INDUCIBLE REPAIR OF UV-DAMAGED DNA IN RODENT CELLS

HOST-CELL REACTIVATION OF A UV-DAMAGED REPORTER GENE IN
UNIRRADIATED AND PRE-UV-IRRADIATED RODENT CELLS

By:

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ABSTRACT

A non-replicating recombinant adenovirus, Ad5MCMVlacZ, which expresses the β-galactosidase (β-gal) reporter gene, was used to examine both constitutive and inducible repair of UVC-damaged DNA in Chinese hamster ovary (CHO) cells. Host cell reactivation (HCR) of β-gal activity for UVC-irradiated Ad5MCMVlacZ was examined in non-irradiated and UVC-irradiated nucleotide excision repair (NER) proficient parental CHO-AA8 and in mutant CHO-UV61 cells which are deficient in the transcription-coupled repair (TCR) pathway of NER. Cells were infected with either UVC-irradiated or non-irradiated Ad5MCMVlacZ and scored for β-gal activity 24 h later. HCR of β-gal activity for UVC-irradiated Ad5MCMVlacZ was significantly reduced in non-irradiated CHO-UV61 cells compared to that in non-irradiated CHO-AA8 cells suggesting that repair in the transcribed strand of the UVC-damaged reporter gene in untreated CHO-AA8 cells utilizes TCR. Prior UVC-irradiation of cells with low UV fluences resulted in a transient enhancement of HCR for expression of the UVC-damaged reporter gene in CHO-AA8 cells but not in TCR deficient CHO-UV61 cells. Pre-UVC-treatment of cells resulted also in an enhanced expression of β-gal for unirradiated Ad5MCMVlacZ in both CHO-AA8 and CHO-UV61 cells. However, compared to CHO-AA8 cells, the CHO-UV61 cells exhibited comparable levels of enhanced β-gal activity following significantly lower UVC exposures to cells suggesting that persistent damage in active genes plays a direct role in enhancing β-gal activity driven by the MCMV promoter in CHO cells. These results suggest that prior UVC

treatment results in a transient enhancement in repair of UVC-damage DNA in the transcribed strand of the active reporter gene in CHO-AA8 cells through an enhancement of TCR or a mechanism that involves the TCR pathway and that the upregulation of reporter gene expression alone is not sufficient for enhanced repair of the reporter gene in CHO-UV61 cells.

The HCR assay was used also to examine both constitutive and inducible repair of UVC-damaged DNA in mouse embryonic fibroblast (MEF) cells. HCR of β-gal activity for UVC-irradiated Ad5MCMVlacZ was examined in non-irradiated and UVC-irradiated NER proficient parental wild type MEF cells and in MEF cells with specific knockouts in the p53 (p53-/-), pRb (pRb-/-), and p107 (p107-/-) genes. Cells were infected with either UVC-irradiated or non-irradiated Ad5MCMVlacZ and scored for β-gal activity 24 h later. HCR of β-gal activity for UVC-irradiated Ad5MCMVlacZ did not show a significant difference in non-irradiated cells for any of the MEF knockouts cells compared to the parental strain suggesting that p53, pRb and p107 does not play a role in repair of the UV-damaged reporter gene in untreated MEF cells. Prior UVC-irradiation of cells with low UVC fluences resulted in an enhancement of HCR for expression of the UVC-damaged reporter gene in MEF wild type cells, low passage pRb-/- and p107-/- MEF cells but not in p53-/- MEF cells or in high passage pRb-/-and p107-/- MEF cells. These results suggest that prior UVC treatment MEF cells results in an induced repair of UVC-damaged DNA that is dependent on p53. The presence of an enhancement of HCR for the UVC-damaged reporter gene in pre-UVC treated cells in low passage, but not in high passage, pRb-/- and p107-/- cells suggests that the lack of pRb or p107 expression per

se does not result in a deficiency in inducible DNA repair. However, these results suggest that the lack of pRb or p107 expression results in alterations in MEF cells at high passage number that abrogate inducible repair of UVC-damaged DNA.

