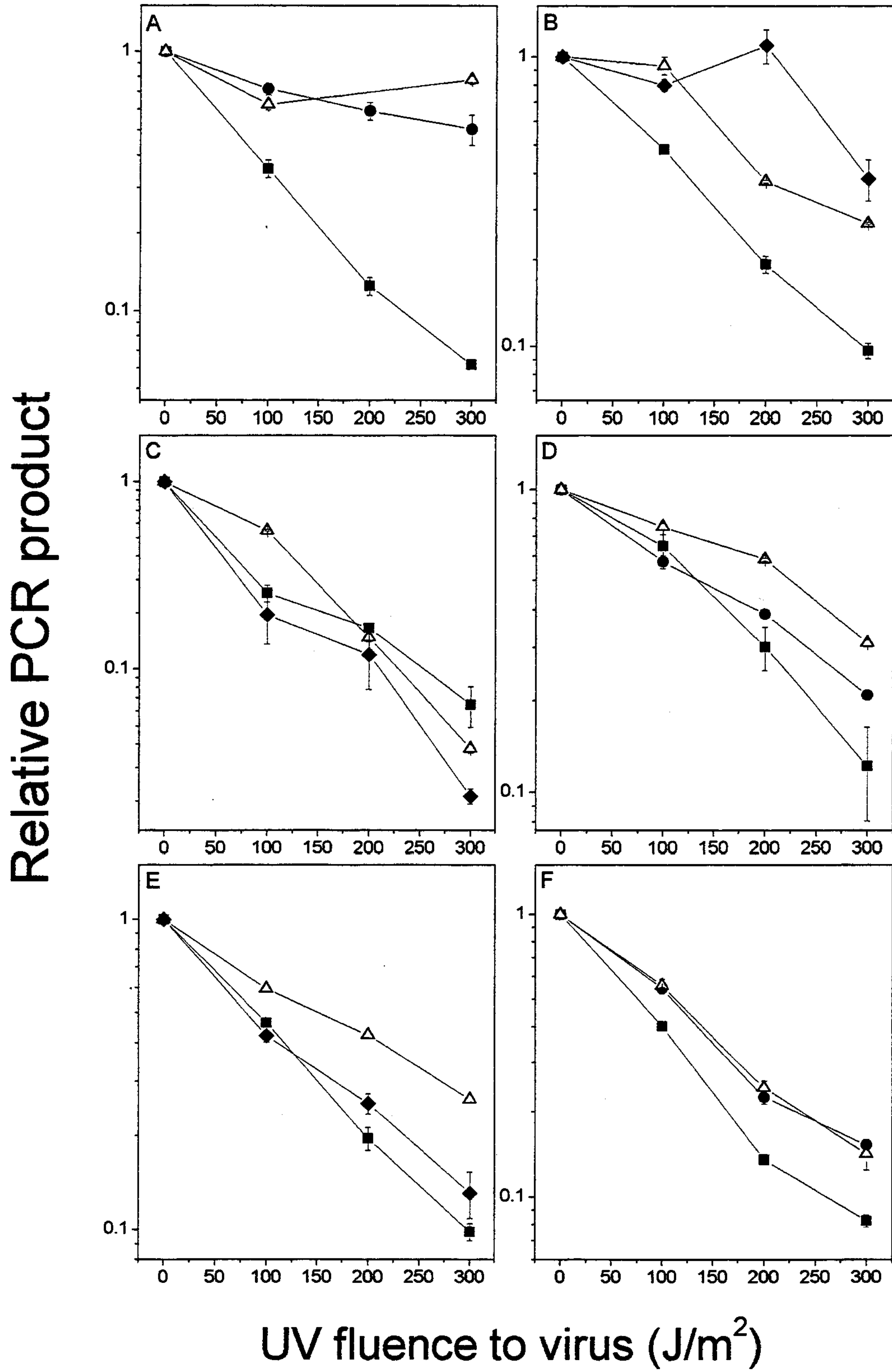
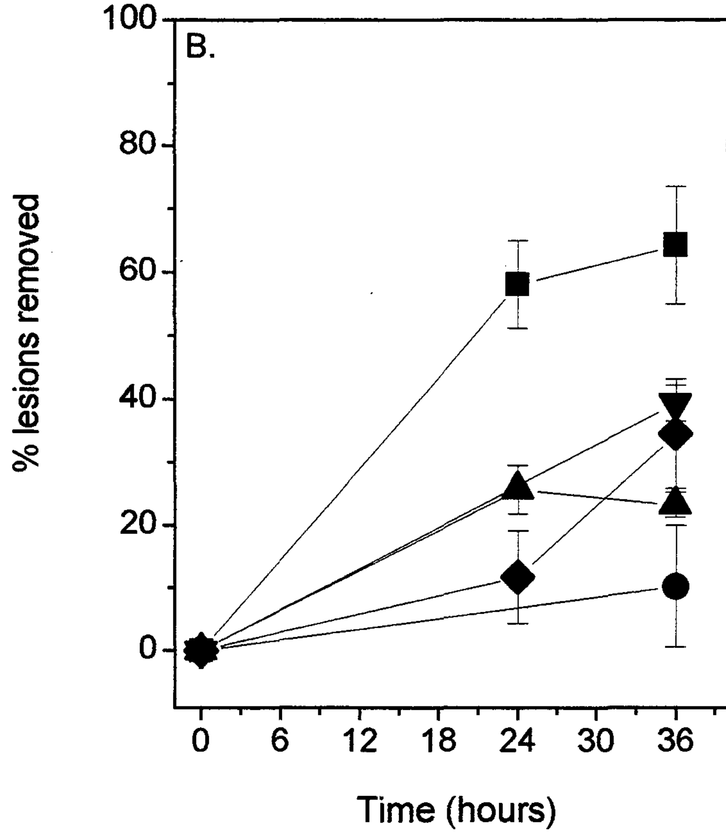

A
B
C
D
E
F
1
0.1
0
50
100
150
200
250
300
Relative PCR product
UV fluence to virus (J/m2)

Figure 2-6. Repair of UV-induced lesions from Ad5HCMVsp1*lacZ*. Lesion removal is shown for (A) the E1 region and (B) the E4 region for the four (pooled) normal fibroblast lines (■), GM00030A (XP-C) (●), GM00510 (XP-C) (◆), GM00082 (XP-A) (▲), and GM01428 (CS-B) (▼). Data points represent the mean (± SE) of relative lesion removal (described in Results section 3.3) for all three doses (two PCR reactions/experimental sample at each dose of 100, 200 & 300 J/m^2) from two or more experiments (except GM00030A E4 region – 1 experiment).


B.
% lesions removed
100
80
60
40
20
0
0
6
12
18
24
30
36
Time (hours)

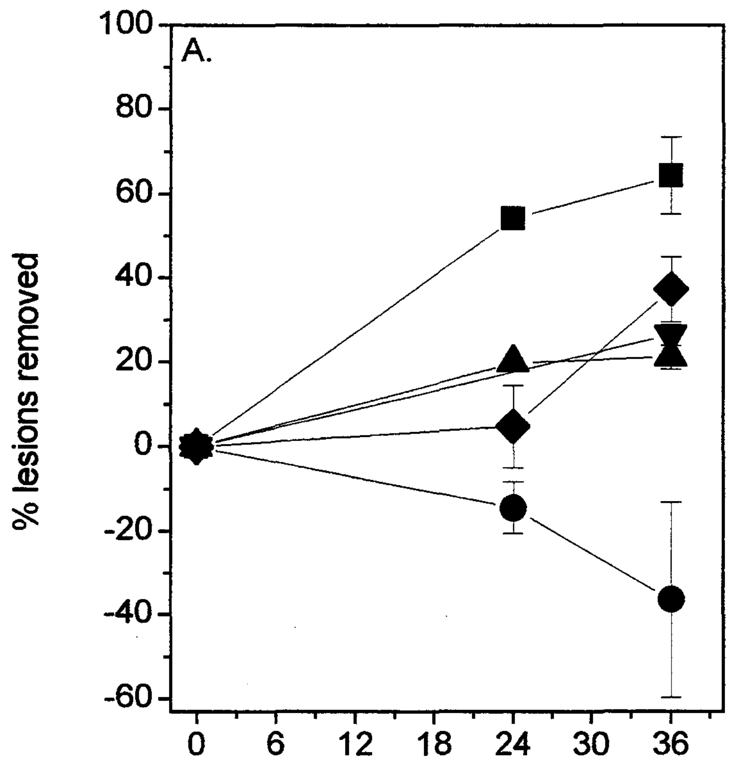

A.
% lesions removed
100
80
60
40
20
0
-20
-40
-60
0
6
12
18
24
30
36

Figure 2-7. UV-enhanced host-cell reactivation of PCR product in normal human lung fibroblasts. Effect of 10 J/m^2 UV treatment of human fibroblast cells (IMR90) 24 hours prior to infection of with UV-irradiated Ad5HCMVsp1*lacZ*. Results shown for actively transcribed *lacZ* gene in deleted E1 region at t=0 h (■), t=24 h in untreated cells (●), t=24 h in UV treated cells (◆), t=36 h in untreated cells (○) and t=36 h in UV treated cells (◇).

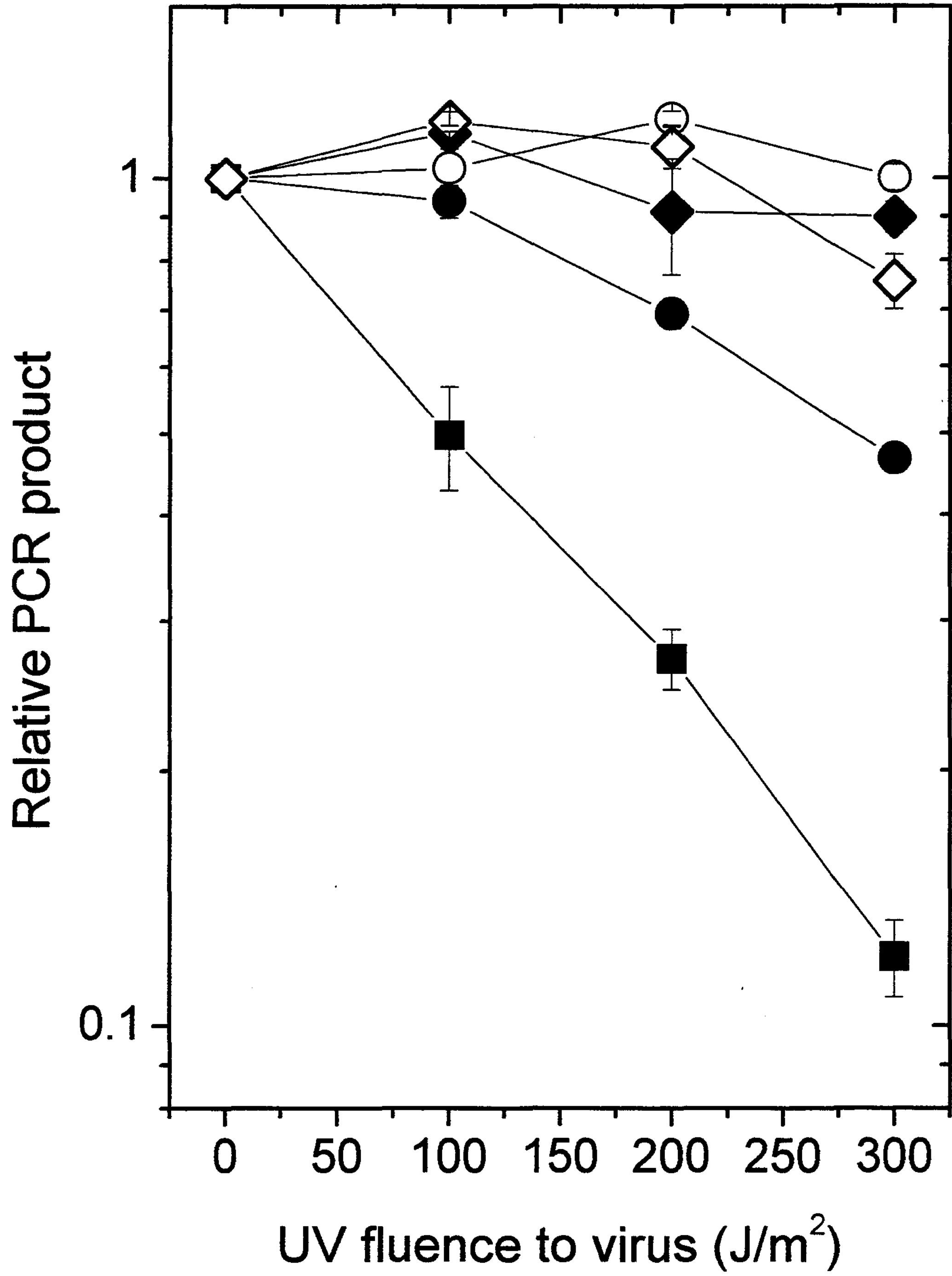
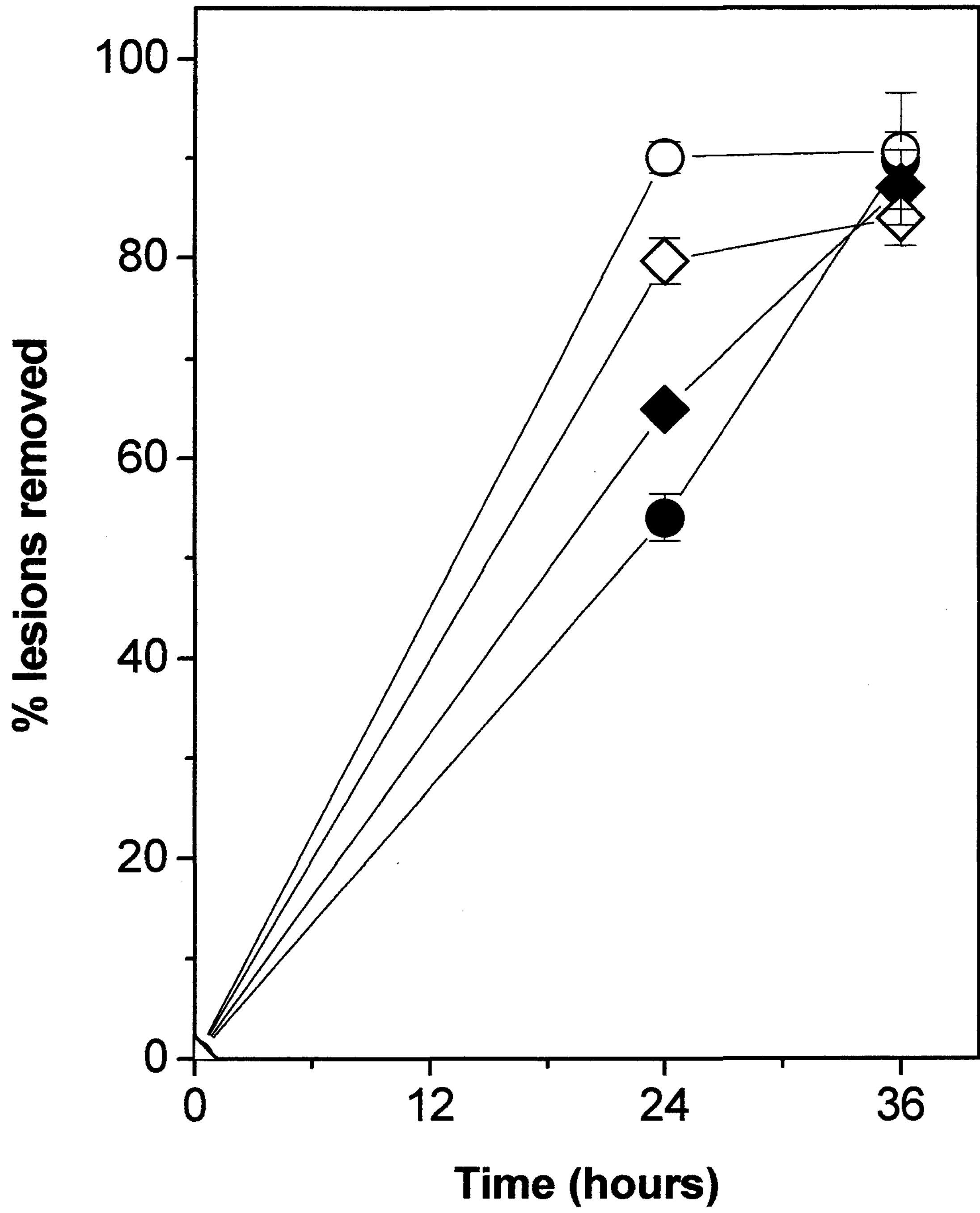

Relative PCR product
1
0.1
0
50
100
150
200
250
300
UV fluence to virus (J/m2)

Figure 2-8. UV-enhanced lesion removal in normal lung fibroblasts. Lesion removal is shown for untreated IMR90 cells from the E1 region (●) and the E4 region (◆), as well as for UV pre-irradiated (10 J/m^2) cells from the E1 region (○) and the E4 region (◇). Data points represent the mean (± SE) of relative lesion removal (described in Results section 3.3) for all three doses (two PCR reactions per sample at each dose of 100, 200 & 300 J/m^2) from two experiments.