UVA produces predominantly single base damage that is repaired through base excision repair (BER), whereas UVC and UVB produce predominantly cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PP) that are repaired through NER. The colony survival following exposure to various UV sources was examined in cells proficient and deficient in (NER). The UV sources were a UVC source from a germicidal lamp emitting predominantly at 254 nm. and a UVA source from a 1KW Hg-Xe arc lamp using either a Band pass filter (BPF) or a 335 Cut-off-filter (335COF). NER deficient CHO-UV5 and CHO-UV61 cells were more sensitive to UVC exposure compared to NER proficient CHO-AA8 cells, consistent with the production of UVC-induced DNA damage predominantly in the form of CPDs and 6-4PPs which are repaired through the NER pathway. NER deficient xeroderma pigmentosum cells from complementation group D (XPD) were more sensitive compared to NER proficient normal human cells following exposure to the UVA-BPF source. In addition XPDdenV cells, which express the denV gene from bacteriophage T4, were more resistant than XPD cells following exposure to the UVA-BPF source. Since the denV protein is specific for excision of CPDs these results indicate a substantial proportion of the induced DNA damage resulting from the UVA-BPF is in the form of CPDs, presumably due to a significant UVB component in the beam. In contrast, the NER deficient CHO-UV5 and CHO-UV61 cells showed a similar sensitivity compared to the NER proficient CHO-AA8 cell line following UVA-335COF

exposures up to 60 KJ/m^2. However, for UVA-335COF exposures greater than 60 KJ/m^2 the NER deficient cells were more sensitive compared to the NER proficient CHO-AA8 cells, although the difference in sensitivity between NER deficient and NER proficient cells was less than that detected following UVA-BPF exposure. These results suggest that the UVA-335COF exposure produces predominantly DNA damage of the single base type for exposures less 60 KJ/m^2. This is consistent with the calculated spectral distribution, which showed a 5.62% UVB component for the UVA-BPF, but only 0.14% UVB component for the UVA-335COF.

ACKNOWLEDGEMENTS

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

Ad – adenovirus strain 5

Ad5HCMVsp1*lacZ* – Ad5MCMV*lacZ* is a non-replicating recombinant adenovirus that contains the *lacZ* gene under control of the mouse cytomegalovirus (MCMV) immediate early promoter.

BER – base excision repair

CHO – chinses hamster ovary cell lines

CPD – cyclobutane pyrimidine dimer

CPRG – chlorophenol red β-D-galactopyranoside

CS – Cockayne's syndrome

DenV – T4 endonuclease V

DNA – deoxyribonucleic acid

DSB – double strand breaks

E1 – early region 1 (of the Ad genome)

E3 – early region 3 (of the Ad genome)

ERCC – excision repair cross complementing (group)

FBS – fetal bovine serum

GADD – growth arrest and DNA damage inducible (genes)

GGR – global genome repair

HCR – host cell reactivation

LFS – Li-Fraumeni syndrome

MMR – mismatch repair

MOI – multiplicity of infection

NER – nucleotide excision repair

PBS – phosphate buffered saline

PCR – polymerase chain reaction

pfu – plaque forming units

pol - polymerase

pRb – retinoblastoma protein

RNA – ribonucleic acid

ROS – reactive oxygen species

SV40 – simian virus 40

TCR – transcription coupled repair

TTD - trichothiodystrophy

UV – ultraviolet radiation

UVER – ultraviolet radiation enhanced reactivation

XP – xeroderma pigmentosum

α-MEM – α minimal essential medium
β-gal - β-galactosidase

Chapter 1

Literature review

Ultraviolet radiation

All living organisms, including humans, are exposed to the sun or to manufactured equipment that emit light. Ultraviolet (UV) light is a well-established mutagenic and carcinogenic agent for most skin cancers (Urbach 1997).

The biological effects and relative occurrence of different UV wavelengths varies considerably across the UV spectrum. This has led to the division of the spectrum into three ranges on the basis of wavelength: UVA (320-400nm), UV-B (280-320nm) and UV-C (200-280nm).UV-A (also referred to as long wave or near-UV) accounts for 95% of UV radiation reach the surface of the earth, it accounts for less than 25% (possibly even less than 1%) of the associated skin cancer risk (IARC 1992). However, it is believed to play a principal role in photoaging of exposed skin (Ryu et al. 1997; Hanson and Simon 1998). At the other of the spectrum is UV-C (short wave, or far UV) which can be directly absorbed by DNA and results in inappropriate bonding between adjacent pyrimidines. Although exposure to UV-C is highly carcinogenic in lab settings, it comprises a minimal contribution to skin cancer in the general population as solar UV-C is almost completely absorbed by ozone in the earth's stratosphere and does not reach the surface of the earth (Barton and Robertson 1975). Rather, it is UVB (mid-UV) that is calculated to account for 75-99% of the skin cancer risk, even though it comprises only 5% of terrestrial UV (IARC 1992).

Ultraviolet radiation and DNA repair

UV radiation effects on DNA

Bulky lesions

UVB and UVC irradiation generates, in cellular DNA, bipyrimidic photoproducts, which mainly produce cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidones (6-4 PPs) at a ratio varying from 4:1 to 10:1 (Figure 1-1). The 6-4 PPs have been found to undergo efficient photoconversion into Dewar valence isomers upon exposure to UVB light (Sage 1993).

Oxidized base

UVA can produce oxidative DNA damage via the production of reactive oxygen species (ROS). Oxidation of DNA by ROS produces a large variety of lesions that have been extensively studied in (oligo) nucleotides (Cadet et al. 1997). In the case of the oxidative stress induced by UVA, mainly 8-oxoguanine as well as oxidized pyrimidines such as thymine glycol or uracil derivatives have been described in cellular systems (Pflaum et al.1994; Kielbassa 1997; Kvam and Tyrrell 1997; Cooke et al. 2000).

Strand breaks

Endogenous photosensitizers (such as melanin, porphyrin, riboflavin) and exogenous drugs (antibiotics, anti-inflammatory drugs...) produce ROS when exposed to
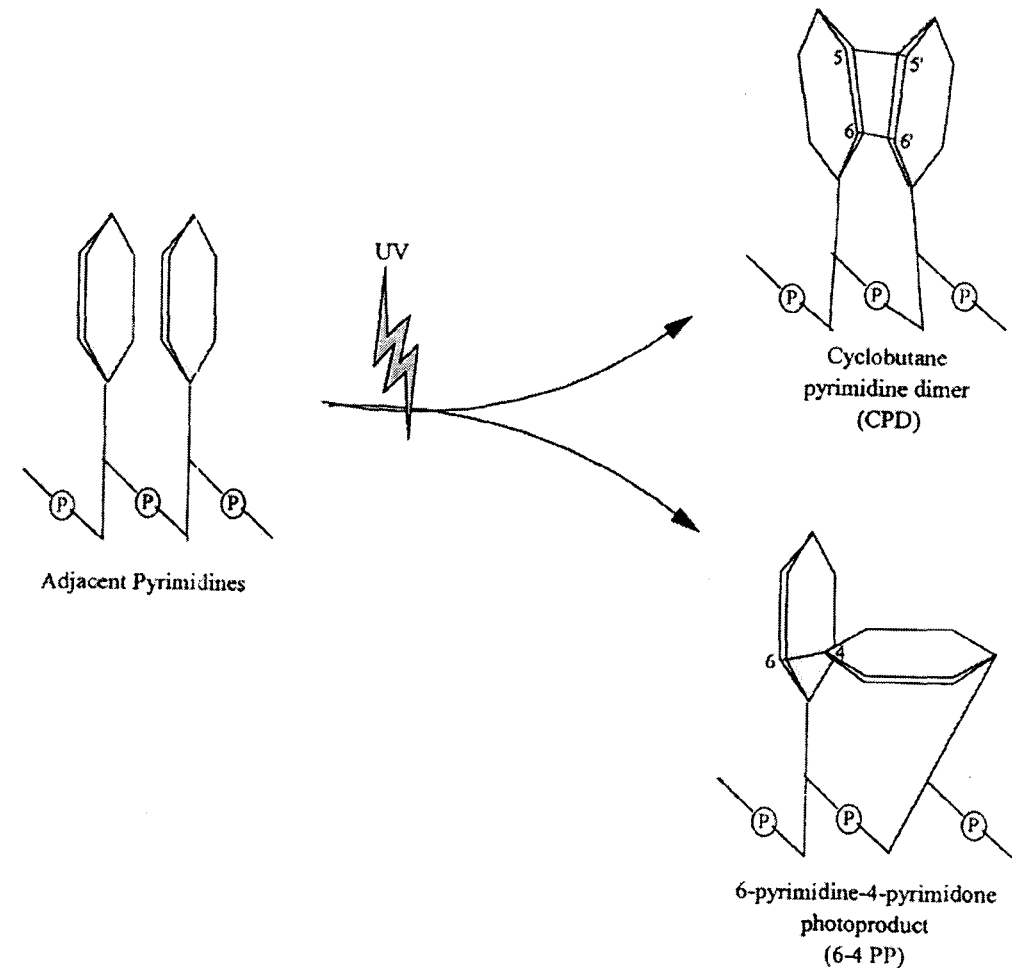
Figure 1-1 Major ultraviolet-induced DNA lesions. Absorption of UV light by DNA results in the production of two major lesions between adjacent pyrimidines. Cyclobutane pyrimidine dimers (CPDs) comprise approximately 75% of the induced photoproducts, while 6-pyrimidine-4-pyrimidone photoproducts (6-4-PPs) account for the bulk of the remaining lesions. While CPDs minimally affect duplex formation (Taylor et al. 1990), 6-4PPs strongly disrupt the DNA double helix (Kim et al. 1995). (Adapted from van Steeg and Kraemer, 1999)

UVA. Some of these ROS, such as hydroxyl-radicals or single oxygen, induce DNA single-strand breaks and alkalilabile sites (Peak et al. 1987).