100
80
60
40
20
0
% lesions removed
0
12
24
36
Time (hours)

References

Barrett, S.F., J.H. Robbins, R.E. Tarone and K.H. Kraemer (1991) Evidence for defective repair of cyclobutane pyrimidine dimers with normal repair of other DNA photoproducts in a transcriptionally active gene transfected into Cockayne syndrome cells, Mutation Research, 255, 281-91.

Bennett, C.B. and A.J. Rainbow (1988) Enhanced reactivation and mutagenesis of UV-irradiated adenovirus in normal human fibroblasts, Mutagenesis, 3, 157-64.

Berk, A.J., F. Lee, T. Harrison, J. Williams and P.A. Sharp (1979) Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs, Cell, 17, 935-44.

Bockstahler, L.E., K.F. Haynes and J.E. Stafford (1976) Investigation of radiation enhanced reactivation of cytoplasmic replicating human virus. pp. 203-9, In: Hazzard Dg, Ed . Symposium on Biological Effects And Measurement Of Light Sources. Qh 651 S983s 1976., United States.

Bregman, D.B., R. Halaban, A.J. van Gool, K.A. Henning, E.C. Friedberg and S.L. Warren (1996) UV-induced ubiquitination of RNA polymerase II: a novel modification deficient in Cockayne syndrome cells, Proceedings Of The National Academy Of Sciences Of The United States Of America, 93, 11586-90.

Brown, T.C. and P.A. Cerutti (1989) UV-enhanced reactivation of UV-damaged SV40 is due to the restoration of viral early gene function, Mutation Research, 218, 211-7.

Chakalova, L. and G. Russev (1998) Quantitative polymerase chain reaction assay for DNA repair within defined genomic regions, Mutation Research, 407, 147-55.

Chang, L.C., H.M. Sheu, Y.S. Huang, T.R. Tsai and K.W. Kuo (1999) A novel function of emodin: enhancement of the nucleotide excision repair of UV- and cisplatin-induced DNA damage in human cells, Biochemical Pharmacology, 58, 49-57.

Christians, F.C. and P.C. Hanawalt (1992) Inhibition of transcription and strand-specific DNA repair by alpha-amanitin in Chinese hamster ovary cells, Mutation Research, 274, 93-101.

Cleaver, J.E. (1968) Defective repair replication of DNA in xeroderma pigmentosum, Nature, 218, 652-6.

Cleaver, J.E. and K.H. Kraemer (1995) Xeroderma Pigmentosum and Cockayne Syndrome, in: C.R. Scriver et al. (Ed.), The Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill, Inc., United States of America.

Drouin, R. and J.P. Therrien (1997) UVB-induced cyclobutane pyrimidine dimer frequency correlates with skin cancer mutational hotspots in p53, Photochemistry and Photobiology, 66, 719-26.

Eller, M.S., T. Maeda, C. Magnoni, D. Atwal and B.A. Gilchrest (1997) Enhancement of DNA repair in human skin cells by thymidine dinucleotides: evidence for a p53-mediated mammalian SOS response., Proceedings Of The National Academy Of Sciences Of The United States Of America, 94, 12627-32.

Francis, M.A. and A.J. Rainbow (1997) UV-enhanced expression of a reporter gene is induced at lower UV fluence in transcription-coupled repair deficient compared with normal human cells and is absent in SV40-transformed human cells, Photochemistry and Photobiology, 65S (Abstr. ThAM-E5), 1028.

Francis, M.A. and A.J. Rainbow (1999) UV-enhanced reactivation of a UV-damaged reporter gene suggests transcription-coupled repair is UV-inducible in human cells, Carcinogenesis, 20, 19-26.

Friedberg, E.C. (1995) DNA repair and mutagenesis, ASM Press, Washington, D.C.

Ganesan, A.K. and P.C. Hanawalt (1994) Removal of cyclobutane pyrimidine dimers from a UV-irradiated shuttle vector introduced into human cells, Somatic Cell And Molecular Genetics, 20, 233-42.

Ganesan, A.K., J. Hunt and P.C. Hanawalt (1999) Expression and nucleotide excision repair of a UV-irradiated reporter gene in unirradiated human cells, Mutation Research, 433, 117-26.

Graham, F.L., J. Smiley, W.C. Russell and R. Nairn (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5, Journal of General Virology, 36, 59-74.

Jeeves, W.P. and A.J. Rainbow (1979a) Gamma-ray enhanced reactivation of gamma-irradiated adenovirus in human cells, Biochemical and Biophysical Research Communications, 90, 567-74.

Jeeves, W.P. and A.J. Rainbow (1979b) Gamma-ray-enhanced reactivation of UV-irradiated adenovirus in normal human fibroblasts, Mutation Research, 60, 33-41.

Jeeves, W.P. and A.J. Rainbow (1983) U.V. enhanced reactivation of U.V.-and gamma-irradiated adenovirus in normal human fibroblasts, International Journal Of Radiation Biology And Related Studies In Physics, Chemistry And Medicine, 43, 599-623.

Jones, N. and T. Shenk (1979) An adenovirus type 5 early gene function regulates expression of other early viral genes, Proceedings Of The National Academy Of Sciences Of The United States Of America, 76, 3665-9.

Klocker, H., R. Schneider, H.J. Burtscher, B. Auer, M. Hirsch-Kauffmann and M. Schweiger (1985) Transient expression of a plasmid gene, a tool to study DNA repair in human cells: defect of DNA repair in Cockayne Syndrome; one thymine cyclobutane dimer is sufficient to block transcription, European Journal of Cell Biology, 39, 346-351.

Leadon, S.A. and D.A. Lawrence (1991) Preferential repair of DNA damage on the transcribed strand of the human metallothionein genes requires RNA polymerase II, Mutation Research, 255, 67-78.

Lehmann, A.R. and A. Oomen (1985) Effect of DNA damage on the expression of the chloramphenicol acetyltransferase gene after transfection into diploid human fibroblasts, Nucleic Acids Research, 13, 2087-95.

Lusky, M., M. Christ, K. Rittner, A. Dieterle, D. Dreyer, B. Mourot, H. Schultz, F. Stoeckel, A. Pavirani and M. Mehtali (1998) In vitro and in vivo biology of recombinant adenovirus vectors with E1, E1/E2A, or E1/E4 deleted, Journal of Virology, 72, 2022-32.

Maeda, T., M.S. Eller, M. Hedayati, L. Grossman and B.A. Gilchrest (1999) Enhanced repair of benzo(a)pyrene-induced DNA damage in human cells treated with thymidine dinucleotides, Mutation Research, 433, 137-145.

McCarthy, M.J., J.I. Rosenblatt and R.S. Lloyd (1996) A modified quantitative polymerase chain reaction assay for measuring gene-specific repair of UV photoproducts in human cells, Mutation Research, 363, 57-66.

McCarthy, M.J., J.I. Rosenblatt and R.S. Lloyd (1997) Kinetics of repair of UV-induced DNA damage in repair-proficient and -deficient cells as determined by quantitative polymerase chain reaction, Photochemistry And Photobiology, 66, 356-62.

McKay, B.C., M.A. Francis and A.J. Rainbow (1997) Wildtype p53 is required for heat shock and ultraviolet light enhanced repair of a UV-damaged reporter gene, Carcinogenesis, 18, 245-9.

McKay, B.C., M.A. Francis and A.J. Rainbow (in press) Use of replication deficient recombinant adenovirus reporter gene constructs to assess repair of UV damaged DNA in mammalian cells, Methods in Molecular Biology.

McKay, B.C. and A.J. Rainbow (1996) Heat-shock enhanced reactivation of a UV-damaged reporter gene in human cells involves the transcription coupled DNA repair pathway, Mutation Research, 363, 125-35.

Morrison, C. and F. Gannon (1994) The impact of the PCR plateau phase on quantitative PCR, Biochimica et Biophysica Acta, 1219, 493-8.

Morsy, M.A., E.L. Alford, A. Bett, F.L. Graham and C.T. Caskey (1993) Efficient adenoviral-mediated ornithine transcarbamylase expression in deficient mouse and human hepatocytes, Journal of Clinical Investigation, 92, 1580-6.

Mullenders, L.H. (1998) Transcription response and nucleotide excision repair, Mutation Research, 409, 59-64.

Nevins, J.R. (1981) Mechanism of activation of early viral transcription by the adenovirus E1A gene product, Cell, 26, 213-20.

Piperakis, S.M. and A.G. McLennan (1984) Hyperthermia enhances the reactivation of irradiated adenovirus in HeLa cells, British Journal of Cancer, 49, 199-205.

Protic, M., E. Roilides, A.S. Levine and K. Dixon (1988) Enhancement of DNA repair capacity of mammalian cells by carcinogen treatment, Somatic Cell and Molecular Genetics, 14, 351-7.

Protic-Sabljic, M. and K.H. Kraemer (1985) One pyrimidine dimer inactivates expression of a transfected gene in xeroderma pigmentosum cells, Proceedings of the National Academy of Sciences of The United States of America, 82, 6622-6.

Rainbow, A.J and Mak, S. (1973) DNA damage and biological function of human adenovirus after u.v.-irradiation, International Journal Of Radiation Biology And Related Studies In Physics, Chemistry And Medicine, 24, 59-72.

Ratner, J.N., B. Balasubramanian, J. Corden, S.L. Warren, and D.B. Bregman, D. B. (1998) Ultraviolet radiation-induced ubiquitination and proteasomal degradation of the large subunit of RNA polymerase II. Implications for transcription-coupled DNA repair, Journal of Biological Chemistry, 273, 5184-9.

Sambrook, J., E.F. Fritsch and T. Maniatis (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, USA.

Setlow, R.B. and Carrier, W.L. (1966) Pyrimidine dimers in ultraviolet-irradiated DNA's, Journal of Molecular Biology, 17, 237-54.

Smith, M.L., I.T. Chen, Q. Zhan, P.M. O'Connor and A.J.J. Fornace (1995) Involvement of the p53 tumor suppressor in repair of u.v.-type DNA damage, Oncogene, 10, 1053-9.

Venema, J., L.H. Mullenders, A.T. Natarajan, A.A. van Zeeland and L.V. Mayne (1990) The genetic defect in Cockayne syndrome is associated with a defect in repair of UV-induced DNA damage in transcriptionally active DNA, Proceedings of the National Academy of Sciences of The United States of America, 87, 4707-11.

Venema, J., A. van Hoffen, V. Karcagi, A.T. Natarajan, A.A. van Zeeland and L.H. Mullenders (1991) Xeroderma pigmentosum complementation group C cells remove pyrimidine dimers selectively from the transcribed strand of active genes, Molecular And Cellular Biology, 11, 4128-34.

Wellinger, R.E. and F. Thoma (1996) Taq DNA polymerase blockage at pyrimidine dimers, Nucleic Acids Research, 24, 1578-9.

Ye, N., M.S. Bianchi, N.O. Bianchi and G.P. Holmquist (1999) Adaptive enhancement and kinetics of nucleotide excision repair in humans., Mutation Research, 435, 43-61.

Chapter 3

Study of the Inducibility of Nucleotide Excision Repair by Gamma Rays

1. Introduction

Ionising radiation (IR) has many effects on mammalian cells. DNA is believed to be the principal target for the biological effects of ionising radiation, including cell killing, mutation, and carcinogenesis (Hall, 1994). The effects of IR on DNA include single-strand breaks, double-strand breaks, crosslinking of DNA (intrastrand and interstrand), crosslinking of DNA with proteins, and base modification (Travis, 1989). In addition to the effect on DNA, IR also activates pathways originating in the cell membrane or cytoplasm, triggering phosphorylation cascades similar to those induced by activation of cell-surface receptors (reviewed in Canman and Kastan, 1996). A range of agents, including UV light, heat shock, carcinogenic drugs and other mutagens triggers several of these "stress response" pathways. The effects of IR and UV in triggering these pathways are best characterised, and appear to be very similar (reviewed in Eckardt-Schupp and Klaus, 1999).

Our laboratory group is interested in the study of DNA repair mechanisms. Of particular interest is the inducibility of DNA repair by various mechanisms, as this has implications in both carcinogenesis and cancer treatment therapies. Previous work with mammalian cells showed that IR can enhance reactivation of UV-damaged virus (Bockstahler and Lytle, 1971; Bockstahler and Lytle, 1977; Hellman, et al., 1976; Jeeves and Rainbow, 1979; Jeeves and

Rainbow, 1983a). The purpose of this study was to examine the potential of IR to induce NER. Using a host cell reactivation (HCR) assay for a UV-damaged reporter gene in an adenovirus vector (McKay, et al., (in press)), it has been shown that both UV light (Francis and Rainbow, 1999) and heat shock (McKay, et al., 1997a; McKay and Rainbow, 1996) stimulate a TP53-dependent DNA repair response in human cells that is dependent on transcription-coupled repair (TCR). Using the same protocol as was used for UV- and heat shock-enhanced reactivation studies, I examined the ability of γ-radiation to enhance HCR of an UV-damaged reporter gene.

2. Materials and Methods

2.1 Cells and Virus

The normal human fibroblast strains GM09503, GM00038A, GM00969 and the NER-deficient XP-C strains GM00030A (XP3BE) and GM00677A (XP2BE) were obtained from the NIGMS Human Genetic Cell Repository (Camden, NJ). The human cervical carcinoma HeLa cells were obtained from the American Type Cultured Cell Collection (Rockville, Maryland). The squamous cell carcinoma SCC-25 cells were obtained from Dr. J. Lazo (University of Pittsburgh School of Medicine, Pittsburgh, PA) and the normal lung epithelial L132 cells were obtained from Dr. J. Arrand (Brunel University, Uxbridge, UK). AS99048 fibroblast cells were obtained by skin biopsy from a patient under Dr. M.

Khalifa's care at the Kingston General Hospital, who is believed to have Cockayne syndrome. The Ad E1 region transformed human 293 cells (Graham, et al., 1977) for propagating virus were obtained from Dr. F. L. Graham (McMaster University). Cultures were maintained in α-minimal essential medium (α-MEM) (Gibco BRL), supplemented with 10% foetal bovine serum (Sigma F-4135) and 1% antibiotic/antimycotic (100μg/ml penicillin G sodium, 100μg/ml streptomycin sulphate and 250 ng/ml amphotericin B; Gibco BRL) in a humidified atmosphere of 5% CO_2 at 37°C.

The vector Ad5HCMVsp1*lacZ* is a recombinant non-replicating adenovirus expressing the bacterial *lacZ* gene under the control of the HCMV immediate early promoter. This virus efficiently infects human cells and expresses β-gal (Morsy, et al., 1993). Virus stocks were prepared as described previously (Graham and Prevec, 1991).

2.2 Irradiation of Virus and Cells

UV irradiation of adenovirus has been described previously (Bennett and Rainbow, 1988). Briefly, viral suspensions in phosphate buffered saline (PBS) were irradiated in 35-mm dishes on ice, with continuous stirring, using a General Electric germicidal lamp (G8T5) at an intensity of 2 J/m^2s at predominantly 254 nm. Aliquots of irradiated virus were removed sequentially during irradiation to vary the fluence, and diluted appropriately with unsupplemented α-MEM.

For UV-enhanced reactivation positive control experiments, confluent host cells were irradiated by the same UV source just prior to infection (20-24 hours after seeding), in 40 μl PBS (warmed to 37°C), at a fluence rate of 1 J/m^2s, for a total fluence of 15 J/m^2. UV fluence was corrected for irradiation conditions in 96-well plates, as described previously (McKay, et al., 1997a).

For γ-ray-enhanced reactivation (γRER) experiments, confluent cells were irradiated by a 1200 Ci ^{137}Cs source (Taylor Source, McMaster University) emitting γ-rays with 660 keV energy. Under the conditions used, the calculated dose rate to the cells was approximately 0.17 Gy per minute, based on a previous calibration of the source by a Farmer dosimeter and a 600 cc ion chamber dosimeter. Cells were infected with virus as soon as possible after γ-irradiation (30-60 minutes).

2.3 Enhanced Host Cell Reactivation Procedure

Cells were seeded in 96-well plates (Falcon) at a density of 1.9×10^4 cells (for fibroblasts) or 3.8×10^4 cells (for tumour lines and L132 cells) per well and left for 20-24 hours. Host cells were either untreated or treated with UV radiation (just before infection) or γ-radiation (30-60 minutes before infection). Confluent cells were infected with either non-irradiated or UV-irradiated virus in a total volume of 40 μl (in α-MEM) at a multiplicity of infection (MOI) of 10

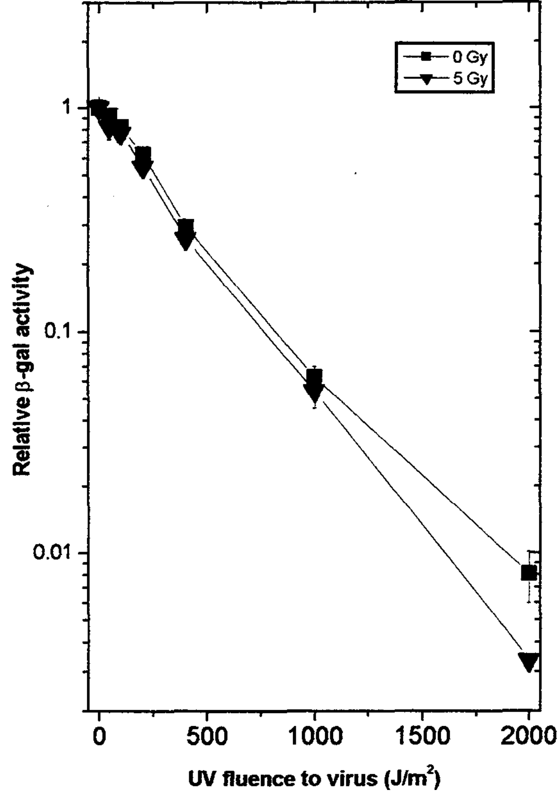
plaque-forming units (pfu) per cell. Following viral adsorption for 60 minutes at 37°C, the viral suspension was aspirated and replaced by 200 μl of supplemented growth medium. Infected cells were harvested 44 hours after infection. Cell layers were incubated with one of two substrates to assess β-gal activity: o-nitrophenol-β-D-galactopyranoside (ONPG) or chlorophenolred-β-D-galactopyranoside (CPRG). ONPG analysis includes: incubation of cell monolayers for 20 minutes with 75μl of 250 mM Tris, 1 μM PMSF, 0.5% NP-40 (pH7.8); addition for 10 minutes of an equal volume of 200mM sodium pyrophosphate, 20 mM KCl, 2mM $MgSO_4$, 100mM 2-mercaptoethanol (pH 7.5); addition of 0.1% ONPG, 200mM sodium pyrophosphate (pH 7.5) and determination of absorbance at OD_{405}. CPRG analysis consists of incubating infected monolayers with 1mM CPRG in 0.01% Triton X-100, 1 mM MgCl2, 100mM sodium pyrophosphate (pH 8.3) and determining absorbance at OD_{570} (Eustice, et al., 1991). OD values were determined at several time points following addition of substrate using a 96-well plate reader (Bio-Tek Instruments EL340 Bio Kinetics reader). In all experiments, background levels of β-gal activity were defined those in cells infected with Ad5HCMVsp1*lacZ* that had been exposed to an UV fluence of 10,000 J/m^2. This background OD value was subtracted from all other OD values for irradiated and unirradiated vector. Experimental values were only accepted if they were at least two-fold higher than the background level.

3. Results

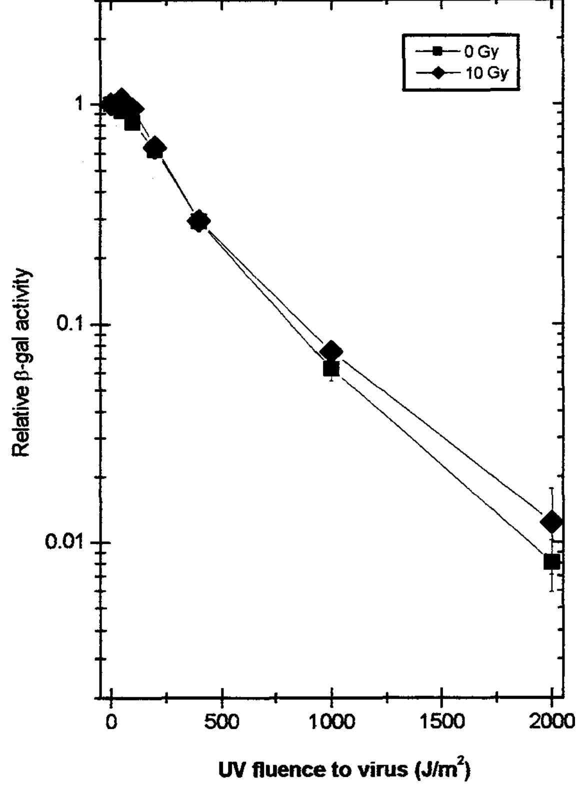
Experiments studying the effect of γ-ray exposure of cells on HCR of UV-damaged reporter gene from Ad5HCMVsp1*lacZ* were carried out with a variety of human cells including normal fibroblasts, NER-deficient fibroblasts as well as tumour cells. Typical results for such experiments are shown in Figures 3-1 and 3-2. Results for the various cell types were pooled and summarised as follows. D_{37} values (dose required to reduce survival to 37%) for β-gal activity were determined for untreated and γ-ray-treated cells. Relative D_{37} (ratio of D_{37} for γ-ray treated cells / D_{37} for untreated cells) values were calculated for each γ-ray dose. For each γ-ray dose to cells, relative D_{37} was not significantly greater than 1. All D_{37} numbers for each cell type were averaged (see Table 3-1). These values represent the net effect of γ-irradiation of cells on survival of β-gal expression from UV-irradiated Ad5HCMVsp1*lacZ*. Results were varied, but no significant enhancement in reactivation of UV-damaged reporter construct was detected.

Since previous work from our lab showed UV-induced enhancement of HCR of UV-irradiated reporter activity (Francis and Rainbow, 1999), UV treatment to cells was carried out in parallel as a positive control in several γRER experiments. Results from such an experiment are shown in Figure 3-3. Exposure of cells to 15 J/m^2 UV light prior to infection with UV-damaged vector enhanced HCR of β-gal activity in both the normal and XP-C cells, whereas

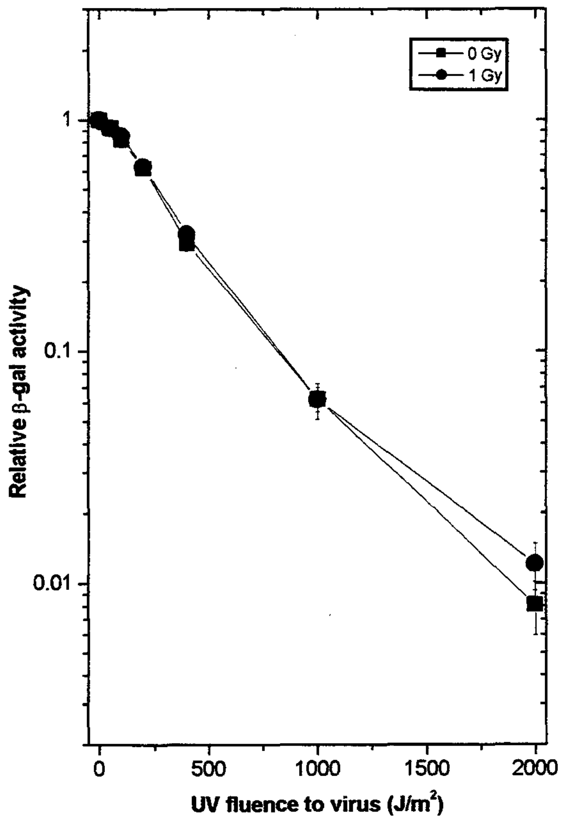
Figure 3-1. Typical result of γRER experiment suggesting no enhanced reactivation due to treatment of cells with γ-rays in L132 cells. Data points represent the mean of three replicates (± SE) from one experiment. Cells were treated with 0 Gy (squares), 1 Gy (circles), 2 Gy (up-triangles), 5 Gy (down-triangles) or 10 Gy (diamonds) γ-rays from a ^{137}Cs source at a dose rate of 0.17 Gy per minute.



0 Gy
5 Gy
1
0.1
0.01
Relative β-gal activity
0
500
1000
1500
2000
UV fluence to virus (J/m2)



0 Gy
10 Gy
1
0.1
0.01
Relative β-gal activity
0
500
1000
1500
2000
UV fluence to virus (J/m2)


0 Gy
1 Gy
Relative β-gal activity
0.01
0.1
1
0
500
1000
1500
2000
UV fluence to virus (J/m²)

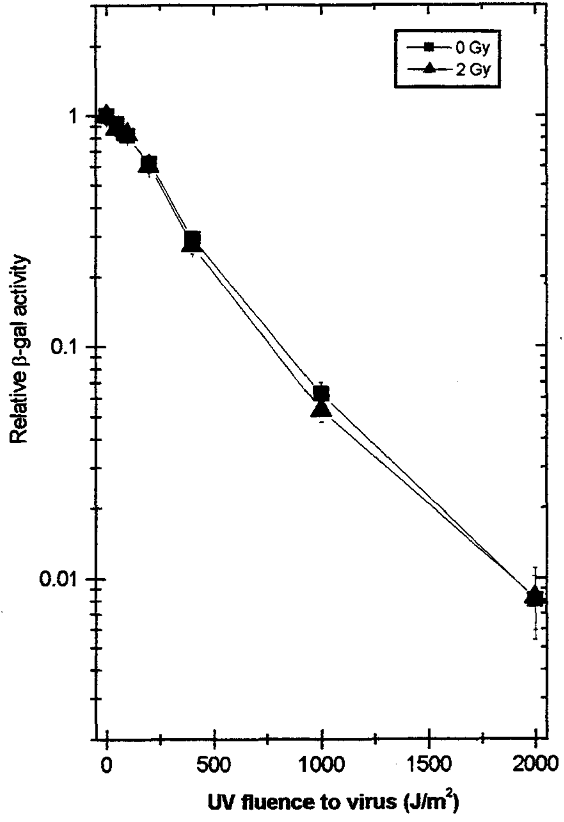
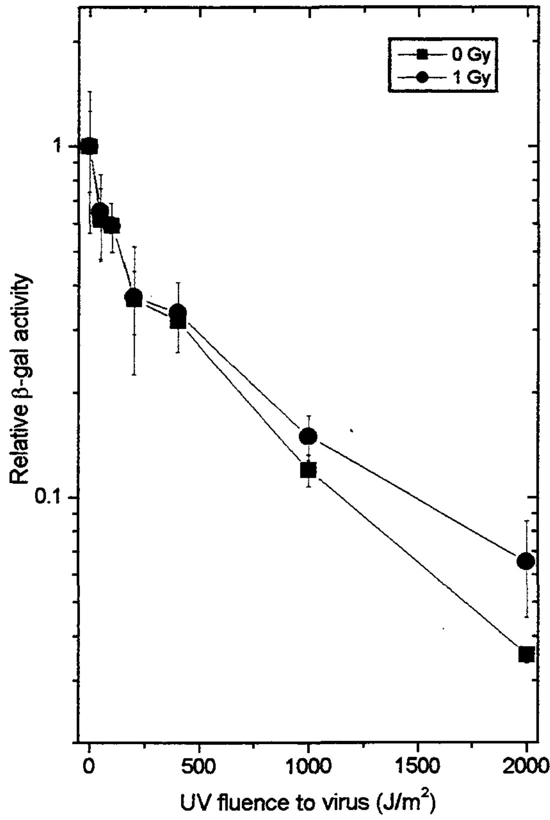
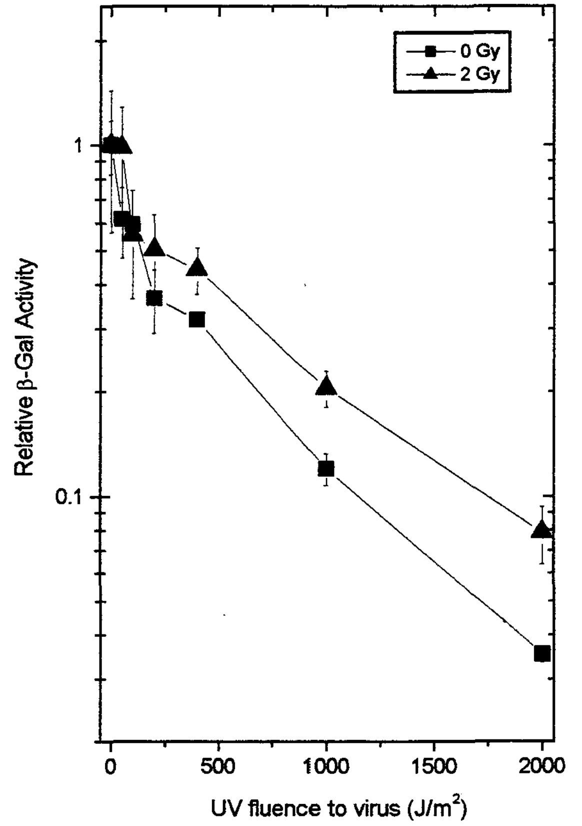
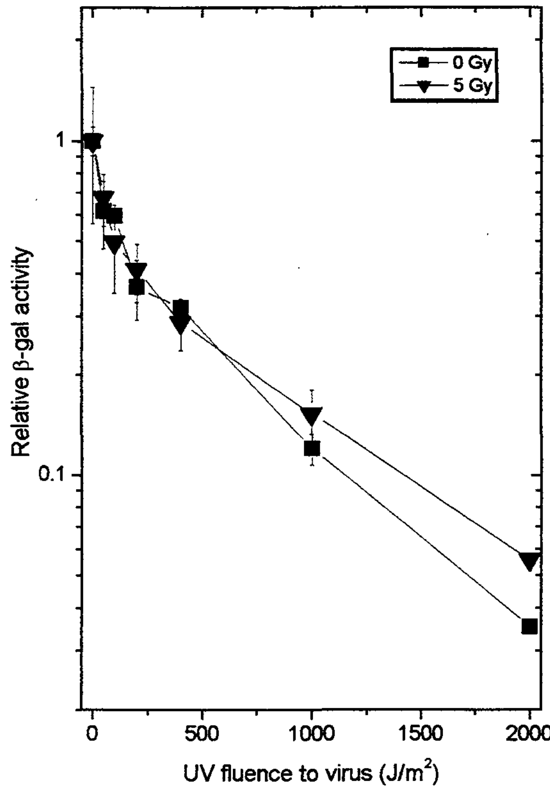

0 Gy
2 Gy
Relative β-gal activity
0.01
0.1
1
0
500
1000
1500
2000
UV fluence to virus (J/m²)

Figure 3-2. Typical result of γRER experiment suggesting possibility of enhanced reactivation in GM38A cells due to treatment of cells with γ-rays. Data points represent the mean of three replicates (± SE) from one experiment. Cells were treated with 0 Gy (squares), 1 Gy (circles), 2 Gy (up-triangles), 5 Gy (down-triangles) or 10 Gy (diamonds) γ-rays from a ^{137}Cs source at a dose rate of 0.17 Gy per minute.