Repair mechanisms for UV damage in DNA

In order to minimise the deleterious potential of these DNA modifications, an array of DNA repair mechanism appeared quite early in evolution and have been highly conserved throughout it. These repair processes can be divided into four main groups: double-strand DNA break repair (DSBR), mismatch repair (MMR), base excision repair (BER), and nucleotide excision repair (NER). Aberrations in each of these pathways are associated with specific cancer and/or developmental defects, but the two which are of particular relevance to the repair of UV-induced damage are the base excision repair and nucleotide excision repair pathways (Taylor and Lehmann 1998; Aravind et al. 1999)

<u>**Nucleotide Excision Repair**</u>

The major contribution of NER is the repair of CPDs and 6-4PPs which produced by the shortwave UV component of sunlight (Friedberg et al. 1995). The NER process in mammals involves the action of at least 30 proteins in a "cut and paste"- like mechanism. Deficiencies in one of the NER proteins lead to three rare recessive human syndromes: Xeroderma pigmentosum(XP), cockayne syndrome(CS) and Trichothidystrophy (TTD) (Friedberg et al. 1995).

NER in prokaryotic cells

NER in E. coli, requires the products of six genes, of which three, UvrA, UvrB and UvrC (Figure 1-2). Initial lesion recognition is performed by a complex consisting of two UvrA and one UvrB subunits which is specific for damage recognition on double-stranded DNA (Seeberg and Fuchs 1990). The binding of UvrC to the UvrB-DNA complex initiates incisions on the damaged DNA strand. The subsequent binding of UvrD (helicase II) dissociates the oligomer from the duplex DNA, making the site accessible to the repair specific DNA polymerase I (pol I) which synthesizes the repair patch. DNA ligase then completes the repair process (Lin and Sancar 1990, Maples and Kushner, 1982; Murray 2000).

NER in eukaryotic cells

Although the general mechanisms of prokaryotic NER are reflected in the eukaryotic model, the processes are considerably more complex. The NER process involves the action of about 20~30 proteins in successive steps of damage recognition, local opening of the DNA double helix around the injury, dual incision, and repair synthesis (Lindahl et al. 1997). A Model for nucleotide excision repair in mammalian cells is presented in Figure 1-3, showing recognition of damage, opening of a region around the lesion, dual incision, and repair synthesis. A helix-altering lesion such as a UV-induced pyrimidine dimmer (represented as an open aquare at top) is recognized by a complex of NER proteins and a region around the damage is opened up by an ATP-dependent process. The damaged strand is then cleaved on the 3' side of the lesion by

XPG nulease and on the 5' side by ERCC1-XPF nuclease, releasing the damage on an oligonucleotide 24-32 residues long. Repair synthesis takes place and the repair patch of 30 nucleotides is ligated to finish the process. In yeast, similar events occur with homologous gene products (Lindahl et al 1997).

Nucleotide excision has two path ways: global genomic NER (GGR), which repairs DNA lesions over the entire genome, and transcription –coupled NER (TCR) which ensures fast and efficient repair of DNA damaged to transcribed regions of the genane. (Friedbery et al. 1995). Both processes are essentially the same except for the initial damage recognition step, which is performed by XPC/HHR23B in GG-NER (Figure1-4).

Instead, the stalled RNA polymeraee II complex itself seems to be the damage recognition signal in TC-NER and attracts the core of the NER machinery (Mu et al 1997). An in vivo interaction between CSB and RNA polymerase II has been reported (Van Gool et al 1997), suggesting that CSA and CSB are involved in the process of the stalled RNA polymerase complex.
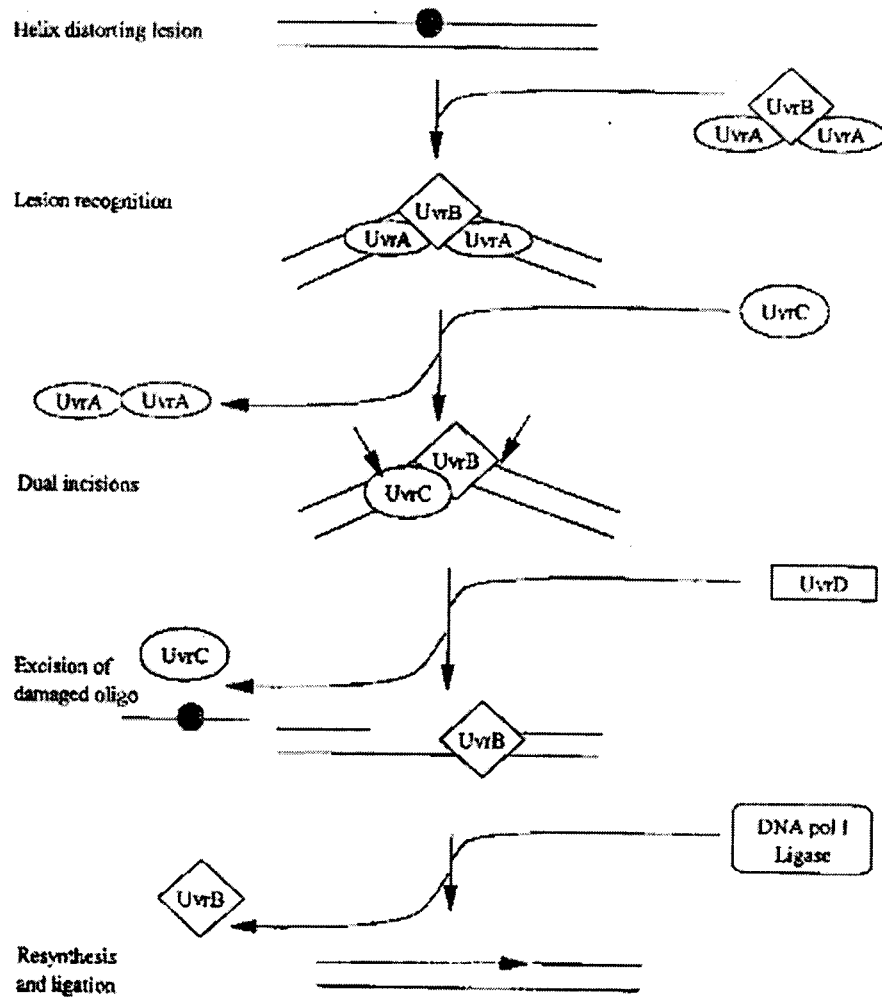
Figure 1-2 Nucleotide excision repair in prokaryotes serves as a model to demonstrate the four main steps in NER. Although the general process is highly conserved throughout evolution, the eukayotic system requires at least 17 polypeptides. (Adapted from Francis, M. A., PhD Thesis, McMaster University, 2000.)
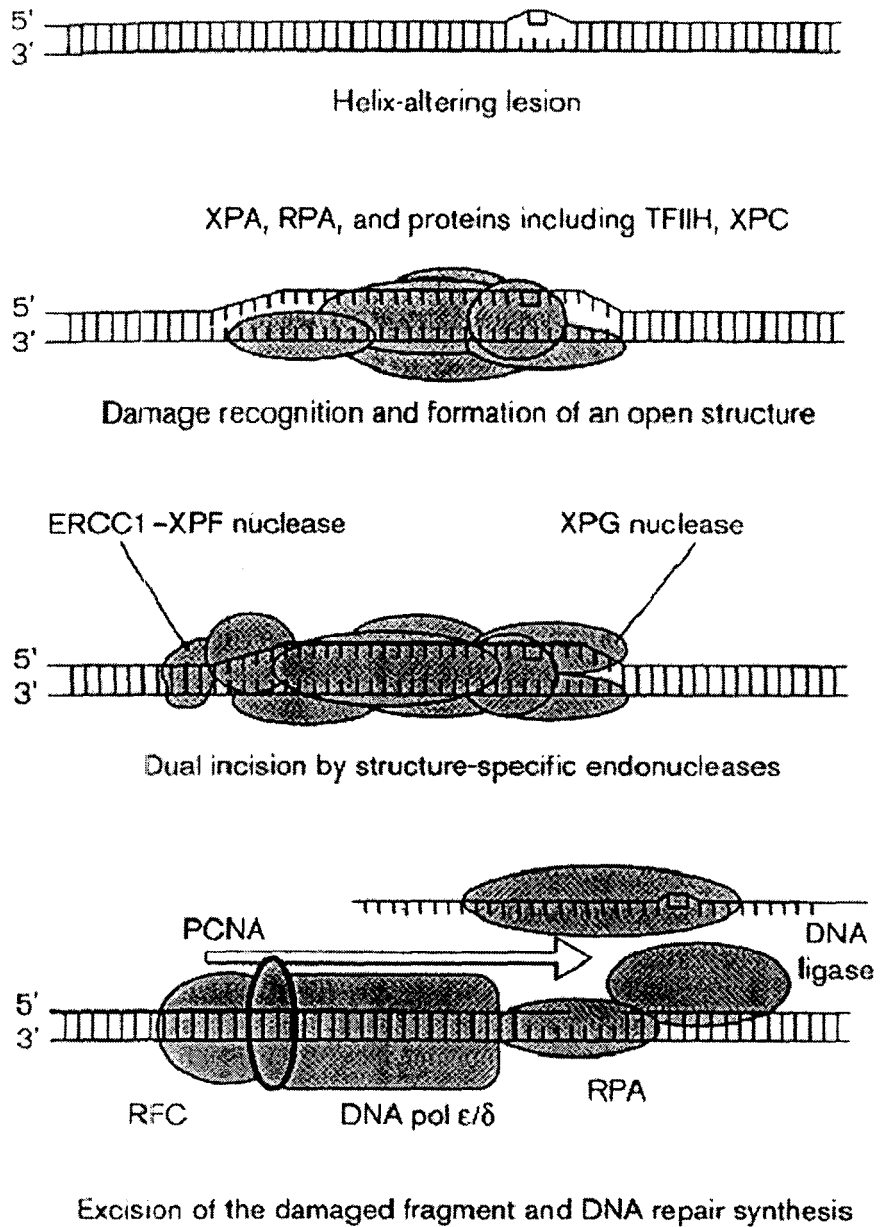