0 Gy
1 Gy
Relative β-gal activity
UV fluence to virus (J/m²)


0 Gy
2 Gy
Relative β-Gal Activity
UV fluence to virus (J/m²)


0 Gy
5 Gy
Relative β-gal activity
UV fluence to virus (J/m²)

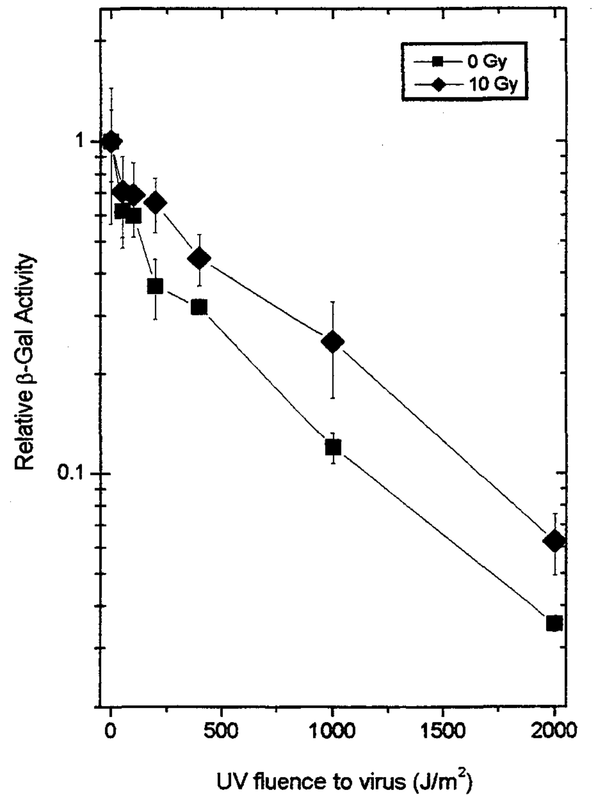
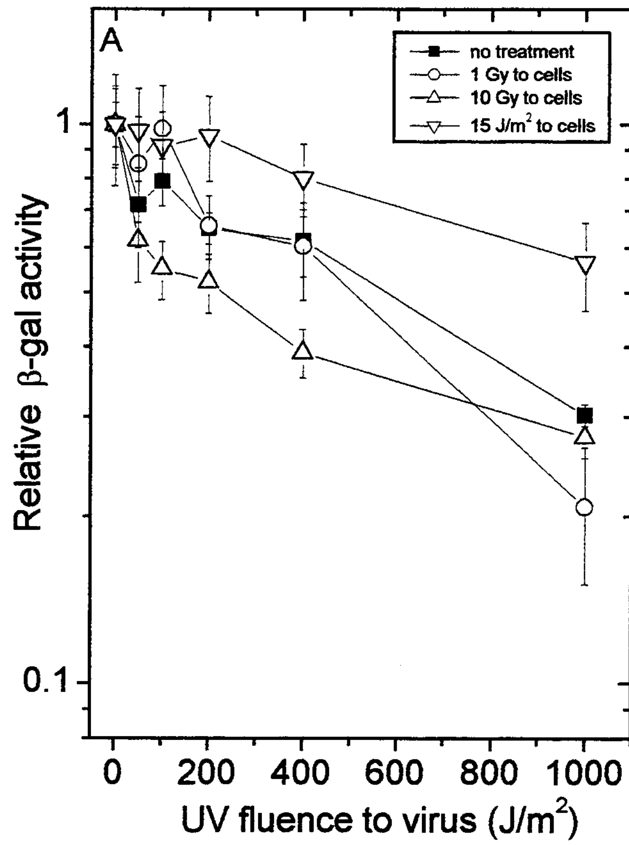

0 Gy
10 Gy
Relative β-Gal Activity
UV fluence to virus (J/m²)

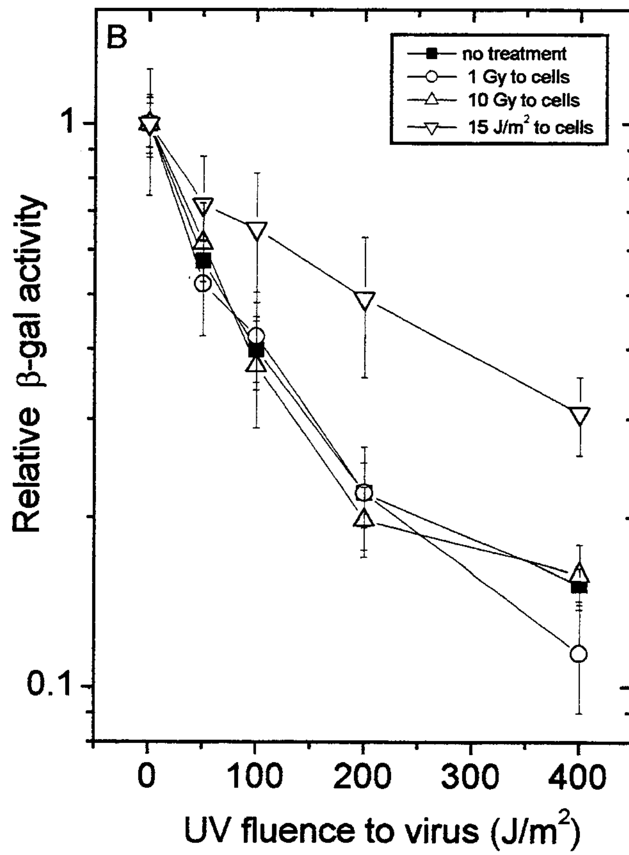
Table 3-1. Relative D_{37} values (± SE) for HCR of reporter gene expression in γ-ray treated cells. Relative D_{37} represents ratio of D_{37} for γ-ray treated cells / D_{37} for untreated cells.

CELL LINE/STRAIN	CELL TYPE	No. OF EXPERIMENTS	RELATIVE D_{37} (±SE)
GM9503	normal	5	0.64±0.06
GM38A	normal	4	0.72±0.09
GM969	normal	1	N/A[2]
GM677	XP-C	2	0.86±0.17
GM30A	XP-C	1	0.73
L132	normal lung epithelial	2	1.02±0.08
SCC25	squamous cell carcinoma	3	1.25±0.11
HeLa	ovarian carcinoma	3	0.79±0.08
AS99048	unknown (skin fibroblast cells)	1	2.27±0.90[3]

[2] survival curves were such that determination of a D_{37} value was not possible
[3] relative D_{37} values for each of two γ-ray doses not significantly > 1

Figure 3-3. Results of γRER experiment with UVER done in parallel (as positive control) suggesting no enhanced reactivation due to treatment of cells with γ-rays. Data points represent the mean of three replicates (± SE) from one experiment with GM9503 (normal fibroblast) cells (panel A) and GM30A (XP-C) cells (panel B). Cells were untreated (squares), treated with 1 Gy (circles), 10 Gy (up-triangles) γ-rays from a ^{137}Cs source at a dose rate of 0.17 Gy per minute, or 15 J/m^2 UV-C (diamonds) at fluence rate of 1 $J/m^2/s$.


A
no treatment
1 Gy to cells
10 Gy to cells
15 J/m2 to cells
Relative β-gal activity
1
0.1
0
200
400
600
800
1000
UV fluence to virus (J/m2)


B
no treatment
1 Gy to cells
10 Gy to cells
15 J/m2 to cells
Relative β-gal activity
1
0.1
0
100
200
300
400
UV fluence to virus (J/m2)

treatment of cells with 1, 5 or 10 Gy of γ-ray did not enhance HCR of β-gal activity from the UV-damaged Ad5HCMVsp1*lacZ*. Subsequent experiments were carried out at a later time with a plaque-purified virus prep (to minimise the proportion of replicating genomes), but they provided uncharacteristic HCR curves with marginal reproducibility. In these follow-up experiments the positive control UV-irradiated cells did not show UVER with either normal cell strains (GM9503, GM0969 or GM00038A) or XP-C cells (GM00677). In AS99048 cells, HCR also did not significantly change in either the UV or γ-treated cells compared to untreated cells. No further experimentation was carried out due to inconsistency of results, uncharacteristic HCR curves and lack of enhanced reactivation in the positive control UV-treated cells. It was later determined (Katharine Sodek, personal communication) that for this type of experiment, residual background β-gal activity in virus preps may play a large role in observed β-gal levels, and may mask reporter gene expression. For best results, preps of non-replicating virus used for reporter gene expression level experiments should be plaque purified to minimise the fraction of replicating genomes, and caesium chloride purified to eliminate residual β-gal activity.

4. Discussion

There are several possible reasons why we did not observe γRER in our experiments; the most obvious one is that there may not be any cross-talk between γ-ray-induced DNA repair mechanisms and UV-induced repair

mechanisms. However, previous work has shown that ionising radiation can enhance reactivation of UV-damaged virus in various mammalian systems (Bockstahler and Lytle, 1971; Bockstahler and Lytle, 1977; Hellman, et al., 1976; Jeeves and Rainbow, 1979; Jeeves and Rainbow, 1983a) suggesting that inducible UV-damage repair in response to γ-rays may indeed exist. Jeeves and Rainbow (1983) showed that both UV and γ-radiation of normal human fibroblast cells can enhance reactivation of UV-damaged adenovirus, however, time course experiments suggested that in normal fibroblasts either the induced mechanism or the induction process itself is, at least in part, different for γRER and UVER.

Other evidence for enhancement of excision repair by ionising radiation comes from Mallya and Sikpi (1998), in a study of γ-radiation-induced DNA repair in human lymphoblast cells. Mutation frequencies (assessed by an endpoint relying on functional gene product expression) were similar after transfecting UV-damaged plasmids for 48 hours into unirradiated $TP53^{wt}$ and $TP53^{mut}$ host cells. In cells irradiated with 0.5 Gy (2 hours prior to transfection with UV-damaged plasmids), mutation frequency of UV-damaged DNA decreased by 50% after transfection into $TP53^{wt}$ host cells, but was unaltered in $TP53^{mu}$ hosts compared to unirradiated cells. These results implicate TP53 (or TP53-regulated gene products) in IR modulation of UV-damage repair.

UV light, ionising radiation and other DNA-damaging agents elicit a complex set of cellular responses. The two responses that are best understood

are the signal transduction pathways initiating in the cell membrane (Canman and Kastan, 1996; Devary, et al., 1992; reviewed in Haimovitz-Friedman, 1998; Schwarz, 1998) and the TP53-mediated response pathway signalled by DNA damage (Kuerbitz, et al., 1992; reviewed in Szumiel, 1998; Ljungman, et al., 1999). TP53 (formerly known as p53) is a multifunctional protein that plays an important role in the integration of stress signalling in cells (Meek, 1998). In response to stress cues, TP53 induction may lead to growth arrest (allowing time for DNA repair) or apoptosis. Regulation of TP53 function is controlled by various phosphorylation events, which determine the protein's participation in site-specific DNA binding, activation and repression of expression of various genes, suppression of colony outgrowth and self-regulation (reviewed in Meek, 1998). The role of TP53 in nucleotide excision repair has been well established (reviewed in McKay, et al., 1999).

Although TP53 activity is increased in response to both UV and γ-ray treatment of mammalian, the timing of the responses to these two types of radiation is quite different (in the dose ranges that were used in this study). In a study of TP53 induction by these two modalities in mouse cells (Lu and Lane, 1993), TP53 activity increased due to ionising radiation (2.5 and 5 Gy) within 1 hour, peaked at 2 hours, and rapidly decreased by 3 hours. By studying the effect in cells with drug-inhibited repair, these investigators concluded that the presence of double-strand breaks is a major factor in the induction of TP53 by IR. Similar results for timing of TP53 induction by IR were seen in normal human

lymphoblasts (Siliciano, et al., 1997), but a delayed response was seen in double-strand break repair-deficient ATM cells, supporting the idea that a persistence of double-strand breaks is important in maintenance of elevated TP53 activity in IR-treated cells. The TP53 response to UV light is quite different. Lu and Lane (1993) exposed cells to 10 and 50 J/m^2 UV radiation, and saw a lag time of 2 hours for TP53 induction, followed by a steady increase in activity to a plateau at 12 hours, with a persistence of high TP53 activity for at least 30 hours. The authors noted that this timing roughly correlated with UV lesion removal. Siliciano and co-workers (1997) noted that TP53 activity after 10 J/m^2 of UV increases after 3 hours and persists at 6 hours (unlike activity due to IR). There is evidence suggesting that increased TP53 activity in UV-irradiated cells is triggered by DNA damage that blocks RNA polymerase II activity (Ljungman, et al., 1999).

Recent work by Hwang and co-workers (1999) has identified a function of TP53 in an inducible NER response. UV damaged DNA-binding protein (UV-DDB) is a protein heterodimer that binds UV-damaged DNA with a 500,000-fold preference over undamaged DNA. It is composed of a p125 subunit, and the inducible p48 subunit (mutated in NER-deficient DDB^- XPE cells) that is primarily controlled at the transcriptional level. There is a gradual increase in p48 mRNA (in a TP53-dependent manner) over a 16-24 hour period after 10 J/m^2 UV irradiation of cells, whereas treatment of cells with 2.5 Gy IR rapidly increases p48 mRNA to maximal levels within 1 hour after treatment. The level

and timing of p48 mRNA in both cases are consistent with accumulation of TP53. In addition, it was found that expression of TP53 increased GGR of CPDs in the primary human fibroblast cells investigated (Ford and Hanawalt, 1995; Ford and Hanawalt, 1997). By transiently expressing TP53 from a transfected vector, Hwang and co-workers (1999) were able to show that p48 transcription increased with induced TP53 expression in the absence of DNA damage. Thus, this study concluded that there is a TP53-dependent activation of GGR, which is mediated by p48 transcription.

In retrospect, evidence suggests that if IR does enhance reactivation of a reporter gene from adenoviral vectors in normal cells, we may not have observed it because it may occur at times earlier than those for UVER. Studies of γRER of V antigen formation of Ad (Jeeves and Rainbow, 1979), TP53 activation kinetics in response to IR and UV (Hwang, et al., 1999; Ljungman, et al., 1999; Lu and Lane, 1993; Siliciano, et al., 1997), and TP53-dependent inducible NER (Hwang, et al., 1999) all suggest that inducible TP53-dependent responses to IR are much faster than those to UV. If such a TP53-dependent enhancement of excision repair (due to treatment of cells with IR) is found in normal cells, this response may be absent in the SCC-25 and HeLa tumour cells. TP53-dependent heat-shock enhanced reactivation of the reporter gene in Ad5HCMVsp1*lacZ*, which is seen in normal fibroblast cells, was not seen in these two cell lines with aberrant TP53 activity (McKay and Rainbow, 1996).

In many of the experiments there was great variability between triplicate samples (at each UV dose to virus) within an experiment, and between experiments. This variability may be too great to determine small increases in HCR due to IR treatment of cells, if present. On the other hand, the QPCR technique described in Chapter 2 was very precise. The variability between duplicate PCR reactions and between experiments was small compared to that seen in experiments with the β-gal reporter gene assay. To illustrate the degree of precision of the QPCR assay in enhanced reactivation studies, lesion removal from the E4 fragment in UV treated normal lung fibroblast cells (mean of two experiments, mean of determinations at three UV doses per experiment) was determined to be 1.2-fold (P=0.002) the amount in untreated cells (see Chapter 2). Thus, the sensitive QPCR technique may be better for investigation of small increases in host-cell reactivation that may be present in γRER studies.

Summary

SUMMARY

Host cell reactivation assays are convenient for the study of DNA repair. Chapter 2 of this thesis describes development and application of a sensitive and convenient assay for DNA repair in a wide variety of mammalian cells, which combines the power of quantitative PCR with the advantages of host cell reactivation assays. The advantages of this assay include the precision, reproducibility and ease of PCR, the ability to specifically investigate several regions of the vector genome simultaneously, and a short period for results (compared to plaque formation, or colony-forming assays). The improvements in this technique compared to other HCR assays include the ability to directly assess initial lesion formation, and an endpoint that is directly based on lesion frequency in the DNA. Using quantitative PCR, the induction and repair of UV photoproducts was measured in a 2.6 kb region of the *lacZ* reporter gene inserted into the deleted E1 region of Ad5HCMVsp1*lacZ* and in a 2.8 kb region of the endogenous E4 region of the virus. Results show a single exponential decrease in PCR product with increasing UV fluence to the virus, which corresponds to approximately 1 cyclobutane pyrimidine dimer per PCR-terminating lesion. This assay was used to show a significant removal of UV photoproducts in Ad5HCMVsp1*lacZ* after infection of normal human fibroblasts, and significantly diminished lesion removal following infection of NER-deficient XP-C, XP-A and CS-B cells. This supports a model in which basal levels of lesion removal from this vector involve both the global genome repair (GGR) and transcription-

coupled repair (TCR) pathways. We also found an increased rate of lesion removal from the vector in irradiated normal lung fibroblast cells compared to unirradiated cells, providing evidence of an UV-inducible repair mechanism in human cells.

Investigation of the inducibility of nucleotide excision repair by ionising radiation, utilising HCR of β-gal reporter expression from Ad5HCMVsp1*lacZ* as an endpoint, did not show a significant enhancement in HCR in γ-ray irradiated cells compared to unirradiated cells. Future study into such a mechanism utilising the β-gal assay should be done with a plaque-purified, caesium chloride purified virus prep to minimise the experimental error. Alternatively, the quantitative PCR technique described in Chapter 2 may provide the necessary sensitivity needed to detect small changed in HCR, if present. However, evidence suggests that if IR does enhance an UV-damage repair mechanism, this enhancement may be short-lived, and it may occur at times earlier than those for UVER. This effect (if present) might not even be detectable by viral HCR studies, since the enhancement may subside by the time the UV-damaged virus becomes accessible to the nuclear repair machinery.

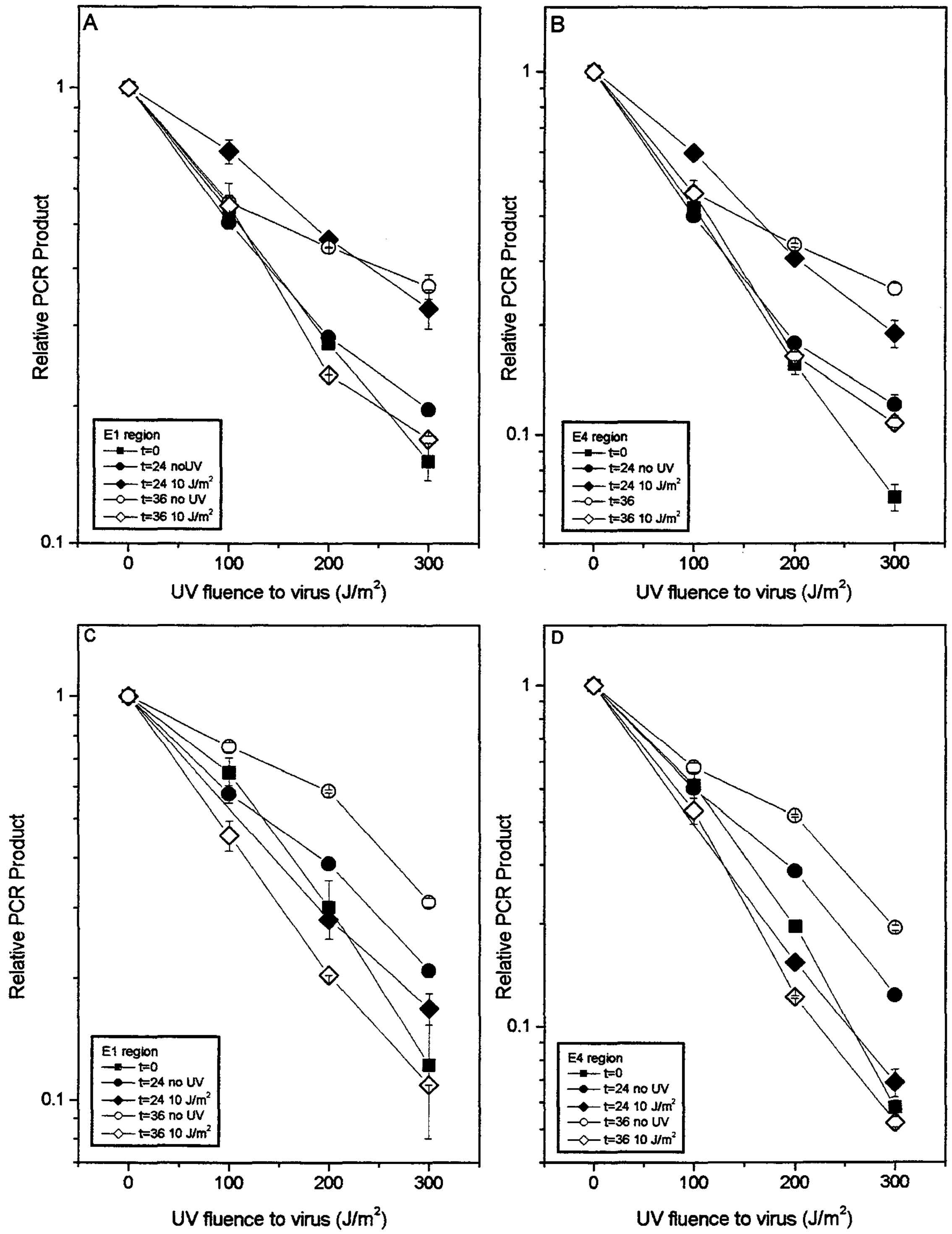
Appendix A

Investigation of UV-Inducible HCR Response in XP-C Cells by QPCR

Previous work from our laboratory group showed that UV-C light enhances reactivation of a reporter gene in XP-C fibroblast cells infected with UV-damaged Ad5HCMVsp1*lacZ* (Francis and Rainbow, 1999), suggesting a TCR-dependent inducible repair response. Two experiments were conducted using the QPCR technique (described in Chapter 2) to investigate the inducibility of lesion removal in XP-C cells (GM00510); these experiments were carried out in parallel with the normal lung fibroblast cells (IMR90). Results for these two experiments with XP-C cells are shown in Figure A-1. Initial survival and HCR of PCR product amount was investigated for both the *lacZ* fragment (in the E1 deleted region) and E4 region of Ad5 HCMVsp1*lacZ*. Results of the first experiment (Figures A-1, panels A & B) showed an inducible response at t=24 h in UV irradiated cells compared to unirradiated cells, but decreased HCR at t=36 h in irradiated cells compared to unirradiated cells. In the second experiment (Figures A-1, panels C & D), UV irradiated cells showed decreased HCR compared to unirradiated cells in UV-irradiated cells at both the t=24 h and t=36 h time points. Both experiments utilised the same virus prep for infection, similar (but independent) UV irradiation of the virus, the same UV source for irradiation, similar experimental conditions, and similar (but independent) UV-irradiation of host cells.

The cause of this observed decreased HCR of PCR product (below levels in unirradiated cells) in UV-irradiated XP-C cells has not been determined. One

Figure A-1. Results of two experiments using the QPCR technique to assess inducibility of lesion removal from Ad5HCMVsp1*lacZ* in GM510 (XP-C) cells by UV light. The first experiment showed an inducible response (increased HCR) in both the E1 (panel A) and E4 (panel B) fragments at t=24 hours after infection of cells (but decreased HCR at t=36 h) with 10 J/m^2 UVC treatment of cells (24 hours prior to infection). In the second experiment, there was a decrease in HCR of PCR product from both the E1 (panel C) and E4 (panel D) fragments in UV-treated cells compared to untreated cells at both time points.


A
Relative PCR Product
UV fluence to virus (J/m²)
E1 region
t=0
t=24 noUV
t=24 10 J/m²
t=36 no UV
t=36 10 J/m²
B
E4 region
t=0
t=24 no UV
t=24 10 J/m²
t=36
t=36 10 J/m²
C
E1 region
t=0
t=24 no UV
t=24 10 J/m²
t=36 no UV
t=36 10 J/m²
D
E4 region
t=0
t=24 no UV
t=24 10 J/m²
t=36 no UV
t=36 10 J/m²

possible explanation for this effect is an apoptotic response in UV-treated cells infected with Ad5HCMVsp1*lacZ* containing UV lesions. There is evidence supporting the idea that UV-light induced apoptosis is triggered by a blockage of RNA polymerase II (Ljungman and Zhang, 1996). Although XP-C cells are NER-deficient (Cleaver, 1968), they retain proficient TCR (Venema, et al., 1991), which specifically recognises and removes lesions in human genes transcribed by RNAPII (Hanawalt, 1994) at a rate faster than the GGR pathway (Bohr, et al., 1985). The TCR pathway also removes UV-induced lesions in actively transcribed genes faster than in the rest of the genome (Bohr, 1987). It is believed that proficient TCR in XP-C cells removes lesions in the transcribed strand sufficiently, so that apoptosis is not as easily triggered (by RNAP II blockage) in these cells as in other NER deficient cells (Ljungman and Zhang, 1996; McKay, et al., 1997b). However, removal of lesions from Ad5HCMVsp1*lacZ* appears to be different from lesion removal in endogenous human genes in XP-C cells. These cells appear to have a deficiency in lesion removal from the actively transcribed *lacZ* gene in Ad5HCMVsp1*lacZ* compared to normal cells (Francis and Rainbow, 1999), and they do not show preferential lesion removal from active genes over less transcribed areas of the vector (Chapter 2). Therefore, there may be a sufficient persistence of lesions in the active *lacZ* gene of Ad5HCMVsp1*lacZ* to sufficiently block RNA polymerase II to induce an apoptotic response in UV-irradiated XP-C cells.

Previous study of UV-enhanced reactivation in XP-C cells (Francis and Rainbow, 1999) assessed reporter gene activity as an endpoint for host-cell reactivation. In this type of experiment, cumulative β-gal protein product is quantitated and used as an indicator of cellular repair activity. Thus, any degradation of DNA due to apoptosis (after repair has occurred, and *lacZ* transcribed) would not affect the observed endpoint. In contrast, a PCR-based technique such as the one presented in Chapter 2 would detect DNA degradation by an apoptotic response. Another difference to note between the two assays is the timing between UV treatment of cells and repair assessment. In the β-gal assay, cells are infected immediately after UV-irradiation. In the QPCR assay, there is a 24-hour delay between treatment of cells, and infection of cells. Thus, β-gal assay assesses repair at the 40-hour time point (post-UV treatment of cells), whereas at the 36-hour time point in the QCR assay, the cells are being harvested 60 hours post-UV treatment, allowing more time for UV-induced apoptosis (if present). Further study is necessary to determine whether apoptosis is the cause of this effect.

Appendix B

Construction of an Internal Standard for Competitive PCR

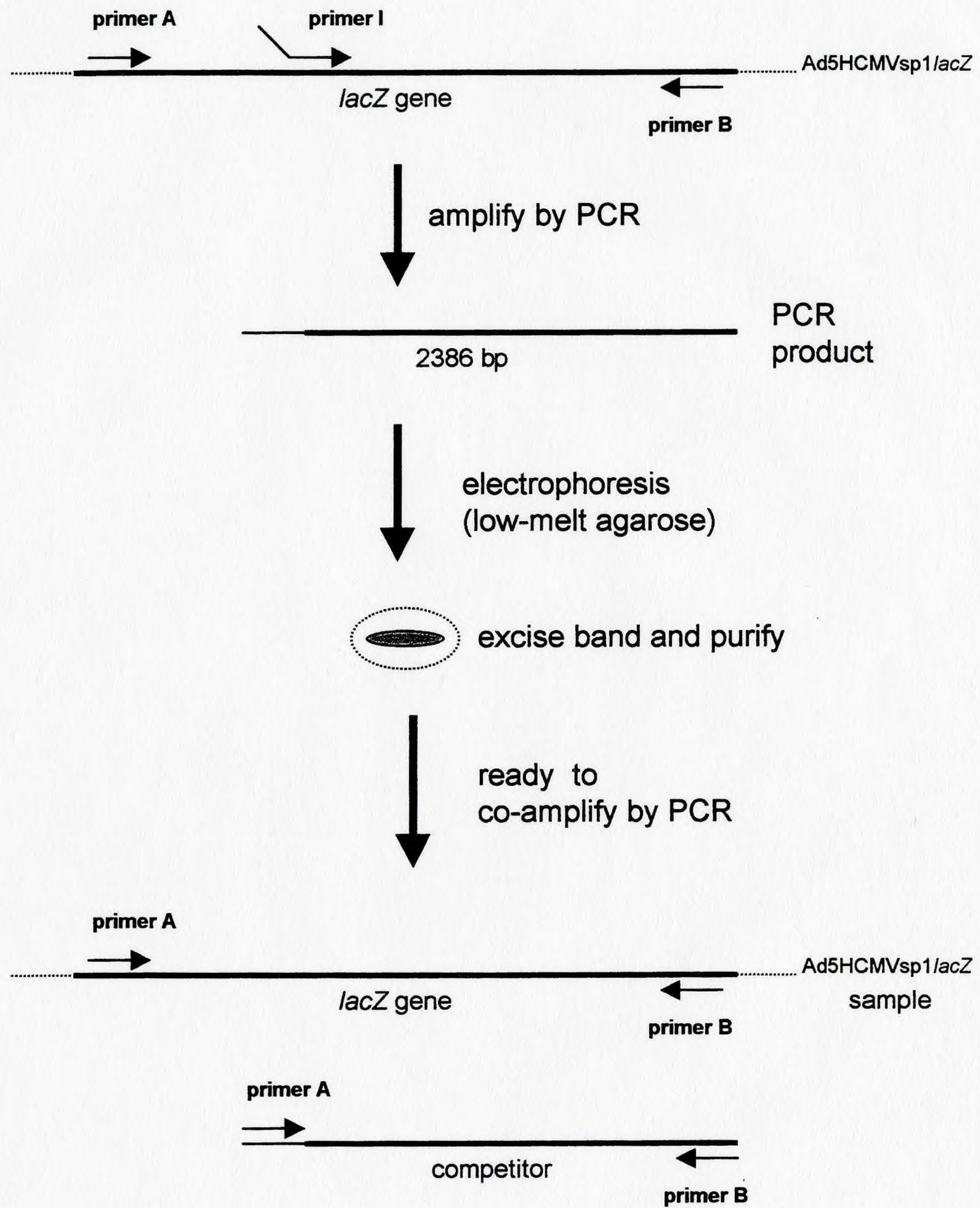
There are a variety of methods to absolutely quantify PCR product, to determine the exact number of copies of nucleic acid target. These techniques commonly include the use of an external standard, an internal standard, or a limiting dilution (Cross, 1995; Ferre, et al., 1994; Orlando, et al., 1998). Techniques utilising an internal standard are particularly useful, since sequence similarity of the internal standard and target, as well as potential use of the same primers for amplification of both fragments, ensure similar amplification efficiencies of the two fragments, even past the exponential phase of PCR amplification (Cottrez, et al., 1994). The use of internal standards for quantitation of virus by PCR has previously been reported (Natarajan, et al., 1994; Telenti, et al., 1992). Several methods for construction of an internal control standard have been described (Jenkins and Parry, 1997; Jin, et al., 1994; Natarajan, et al., 1994). I have adapted the method of Jin and co-workers (1994) to construct an internal control fragment for amplification with *lacZ* (in deleted E1 region of Ad5HCMVsp1*lacZ*) due to the simplicity of this technique, as well as the ability to co-amplify the internal standard and template of interest using the same primer pair.