Figure 1-3 Model for nucleotide excision repair in mammalian cells. (Adapted from Lindahl et al. 1997).
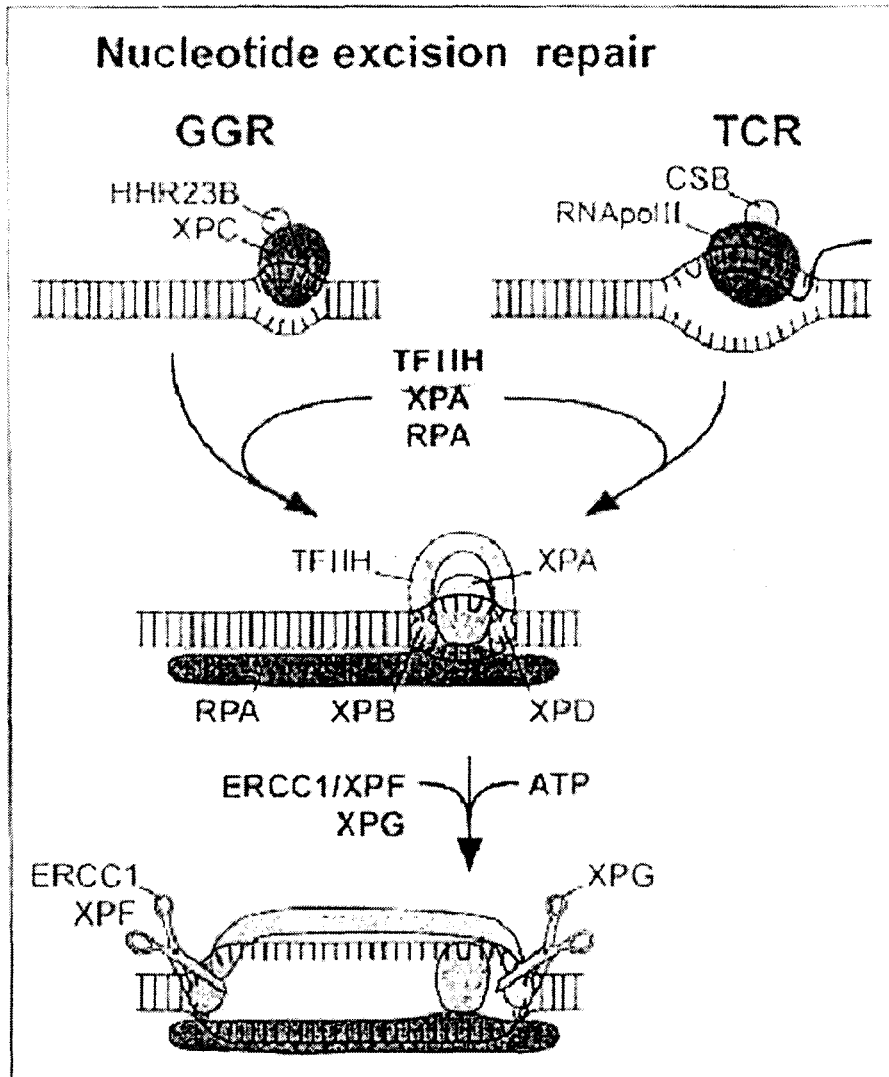
Figure 1-4 Model for TC-NER and GG-NER. Model for TC-NER. Recognition of DNA damage can occur either by the XPC/HHR23B complex (specific for GG-NER) or by RNA poymerase and the CSA and CSB proterns (specifically engaged in TC-NER). Subsequently, DNA around the lesion is opened by the concerted action of RPA, XPA and the bi-directional XPB/XPD helicase subunits of TFIIH. This allows incisions of the damaged strand on both sides of the injury by the repair endonucleases ERCC1/XPF and XPG, excision of the lesion-containing oligonucleotide and gap-filling DNA synthesis. (Adapted from Winkler, G. S. and Hoeijmakers, J. H. J.)