The absolute quantitation of Ad5HCMVsp1*lacZ* has several applications. These include determination of infectivity of virus in various cell strains, and comparisons of infectivity under various conditions (e.g. non-treated cells vs. UV-irradiated cells). Also, use of an internal standard has a potential application

in future DNA repair studies similar to those outlined in Chapter 2. By including the same amount of internal standard in all PCR amplifications of experimental samples, any differences in amplification efficiency between samples could be corrected for by comparison of internal standard amplification. However, for UV irradiated samples, it is possible that amplification efficiency of the internal control may increase as amplification of viral DNA decreases due to PCR-blocking lesions induced by UV light. A solution to this potential problem may be to UV irradiate the internal standard with the same fluence as the viral samples.

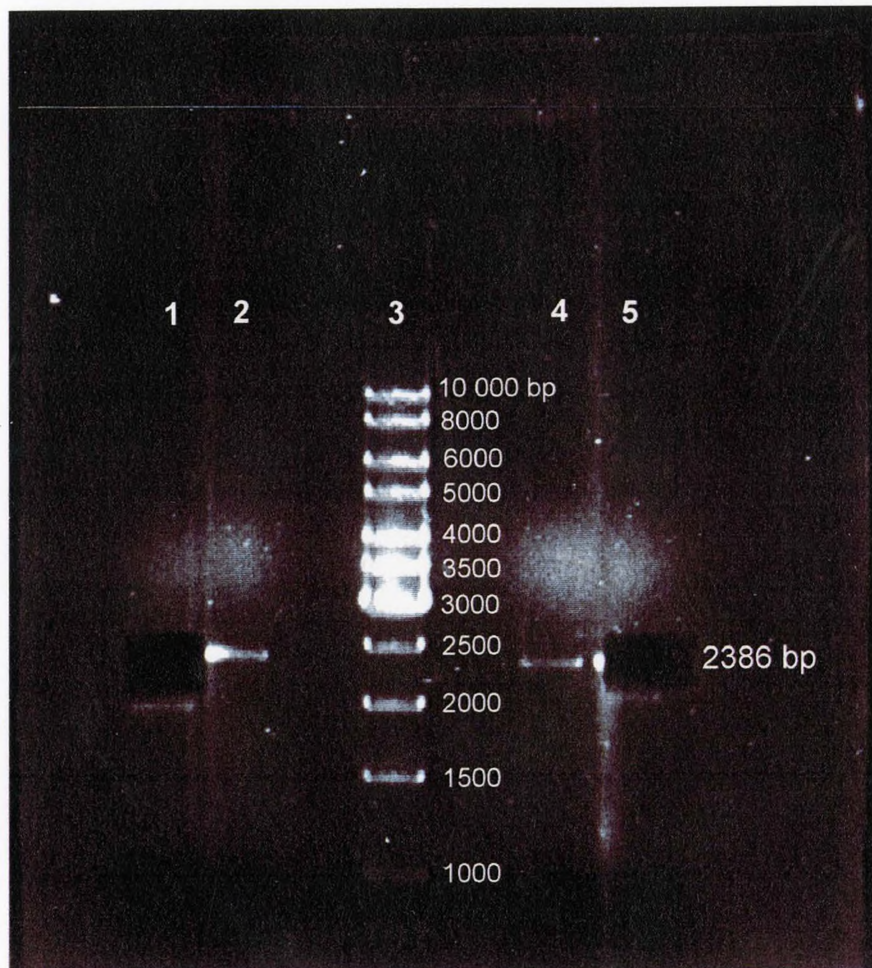
The method I used to construct the internal standard is outlined in Figure B-1. An internal primer (primer I) was designed to anneal to the *lacZ* sequence between primers A and B (used for QPCR described in Chapter 2) to generate a fragment for use in competitive PCR. Primer I was designed to produce an internal control fragment that can be distinguished from the *lacZ* fragment (amplified utilising primer set AB) when electrophoresed on a 0.8% agarose gel. The 38 base long primer I has the sequence: 5′-GGT GTG GGC CAT AAT TCA ATT CCA TCA ACG GTA ATC GCC A-3′. The 18 bases at the 3′ end of this primer are identical to bases 1022 through 1039 in Ad5HCMVsp1*lacZ*, while the 22 bases at the 5′ end have the same sequence as primer A. Thus, primers I and B were included in a PCR reaction utilising Ad5HCMVsp1*lacZ* as a template, to generate the competitor template. PCR amplification was performed using a master mix as described in Chapter 2, substituting Primer I for Primer A.

Figure B-1. Scheme for the synthesis of deleted internal standard for quantitative PCR of Ad5HCMVsp1*lacZ*. Individual steps are described in the text. (Adapted from Jin *et al*, 1994).


primer A
primer I
Ad5HCMVsp1*lacZ*
lacZ gene
primer B
amplify by PCR
PCR
product
2386 bp
electrophoresis
(low-melt agarose)
excise band and purify
ready to
co-amplify by PCR
primer A
Ad5HCMVsp1*lacZ*
sample
lacZ gene
primer B
primer A
competitor
primer B

The thermal cycler was programmed to operate with: an initial 94°C for 1 min, followed by 27 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 3 min and ending with a final 3 minutes at 72°C. Reactions were kept at 4°C until purification and analysis. PCR product was purified (Qiagen QIAquick PCR purification kit) and electrophoresed on a 0.8% low-melt agarose gel (see figure B-2) to verify PCR amplification and product size (2386 bp). PCR product was excised from the gel, and purified using a phenol:chloroform extraction (Sambrook, et al., 1989). Upon quantitation of product yield, this competitive template is ready to be used in QPCR to quantify the number of copies of *lacZ* gene in a sample, carried in Ad5HCMVsp1*lacZ*.

Figure B-2. Agarose gel analysis of *lacZ* deletion construct. Lanes 1, 2, 4 and 5 contained the 2386 bp product. Lane 3 contains a 1 kb ladder (MBI Fermentas). Construct was isolated from lanes 1 and 5 by lining up the lanes with ethidium bromide (EtBr) stained (and visualised on UV transilluminator) middle gel fragment, containing lanes 2 and 4. Outside gel segments containing Lanes 1 and 5 were EtBr stained after excision to ensure that the construct was retrieved.


1
2
3
4
5
10 000 bp
8000
6000
5000
4000
3500
3000
2500
2000
1500
1000
2386 bp

References

Abrahams, P.J., B.A. Huitema and A.J. van der EB (1984) Enhanced reactivation and enhanced mutagenesis of herpes simplex virus in normal human and xeroderma pigmentosum cells, Molecular And Cellular Biology, 4, 2341-6.

Abrahams, P.J., A.A. van der Kleij, R. Schouten and A.J. van der Eb (1988) Absence of induction of enhanced reactivation of herpes simplex virus in cells from xeroderma pigmentosum patients without skin cancer, Cancer Research, 48, 6054-7.

Adler, V., S.Y. Fuchs, J. Kim, A. Kraft, M.P. King, J. Pelling and Z. Ronai (1995) jun-NH2-terminal kinase activation mediated by UV-induced DNA lesions in melanoma and fibroblast cells, Cell Growth And Differentiation, 6, 1437-46.

Andrews, A., S. Barrett and J. Robbins (1978) Xeroderma pigmentosum neurological abnormalities correlate with colony-forming ability after ultraviolet radiation., Proceeding of the National Academies of Science of the United States of America, 75, 1984-8.

Araujo, S., F. Tirode, F. Coin, H. Pospiech, J. Syvaoja, M. Stucki, U. Hubscher, J. Egly and R. Wood (2000) Nucleotide excision repair of DNA with recombinant human proteins: definition of the minimal set of factors, active forms of TFIIH, and modulation by CAK, Genes and Development, 14(3), 349-59.

Arnold, W.R. and A.J. Rainbow (1996) Host cell reactivation of irradiated adenovirus in UV-sensitive Chinese hamster ovary cell mutants, Mutagenesis, 11, 89-94.

Asami, S., H. Manabe, J. Miyake, Y. Tsurudome, T. Hirano, R. Yamaguchi, H. Itoh and H. Kasai (1997) Cigarette smoking induces an increase in oxidative DNA damage, 8-hydroxydeoxyguanosine, in a central site of the human lung, Carcinogenesis, 18, 1763-6.

Balajee, A.S., A. May, G.L. Dianov, E.C. Friedberg and V.A. Bohr (1997) Reduced RNA polymerase II transcription in intact and permeabilized Cockayne syndrome group B cells, Proceedings of The National Academy of Sciences of The United States of America, 94, 4306-11.

Bender, K., C. Blattner, A. Knebel, M. Iordanov, P. Herrlich and H.J. Rahmsdorf (1997) UV-induced signal transduction, Journal of Photochemistry and Photobiology. B, Biology, 37, 1-17.

Bennett, C.B. and A.J. Rainbow (1988) Enhanced reactivation and mutagenesis of UV-irradiated adenovirus in normal human fibroblasts, Mutagenesis, 3, 157-64.

Bockstahler, L.E., K.F. Haynes and J.E. Stafford (1976) Investigation of radiation enhanced reactivation of cytoplasmic replicating human virus. pp. 203-9, In: Hazzard Dg, Ed . Symposium on Biological Effects and Measurement of Light Sources. Qh 651 S983s 1976., UNITED STATES.

Bockstahler, L.E. and C.D. Lytle (1970) Ultraviolet light enhanced reactivation of a mammalian virus, Biochemical and Biophysical Research Communications, 41, 184-9.

Bockstahler, L.E. and C.D. Lytle (1971) X-ray-enhanced reactivation of ultraviolet-irradiated human virus, Journal of Virology, 8, 601-2.

Bockstahler, L.E. and C.D. Lytle (1977) Radiation enhanced reactivation of nuclear replicating mammalian viruses, Photochemistry and Photobiology, 25, 477-82.

Bohr, V.A. (1987) Preferential DNA repair in active genes, Danish Medical Bulletin, 34, 309-20.

Bohr, V.A. (1991) Gene specific damage and repair after treatment of cells with UV and chemotherapeutical agents, Advances in Experimental Medicine and Biology, 283, 225-33.

Bohr, V.A., C.A. Smith, D.S. Okumoto and P.C. Hanawalt (1985) DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall, Cell, 40, 359-69.

Boyce, R.P. and P. Howard-Flanders (1964) Proceedings of The National Academy of Sciences of The United States of America, 51, 293-300.

Boyle, J.M. and R.B. Setlow (1970) Correlations between host-cell reactivation, ultraviolet reactivation and pyrimidine dimer excision in the DNA of bacteriophage lambda, Journal of Molecular Biology, 51, 131-44.

Bregman, D.B., R. Halaban, A.J. van Gool, K.A. Henning, E.C. Friedberg and S.L. Warren (1996) UV-induced ubiquitination of RNA polymerase II: a novel modification deficient in Cockayne syndrome cells, Proceedings of The National Academy of Sciences of The United States of America, 93, 11586-90.

Bronner, C.E., S.M. Baker, P.T. Morrison, G. Warren, L.G. Smith, M.K. Lescoe, M. Kane, C. Earabino, J. Lipford, A. Lindblom and e. al (1994) Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer, Nature, 368, 258-61.

Canman, C.E. and M.B. Kastan (1996) Signal transduction. Three paths to stress relief [news; comment], Nature, 384, 213-4.

Chakalova, L. and G. Russev (1998a) Comparison of repair activity in different genomic regions, Acta Biochimica Polonica, 45, 173-81.

Chakalova, L. and G. Russev (1998b) Quantitative polymerase chain reaction assay for DNA repair within defined genomic regions, Mutation Research, 407, 147-55.

Chandrasekhar, D. and B. Van Houten (1994) High resolution mapping of UV-induced photoproducts in the Escherichia coli lacI gene. Inefficient repair of the non-transcribed strand correlates with high mutation frequency, Journal of Molecular Biology, 238, 319-32.

Chang, L.C., H.M. Sheu, Y.S. Huang, T.R. Tsai and K.W. Kuo (1999) A novel function of emodin: enhancement of the nucleotide excision repair of UV- and cisplatin-induced DNA damage in human cells, Biochemical Pharmacology, 58, 49-57.

Chu, G. (1997) Double strand break repair, Journal of Biological Chemistry, 272, 24097-100.

Cleaver, J.E. (1968) Defective repair replication of DNA in xeroderma pigmentosum, Nature, 218, 652-6.

Cleaver, J.E. and K.H. Kraemer (1995) Xeroderma Pigmentosum and Cockayne Syndrome, in: C.R. Scriver et. al. (Eds.), The Metabolic and Molecular Bases of Inherited Disease, Mcgraw-Hill, Inc., United States of America.

Coin, F., E. Bergmann, A. Tremeau-Bravard and J.M. Egly (1999) Mutations in XPB and XPD helicases found in xeroderma pigmentosum patients impair the transcription function of TFIIH, EMBO Journal, 18, 1357-66.

Cottrez, F., C. Auriault, A. Capron and H. Groux (1994) Quantitative PCR: validation of the use of a multispecific internal control, Nucleic Acids Research, 22, 2712-3.

Cross, N.C.P. (1995) Quantitative PCR Techniques and Applications, British Journal Of Haematology, 89, 693-697.

Davis, K., D.J. Tomkins and A.J. Rainbow (1996) Roberts syndrome fibroblasts showing cisplatin hypersensitivity have normal host cell reactivation of cisplatin-treated adenovirus and normal capacity of cisplatin-treated cells for adenovirus DNA synthesis, Somatic Cell and Molecular Genetics, 22, 393-402.

Day, R.S. (1974a) Cellular reactivation of ultraviolet-irradiated human adenovirus 2 in normal and xeroderma pigmentosum fibroblasts, Photochemistry and Photobiology, 19, 9-13.

Day, R.S. (1974b) Studies on repair of adenovirus 2 by human fibroblasts using normal, xeroderma pigmentosum, and xeroderma pigmentosum heterozygous strains, Cancer Research, 34, 1965-70.

Day, R.S., C.H. Ziolkowski and M. DiMattina (1981) Decreased host cell reactivation of UV-irradiated adenovirus 5 by fibroblasts from Cockayne's syndrome patients, Photochemistry and Photobiology, 34, 603-7.

Defais, M.J., P.C. Hanawalt and A.R. Sarasin (1983) Viral Probes for DNA Repair, Advances in Radiation Biology, Academy Press, Inc., pp. 1-37.

Devary, Y., R.A. Gottlieb, T. Smeal and M. Karin (1992) The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases, Cell, 71, 1081-91.

Dianov, G.L., J.F. Houle, N. Iyer, V.A. Bohr and E.C. Friedberg (1997) Reduced RNA polymerase II transcription in extracts of cockayne syndrome and xeroderma pigmentosum/Cockayne syndrome cells, Nucleic Acids Research, 25, 3636-42.

Drouin, R. and J.P. Therrien (1997) UVB-induced cyclobutane pyrimidine dimer frequency correlates with skin cancer mutational hotspots in p53, Photochemistry and Photobiology, 66, 719-26.

Eckardt-Schupp, F. and C. Klaus (1999) Radiation inducible DNA repair processes in eukaryotes, Biochimie, 81, 161-71.

Eller, M.S., T. Maeda, C. Magnoni, D. Atwal and B.A. Gilchrest (1997) Enhancement of DNA repair in human skin cells by thymidine dinucleotides: evidence for a p53-mediated mammalian SOS response., Proceedings of The National Academy of Sciences of The United States of America, 94, 12627-32.

Eller, M.S., K. Ostrom and B.A. Gilchrest (1996) DNA damage enhances melanogenesis, Proceedings of The National Academy of Sciences of The United States of America, 93, 1087-92.

Eustice, D.C., P.A. Feldman, A.M. Colberg-Poley, R.M. Buckery and R.H. Neubauer (1991) A sensitive method for the detection of beta-galactosidase in transfected mammalian cells, Biotechniques, 11, 739-40, 742-3.

Evans, M.K., J.H. Robbins, M.B. Ganges, R.E. Tarone, R.S. Nairn and V.A. Bohr (1993) Gene-specific DNA repair in xeroderma pigmentosum complementation groups A, C, D, and F. Relation to cellular survival and clinical features, Journal of Biological Chemistry, 268, 4839-47.

Ferre, F., A. Marchese, P. Pezzoli, S. Griffin, E. Buxton and V. Boyer (1994) Quantitative PCR: An Overview, in: K.B. Mullis, F. Ferre and R.A. Gibbs (Eds.), The Polymerase Chain Reaction, Birkhauser, Boston, pp. 67-88.

Fishel, R. and R.D. Kolodner (1995) Identification of mismatch repair genes and their role in the development of cancer, Current Opinion In Genetics and Development, 5, 382-95.

Ford, J.M. and P.C. Hanawalt (1995) Li-Fraumeni syndrome fibroblasts homozygous for p53 mutations are deficient in global DNA repair but exhibit normal transcription-coupled repair and enhanced UV resistance, Proceedings of The National Academy of Sciences of the United States of America, 92, 8876-80

Ford, J.M. and P.C. Hanawalt (1995) Expression of wild-type p53 is required for efficient global genomic nucleotide excision repair in UV-irradiated human fibroblasts, Journal of Biological Chemistry, 272, 28073-80.

Foray, N., A. Priestley, G. Alsbeih, C. Badie, E.P. Capulas, C.F. Arlett and E.P. Malaise (1997) Hypersensitivity of ataxia telangiectasia fibroblasts to ionizing radiation is associated with a repair deficiency of DNA double-strand breaks, International Journal of Radiation Biology, 72, 271-83.

Francis, M.A. and A.J. Rainbow (1999) UV-enhanced reactivation of a UV-damaged reporter gene suggests transcription-coupled repair is UV-inducible in human cells, Carcinogenesis, 20, 19-26.

Friedberg, E.C. (1995) DNA repair and mutagenesis, ASM Press, Washington, D.C.

Friedberg, E.C. (1996) Relationships between DNA repair and transcription, Annual Review of Biochemistry, 65, 15-42.

Frit, P., E. Bergmann and J.M. Egly (1999) Transcription factor IIH: a key player in the cellular response to DNA damage, Biochimie, 81, 27-38.

Gao, S., R. Drouin and G.P. Holmquist (1994) DNA repair rates mapped along the human PGK1 gene at nucleotide resolution [see comments], Science, 263, 1438-40.

Gözükara, E.M., C.N. Parris, C.A. Weber, E.P. Salazar, M.M. Seidman, J.F. Watkins, L. Prakash and K.H. Kraemer (1994) The human DNA repair gene, ERCC2 (XPD), corrects ultraviolet hypersensitivity and ultraviolet hypermutability of a shuttle vector replicated in xeroderma pigmentosum group D cells, Cancer Research, 54, 3837-44.

Graham, F.L. and L. Prevec (1991) Manipulation of Adenovirus Vectors, in: E.J. Murray (Ed.), Methods in Molecular Biology, The Humana Press Inc., Clifton, NJ, pp. 109-127.

Graham, F.L., J. Smiley, W.C. Russell and R. Nairn (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5, Journal of General Virology, 36, 59-74.

Grawunder, U., N. Finnie, S.P. Jackson, B. Riwar and R. Jessberger (1996) Expression of DNA-dependent protein kinase holoenzyme upon induction of lymphocyte differentiation and V(D)J recombination, European Journal of Biochemistry, 241, 931-40.

Haimovitz-Friedman, A. (1998) Radiation-induced signal transduction and stress response, Radiation Research, 150, S102-8.

Hall, E.J. (1994) Radiobiology for the Radiologist, J.B. Lippincott Company, Philadelphia.

Hanawalt, P.C. (1991) Heterogeneity of DNA repair at the gene level, Mutation Research, 247, 203-11.

Hanawalt, P.C. (1994) Transcription-coupled repair and human disease, Science, 266, 1957-8.

He, Z., L.A. Henricksen, M.S. Wold and C.J. Ingles (1995) RPA involvement in the damage-recognition and incision steps of nucleotide excision repair, Nature, 374, 566-9.

Hellman, K.B., C.D. Lytle and L.E. Bockstahler (1976) Radiation enhanced reactivation of herpes simplex virus: effect of caffeine, Mutation Research, 36, 249-56.

Henning, K.A., L. Li, N. Iyer, L.D. McDaniel, M.S. Reagan, R. Legerski, R.A. Schultz, M. Stefanini, A.R. Lehmann, L.V. Mayne and e. al (1995) The Cockayne syndrome group A gene encodes a WD repeat protein that interacts with CSB protein and a subunit of RNA polymerase II TFIIH, Cell, 82, 555-64.

Hwang, B.J., J.M. Ford, P.C. Hanawalt and G. Chu (1999) Expression of the p48 xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair, Proceedings of The National Academy of Sciences of The United States of America, 96, 424-8.

Hwang, B.J., S. Toering, U. Francke and G. Chu (1998) p48 Activates a UV-damaged-DNA binding factor and is defective in xeroderma pigmentosum group E cells that lack binding activity, Molecular and Cellular Biology, 18, 4391-9.

Jeeves, W.P. and A.J. Rainbow (1979) Gamma-ray-enhanced reactivation of UV-irradiated adenovirus in normal human fibroblasts, Mutation Research, 60, 33-41.

Jeeves, W.P. and A.J. Rainbow (1983a) Gamma-ray-enhanced reactivation of irradiated adenovirus in Xeroderma pigmentosum and Cockayne syndrome fibroblasts, Radiation Research, 94, 480-98.

Jeeves, W.P. and A.J. Rainbow (1983b) U.V. enhanced reactivation of U.V.-and gamma-irradiated adenovirus in normal human fibroblasts, International Journal of Radiation Biology and Related Studies in Physics, Chemistry and Medicine, 43, 599-623.

Jenkins, G.J.S. and J.M. Parry (1997) A novel method for the construction of an internal standard molecule for quantitative PCR, Elsevier Trends Journals Technical Tips Online.

Jennerwein, M.M. and A. Eastman (1991) A polymerase chain reaction-based method to detect cisplatin adducts in specific genes, Nucleic Acids Research, 19, 6209-14.

Jin, C.F., M. Mata and D.J. Fink (1994) Rapid construction of deleted DNA fragments for use as internal standards in competitive PCR, PCR Methods and Applications, 3, 252-5.

Jiricny, J. (1998) Replication errors: cha(lle)nging the genome, EMBO Journal, 17, 6427-36.

Kalinowski, D.P., S. Illenye and B. Van Houten (1992) Analysis of DNA damage and repair in murine leukemia L1210 cells using a quantitative polymerase chain reaction assay, Nucleic Acids Research, 20, 3485-94.

Karmarkar, P., A. Leer-van Hoffen, A.T. Natarajan, A.A. van Zeeland and L.H. Mullenders (1998) UV-induced alterations in the spatial distribution of the basal transcription factor TFIIH: an early event in nucleotide excision repair, Mutation Research, 404, 129-31.

Kraemer, K.H. (1997) Sunlight and skin cancer: another link revealed [comment], Proceedings of The National Academy of Sciences of The United States of America, 94, 11-4.

Kraemer, K.H., M.M. Lee and J. Scotto (1984) DNA repair protects against cutaneous and internal neoplasia: evidence from xeroderma pigmentosum, Carcinogenesis, 5, 511-4.

Kuerbitz, S.J., B.S. Plunkett, W.V. Walsh and M.B. Kastan (1992) Wild-type p53 is a cell cycle checkpoint determinant following irradiation, Proceedings of The National Academy of Sciences of The United States of America, 89, 7491-5.

Lambert, W.C. and M.W. Lambert (1991) Diseases Associated With DNA and Chromosomal Instability, in: J.A. Alper (Ed.), Genetic Disorders of the Skin, Mosby-Year Book, USA, pp. 320-354.

Léveillard, T., L. Andera, N. Bissonnette, L. Schaeffer, L. Bracco, J.M. Egly and B. Wasylyk (1996) Functional interactions between p53 and the TFIIH complex are affected by tumour-associated mutations, EMBO Journal, 15, 1615-24.

Li, L., X. Lu, C.A. Peterson and R.J. Legerski (1995) An interaction between the DNA repair factor XPA and replication protein A appears essential for nucleotide excision repair, Molecular and Cellular Biology, 15, 5396-402.

Ljungman, M. and F. Zhang (1996) Blockage of RNA polymerase as a possible trigger for u.v. light-induced apoptosis, Oncogene, 13, 823-31.

Ljungman, M., F. Zhang, F. Chen, A.J. Rainbow and B.C. McKay (1999) Inhibition of RNA polymerase II as a trigger for the p53 response, Oncogene, 18, 583-92.

Lu, X. and D.P. Lane (1993) Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes?, Cell, 75, 765-78.

Lytle, C.D., J. Coppey and W.D. Taylor (1978) Enhanced survival of ultraviolet-irradiated herpes simplex virus in carcinogen-pretreated cells, Nature, 272, 60-2.

Maeda, T., M.S. Eller, M. Hedayati, L. Grossman and B.A. Gilchrest (1999) Enhanced repair of benzo(a)pyrene-induced DNA damage in human cells treated with thymidine dinucleotides, Mutation Research, 433, 137-145.

Mallya, S.M. and M.O. Sikpi (1998) Evidence of the involvement of p53 in gamma-radiation-induced DNA repair in human lymphoblasts, International Journal of Radiation Biology, 74, 231-8.

Matsuda, T., M. Saijo, I. Kuraoka, T. Kobayashi, Y. Nakatsu, A. Nagai, T. Enjoji, C. Masutani, K. Sugasawa, F. Hanaoka and e. al (1995) DNA repair protein XPA binds replication protein A (RPA), Journal of Biological Chemistry, 270, 4152-7.

Mayne, L.V. and A.R. Lehmann (1982) Failure of RNA synthesis to recover after UV irradiation: an early defect in cells from individuals with Cockayne's syndrome and xeroderma pigmentosum, Cancer Research, 42, 1473-8.

McCarthy, M.J., J.I. Rosenblatt and R.S. Lloyd (1996) A modified quantitative polymerase chain reaction assay for measuring gene-specific repair of UV photoproducts in human cells, Mutation Research, 363, 57-66.

McCarthy, M.J., J.I. Rosenblatt and R.S. Lloyd (1997) Kinetics of repair of UV-induced DNA damage in repair-proficient and -deficient cells as determined by quantitative polymerase chain reaction, Photochemistry and Photobiology, 66, 356-62.

McGregor, W. (1999) DNA repair, DNA replication, and UV mutagenesis, Journal of Investigative Dermatology Symposium Proceedings, 4(1), 1-5.

McKay, B.C., M.A. Francis and A.J. Rainbow (1997a) Wildtype p53 is required for heat shock and ultraviolet light enhanced repair of a UV-damaged reporter gene, Carcinogenesis, 18, 245-9.

McKay, B.C., M.A. Francis and A.J. Rainbow (in press) Use of replication deficient recombinant adenovirus reporter gene constructs to assess repair of UV damaged DNA in mammalian cells, Methods in Molecular Biology.

McKay, B.C., M. Ljungman and A.J. Rainbow (1999) Potential roles for p53 in nucleotide excision repair, Carcinogenesis, 20, 1389-96.

McKay, B.C. and A.J. Rainbow (1996) Heat-shock enhanced reactivation of a UV-damaged reporter gene in human cells involves the transcription coupled DNA repair pathway, Mutation Research, 363, 125-35.

McKay, B.C., C. Winrow and A.J. Rainbow (1997b) Capacity of UV-irradiated human fibroblasts to support adenovirus DNA synthesis correlates with transcription-coupled repair and is reduced in SV40-transformed cells and cells expressing mutant p53, Photochemistry and Photobiology, 66, 659-64.

Meek, D.W. (1998) New developments in the multi-site phosphorylation and integration of stress signalling at p53, International Journal of Radiation Biology, 74, 729-37.

Mellon, I., G. Spivak and P.C. Hanawalt (1987) Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene, Cell, 51, 241-9.

Mitchell, D.L. and R.S. Nairn (1989) The biology of the (6-4) photoproduct, Photochemistry and Photobiology, 49, 805-19.

Morsy, M.A., E.L. Alford, A. Bett, F.L. Graham and C.T. Caskey (1993) Efficient adenoviral-mediated ornithine transcarbamylase expression in deficient mouse and human hepatocytes, Journal of Clinical Investigation, 92, 1580-6.

Mu, D., M. Wakasugi, D.S. Hsu and A. Sancar (1997) Characterization of reaction intermediates of human excision repair nuclease, Journal of Biological Chemistry, 272, 28971-9.

Mullenders, L.H. (1998) Transcription response and nucleotide excision repair, Mutation Research, 409, 59-64.

Murata, T., H. Hibasami, S. Maekawa, T. Tagawa and K. Nakashima (1990) Preferential binding of cisplatin to mitochondrial DNA and suppression of ATP generation in human malignant melanoma cells, Biochemistry International, 20, 949-55.