NER: mammalian proteins

XPC-hHR23B

XPC-hHR23B complex is the first NER factor to detect a lesion and recruit the rest of the repair machinery to the damaged site in GG-NER, but XPC-hHR23B is the sole XP factor dispensable for TC-NER (Venema et al. 1991). hHR23B stimulates XPC activity in vitro (Sugasawa et al. 1996), probably in a structural rather than a catalytic fashion, as the XPC-binding domain of hHR23B is already for XPC stimulation (Masutani et al. 1997, de Laat et al.1999).

TFIIH

TFIIH is a nine –subunit protein complex (Figure1-5) (Winkler et al. 1998) involved in initiation of RNA polymerase II (Pol II) transcription, NER and possibly in cell cycle regulation (.de Laat et al.1999). The function of TFIIH in NER is to catalyze open complex formation around the lesion and to facilitates repair complex assembly. TFIIH has multiple enzymatic activities. XPB and XPD exhibit DNA dependent ATPase and helicase functions; XPB can unwind DNA in a 3'-5' direction, and XPD in the opposite direction (Schaeffer et al. 1993, 1994; Roy et al. 1994a, de Laat et al. 1999).

XPA

XPA has a important role at an early process of both TCR and GCR in NER. XPA is a DNA binding protein (Figure1-5) with a marked preference for damaged DNA. XPA has been considered the damage-sensing and repair-recruitment factor of NER. XPA is

anticipated to verify NER lesion and to play a important role in positioning the repair machinery correctly around the lesion (de Laat et al.1999).

RPA

RPA is a ssDNA-binding protein composed of three subunits of 70,30 and 14Kb. (Figure1-5). In NER, full opening around the lesion requires RPA (Evans et al. 1997b; Mu et al. 1997), which probably binds to the undamaged DNA strand (de Latt et al 1998b). RPA may not only stabilize a fully open repair complex, but also facilitate its creation. RPA is also crucial for coordinating the NER nucleases (de Latt et al 1999)

XPG

XPG can catalyzes 3' incision and XPG protein is a member of the FEN-1 family of structure-specific endonucleases, which all cut with similar polarity at junctions of duplex and unpaired DNA. (Lieber 1997, de Laat et al. 1999).

ERCC1-XPF

The gene products of ERCC1 and XPF form a stable complex in vivo and in vitro. ERCC1-XPF can catalyzes 5' incision in NER. XPA interacts with the complex mainly via ERCC1 (Fig 1-5), although a weak affinity for XPF also has been reported. RPA also interacts with ERCC1-XPF presumably via XPF (de Laat et al 1999).

I. XPC-hHR23B — XPC — 940 aa

114 311 503
ScRad4-hom ScRad4-hom

hHR23B — 409 aa
XPC-BD
275 332
1 80 178 232 357 409
ubiquitin UBA UBA

hHR23A — 363 aa
XPC-BD
231 288
1 82 151 205 311 363
ubiquitin UBA UBA

II. TFIIH — XPB — 782 aa
XPG-BD
XPD, p34-BD p62, p44-BD needed for 5' incision
1 198 305 387 741 762
I I⁰ II III IV V VI
DNA unwinding domains

XPD — 760 aa
p34-BD
p44, XPB, XPG-BD p62-BD
XPG-BD
1 162 248 342 434
I I⁰ II III IV V VI
DNA unwinding domains

III. XPA — XPA — 273 aa
DNA-BD
RPA32-BD ERCC1-BD RPA70-BD TFIIH-BD
4 29 72 84 98 187 219 226 273
30 42 78 84 105-6 126-9
NLS E-cluster Zn Zn