Natarajan, V., R.J. Plishka, E.W. Scott, H.C. Lane and N.P. Salzman (1994) An internally controlled virion PCR for the measurement of HIV-1 RNA in plasma, PCR Methods and Applications, 3, 346-50.

Nichols, A.F., P. Ong and S. Linn (1996) Mutations specific to the xeroderma pigmentosum group E Ddb- phenotype, Journal of Biological Chemistry, 271, 24317-20.

Nichols, A.F. and A. Sancar (1992) Purification of PCNA as a nucleotide excision repair protein [corrected and republished with original paging, article originally printed in Nucleic Acids Res 1992 May 25;20(10):2441-6], Nucleic Acids Research, 20, 2441-6.

Nikolov, D.B. and S.K. Burley (1997) RNA polymerase II transcription initiation: a structural view, Proceedings of The National Academy of Sciences of The United States of America, 94, 15-22.

Nyström-Lahti, M., R. Parsons, P. Sistonen, L. Pylkkänen, L.A. Aaltonen, F.S. Leach, S.R. Hamilton, P. Watson, E. Bronson, R. Fusaro and e. al (1994) Mismatch repair genes on chromosomes 2p and 3p account for a major share of hereditary nonpolyposis colorectal cancer families evaluable by linkage, American Journal of Human Genetics, 55, 659-65.

Orlando, C., P. Pinzani and M. Pazzagli (1998) Developments in Quantitative PCR, Clin Chem Lab Med, 36, 255-69.

Otrin, V.R., M. McLenigan, M. Takao, A.S. Levine and M. Protic (1997) Translocation of a UV-damaged DNA binding protein into a tight association with chromatin after treatment of mammalian cells with UV light, Journal of Cell Science, 110 (Pt 10), 1159-68.

Petit, C. and A. Sancar (1999) Nucleotide excision repair: from E. coli to man, Biochimie, 81, 15-25.

Ploskonosova, I.I., V.I. Baranov and A.I. Gaziev (1999a) Estimation of DNA damage and repair in tissues of gamma-irradiated animals using the polymerase chain reaction, Biochemistry (Mosc), 64(11), 1320-5.

Ploskonosova, I.I., V.I. Baranov and A.I. Gaziev (1999b) PCR assay of DNA damage and repair at the gene level in brain and spleen of gamma-irradiated young and old rats, Mutation Research, 434(2), 109-17.

Protic, M., S. Hirschfeld, A.P. Tsang, M. Wagner, K. Dixon and A.S. Levine (1989) Induction of a novel damage-specific DNA binding protein correlates with enhanced DNA repair in primate cells, Molecular Toxicology, 2, 255-70.

Protic, M., E. Roilides, A.S. Levine and K. Dixon (1988) Enhancement of DNA repair capacity of mammalian cells by carcinogen treatment, Somatic Cell and Molecular Genetics, 14, 351-7.

Rainbow, A.J. (1981) , in: H.F. Stich and R.H.C. San (Eds.), Short Term Test for Chemical Carcinogens, Springer-Verlag, Berlin and New York, pp. 20-35.

Rainbow, A.J. and M. Howes (1982) A deficiency in the repair of UV and gamma-ray damaged DNA in fibroblasts from Cockayne's syndrome, Mutation Research, 93, 235-47.

Ratner, J.N., B. Balasubramanian, J. Corden, S.L. Warren, and D.B. Bregman, D. B. (1998) Ultraviolet radiation-induced ubiquitination and proteasomal degradation of the large subunit of RNA polymerase II. Implications for transcription-coupled DNA repair, Journal of Biological Chemistry, 273, 5184-9.

Riou, L., L. Zeng, O. Chevallier-Lagente, A. Stary, O. Nikaido, A. Taüeb, G. Weeda, M. Mezzina and A. Sarasin (1999) The relative expression of mutated XPB genes results in xeroderma pigmentosum/ Cockayne's syndrome or trichothiodystrophy cellular phenotypes, Human Molecular Genetics, 8, 1125-33.

Robbins, J.H., R.A. Brumback, M. Mendiones, S.F. Barrett, J.R. Carl, S. Cho, M.B. Denckla, M.B. Ganges, L.H. Gerber, R.A. Guthrie and e. al (1991) Neurological disease in xeroderma pigmentosum. Documentation of a late onset type of the juvenile onset form, Brain, 114 (Pt 3), 1335-61.

Rossignol, M., I. Kolb-Cheynel and J.M. Egly (1997) Substrate specificity of the cdk-activating kinase (CAK) is altered upon association with TFIIH, EMBO Journal, 16, 1628-37.

Sage, E. (1993) Distribution and repair of photolesions in DNA: genetic consequences and the role of sequence context, Photochemistry and Photobiology, 57, 163-74.

Sambrook, J., E.F. Fritsch and T. Maniatis (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, USA.

Sancar, A. (1994) Mechanisms of DNA excision repair, Science, 266, 1954-6.

Sancar, A. (1996) DNA excision repair, Annual Review of Biochemistry, 65, 43-81.

Sarasin, A. (1978) Induced DNA repair processes in eucaryotic cells, Biochimie, 60(10), 1141-4.

Sarasin, A.R. and P.C. Hanawalt (1978) Carcinogens enhance survival of UV-irradiated simian virus 40 in treated monkey kidney cells: induction of a recovery pathway?, Proceedings of The National Academy of Sciences of The United States of America, 75, 346-50.

Savitsky, K., A. Bar-Shira, S. Gilad, G. Rotman, Y. Ziv, L. Vanagaite, D.A. Tagle, S. Smith, T. Uziel, S. Sfez and e. al (1995) A single ataxia telangiectasia gene with a product similar to PI-3 kinase, Science, 268, 1749-53.

Schwarz, T. (1998) UV light affects cell membrane and cytoplasmic targets, Journal of Photochemistry And Photobiology. B, Biology, 44, 91-6.

Selby, C.P. and A. Sancar (1993) Molecular mechanism of transcription-repair coupling, Science, 260, 53-8.

Selby, C.P. and A. Sancar (1995) Structure and function of transcription-repair coupling factor. I. Structural domains and binding properties, Journal of Biological Chemistry, 270, 4882-9.

Selby, C.P. and A. Sancar (1997) Cockayne syndrome group B protein enhances elongation by RNA polymerase II, Proceedings of The National Academy of Sciences of The United States of America, 94, 11205-9.

Setlow, R.B. and W.L. Carrier (1964) Proceedings of The National Academy of Sciences of The United States of America, 51, 226-231.

Shenk, T. (1996) Adenoviridae: The Viruses and Their Replication, in: B.N. Fields, D.M. Knipe, P.M. Howley and e. al. (Eds.), Fields Virology, Lippincott-Raven Publishers, Philadelphia, pp. 2111-2148.

Shivji, K.K., M.K. Kenny and R.D. Wood (1992) Proliferating cell nuclear antigen is required for DNA excision repair, Cell, 69, 367-74.

Siliciano, J.D., C.E. Canman, Y. Taya, K. Sakaguchi, E. Appella and M.B. Kastan (1997) DNA damage induces phosphorylation of the amino terminus of p53, Genes and Development, 11, 3471-81.

Stefanini, M., P. Lagomarsini, S. Giliani, T. Nardo, E. Botta, A. Peserico, W.J. Kleijer, A.R. Lehmann and A. Sarasin (1993a) Genetic heterogeneity of the excision repair defect associated with trichothiodystrophy, Carcinogenesis, 14, 1101-5.

Stefanini, M., W. Vermeulen, G. Weeda, S. Giliani, T. Nardo, M. Mezzina, A. Sarasin, J.I. Harper, C.F. Arlett, J.H. Hoeijmakers and e. al (1993b) A new nucleotide-excision-repair gene associated with the disorder trichothiodystrophy, American Journal of Human Genetics, 53, 817-21.

Stewart, P.L., R.M. Burnett, M. Cyrklaff and S.D. Fuller (1991) Image reconstruction reveals the complex molecular organization of adenovirus, Cell, 67, 145-154.

Szumiel, I. (1998) Monitoring and signaling of radiation-induced damage in mammalian cells, Radiation Research, 150, S92-101.

Tantin, D., A. Kansal and M. Carey (1997) Recruitment of the putative transcription-repair coupling factor CSB/ERCC6 to RNA polymerase II elongation complexes, Molecular and Cellular Biology, 17, 6803-14.

Taylor, E.M., B.C. Broughton, E. Botta, M. Stefanini, A. Sarasin, N.G. Jaspers, H. Fawcett, S.A. Harcourt, C.F. Arlett and A.R. Lehmann (1997) Xeroderma pigmentosum and trichothiodystrophy are associated with different mutations in the XPD (ERCC2) repair/transcription gene, Proceedings of The National Academy of Sciences of The United States of America, 94, 8658-63.

Taylor, E.M. and A.R. Lehmann (1998) Conservation of eukaryotic DNA repair mechanisms, International Journal of Radiation Biology, 74, 277-86.

Telenti, A., P. Imboden and D. Germann (1992) Competitive polymerase chain reaction using an internal standard: application to the quantitation of viral DNA, Journal of Virological Methods, 39, 259-68.

Tornaletti, S. and G.P. Pfeifer (1994) Slow repair of pyrimidine dimers at p53 mutation hotspots in skin cancer, Science, 263, 1436-8.

Tornaletti, S. and G.P. Pfeifer (1996) UV damage and repair mechanisms in mammalian cells, Bioessays, 18, 221-8.

Travis, E.L. (1989) Primer of Medical Radiobiology, Mosby-Year Book, St. Louis, MO, USA.

Troelstra, C., H. Odijk, J. de Wit, A. Westerveld, L.H. Thompson, D. Bootsma and J.H. Hoeijmakers (1990) Molecular cloning of the human DNA excision repair gene ERCC-6, Molecular and Cellular Biology, 10, 5806-13.

Tu, Y., S. Bates and G.P. Pfeifer (1998) The transcription-repair coupling factor CSA is required for efficient repair only during the elongation stages of RNA polymerase II transcription, Mutation Research, 400, 143-51.

Tyrrell, R.M. (1984) Exposure of nondividing populations of primary human fibroblasts to UV (254 nm) radiation induces a transient enhancement in capacity to repair potentially lethal cellular damage, Proceedings of The National Academy of Sciences of The United States of America, 81, 781-4.

van Gool, A.J., E. Citterio, S. Rademakers, R. van Os, W. Vermeulen, A. Constantinou, J.M. Egly, D. Bootsma and J.H. Hoeijmakers (1997a) The Cockayne syndrome B protein, involved in transcription-coupled DNA repair, resides in an RNA polymerase II-containing complex, EMBO Journal, 16, 5955-65.

van Gool, A.J., G.T. van der Horst, E. Citterio and J.H. Hoeijmakers (1997b) Cockayne syndrome: defective repair of transcription?, EMBO Journal, 16, 4155-62.

van Hoffen, A., A.T. Natarajan, L.V. Mayne, A.A. van Zeeland, L.H. Mullenders and J. Venema (1993) Deficient repair of the transcribed strand of active genes in Cockayne's syndrome cells, Nucleic Acids Research, 21, 5890-5.

van Hoffen, A., J. Venema, R. Meschini, A.A. van Zeeland and L.H. Mullenders (1995) Transcription-coupled repair removes both cyclobutane pyrimidine dimers and 6-4 photoproducts with equal efficiency and in a sequential way from transcribed DNA in xeroderma pigmentosum group C fibroblasts, EMBO Journal, 14, 360-7.

van Steeg, H. and K.H. Kraemer (1999) Xeroderma pigmentosum and the role of UV-induced DNA damage in skin cancer, Molecular Medicine Today, 5, 86-94.

Venema, J., L.H. Mullenders, A.T. Natarajan, A.A. van Zeeland and L.V. Mayne (1990a) The genetic defect in Cockayne syndrome is associated with a defect in repair of UV-induced DNA damage in transcriptionally active DNA, Proceedings of The National Academy of Sciences of The United States of America, 87, 4707-11.

Venema, J., A. van Hoffen, V. Karcagi, A.T. Natarajan, A.A. van Zeeland and L.H. Mullenders (1991) Xeroderma pigmentosum complementation group C cells remove pyrimidine dimers selectively from the transcribed strand of active genes, Molecular and Cellular Biology, 11, 4128-34.

Venema, J., A. van Hoffen, A.T. Natarajan, A.A. van Zeeland and L.H. Mullenders (1990b) The residual repair capacity of xeroderma pigmentosum complementation group C fibroblasts is highly specific for transcriptionally active DNA, Nucleic Acids Research, 18, 443-8.

Vermeulen, W., J. de Boer, E. Citterio, A.J. van Gool, G.T. van der Horst, N.G. Jaspers, W.L. de Laat, A.M. Sijbers, P.J. van der Spek, K. Sugasawa, G. Weeda, G.S. Winkler, D. Bootsma, J.M. Egly and J.H. Hoeijmakers (1997) Mammalian nucleotide excision repair and syndromes, Biochemical Society Transactions, 25, 309-15.

Vermeulen, W., A.J. van Vuuren, M. Chipoulet, L. Schaeffer, E. Appeldoorn, G. Weeda, N.G. Jaspers, A. Priestley, C.F. Arlett, A.R. Lehmann and e. al (1994) Three unusual repair deficiencies associated with transcription factor BTF2(TFIIH): evidence for the existence of a transcription syndrome, Cold Spring Harbor Symposia On Quantitative Biology, 59, 317-29.

Wakasugi, M. and A. Sancar (1998) Assembly, subunit composition, and footprint of human DNA repair excision nuclease, Proceedings of The National Academy of Sciences of The United States of America, 95, 6669-74.

Wang, X.W., W. Vermeulen, J.D. Coursen, M. Gibson, S.E. Lupold, K. Forrester, G. Xu, L. Elmore, H. Yeh, J.H. Hoeijmakers and C.C. Harris (1996) The XPB and XPD DNA helicases are components of the p53-mediated apoptosis pathway, Genes and Development, 10, 1219-32.

Wang, X.W., H. Yeh, L. Schaeffer, R. Roy, V. Moncollin, J.M. Egly, Z. Wang, E.C. Freidberg, M.K. Evans, B.G. Taffe and e. al (1995) p53 modulation of TFIIH-associated nucleotide excision repair activity, Nature Genetics, 10, 188-95.

Winkler, G.S., S.J. Araujo, U. Fiedler, W. Vermeulen, F. Coin, J.M. Egly, J.H. Hoeijmakers, R.D. Wood, H.T. Timmers and G. Weeda (2000) TFIIH with inactive XPD helicase functions in transcription initiation but is defective in DNA repair, Journal of Biological Chemistry, 275(6), 4258-66.

Wood, R.D. (1999) DNA damage recognition during nucleotide excision repair in mammalian cells, Biochimie, 81, 39-44.

Xiao, H., A. Pearson, B. Coulombe, R. Truant, S. Zhang, J.L. Regier, S.J. Triezenberg, D. Reinberg, O. Flores, C.J. Ingles and e. al (1994) Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53, Molecular and Cellular Biology, 14, 7013-24.

Yankulov, K.Y. and D.L. Bentley (1997) Regulation of CDK7 substrate specificity by MAT1 and TFIIH, EMBO Journal, 16, 1638-46.

Ye, N., M.S. Bianchi, N.O. Bianchi and G.P. Holmquist (1999) Adaptive enhancement and kinetics of nucleotide excision repair in humans., Mutation Research, 435, 43-61.

Zdzienicka, M.Z. (1995) Mammalian mutants defective in the response to ionizing radiation-induced DNA damage, Mutation Research, 336, 203-13.