IV. RPA — RPA70 — 616 aa
DNA-BD (A) DNA-BD (B) subunit interactions
198 291 305 402

RPA32 — 270 aa
phosphorylation XPA-BD
35 237 270
63 150
SBD

RPA14 — 121 aa
5 121
SBD

V. XPG — XPG — 1186 aa
RPA-BD
XPB, XPD-BD XPB, XPD-BD
1 377 688 747 928
1 95 753 889 1166-86
N I C

VI. ERCC1-XPF — XPF — 905 aa
ERCC1-BD
813 905

ERCC1 — 297 aa
XPA-BD XPF-BD
93 120 224 297
236 269
2xHhH-motif

Figure 1-5 Functional domains in mammailian NER proterns. Schematic presentation of identified functional domains in NER proteins. (Adapted from De Laat et al. 1999).

NER deficient rodent cells and mouse models

In the field of DNA repair research, the interest in non-human mammalian cells has increased greatly in the past decade or so, due in part to use of laboratory-isolated rodent cell DNA repair mutants in the identification and isolation of several human DNA repair genes. In particular, a number of different Chinese hamster ovary (CHO) cell DNA repair mutants have been isolated (Collins and Johnson 1987; Collins 1993; Jones 1994), and many of these have been transfected with repair-competent human DNA in efforts to isolate human DNA repair genes. These efforts have yielded a number of human excision repair cross-complementing (ERCC) genes, including ERCC1 (Weaterveld et al. 1984), ERCC2 (Weber et al.1988), ERCC3 (Weeda. et al. 1990), ERCC4 (Thompson. et al. 1994), ERCC5 (Mudgett and MacInnes 1990) and ERCC6 (Troelstra et al. 1990) (Table 1-1). In particular, the CSB (earlier know as ERCC6) gene, mutations in which have been shown in a CSB patient (Orren et al. 1996), was cloned by virtue of its ability to correct the UV sensitivity of UV61 cells (Troelstra et al. 1990). UV61 cells were isolated from NER proficient CHOAA8 parental cells and are the hamster homologue of human CSB cells. Unlike CHOAA8 cells, UV61 cells are deficient in the TCR and transfection with CSB cDNA fully restores their ability to survive after UV irradiation (Balajee et al. 2000, Orren et al. 1996).

In order to better examine NER in vivo, mice carrying alterations or deletions of several genes involved in NER have been derived (de Boer and Hoeijmakers 1999). Deletion of the XPD gene resulted in pre-implantation lethality (de Boer et al. 1998), which is consistent with an essential role of this gene in yeast (Naumovski and Friedberg

1983). Similarly, alteration of the NER gene ERCC1 resulted in mice that died of liver failure before weaning (McWhir et al. 1993). However, deletion of the XP-A gene does yield viable mice (de Vries et al. 1995). Mouse models for XP-C (Sand et al. 1995), CS-B (van der Horst et al. 1997) and TTD (de Boer et al. 1999) have also been constructed by creating mice homozygous for characteristic mutations in the XPC, CSB and XPD genes, respectively.

NER deficient human cells and syndromes

The consequences of a defect in one of the NER proteins are apparent from three rare recessive photosensitive human syndromes: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and the photosensitive from of the brittle hair disorder trichothiodystrophy (TTD). The specific repair defects associated with each of the complementation groups of these disorders are summarized in Table 1-2.

Xeroderma pigmentosum (XP)
XP patient extremely sensitive to sunlight, 100% chance of develope skin cancer, most don't survive past age 30. Cell fusion experiments have led to the identification of seven complementation groups with in the NER-deficient class of XP patients (XP-A to XPG). Many of the XPA, XPB, XP-D and XP-G individuals exhibit a severe NER deficiency. XPC protein is required only for GG-NER. So XP-C patients display susceptibility to sunburn in the wild-type rang because the causative transcription-blocking lesions are removed normally. XP-C cells have a residual repair synthesis of 15-

30% derived from TC-NER, and are less sensitive to UV than XP-A or XP-D cells. (Boer et al 2000).

Cockayne syndrome (CS)

CS cells display increased sensitivity to a number of DNA-damaging agents including UV, due to a defect in TC-NER. Surprisingly, CS patients are apparently not predisposed to develop skin cancer. CS is primarily a developmental disorder. It is characterized by profound physical and mental retardation, progressive pigmenary retinopathy, characteristic wizened facies with sunken eyes, a thin prominent nose, and the appearance of premature aging (Bootsma et al. 1998; Nance and Berry 1992). The mean age of death is 12.5 years and the main causes of death are pneumonia and respiratory infections, which could well be due to the generally poor condition of the patients (Nance and Berry 1992).

Trichothiodystrophy (TTD)

Sulfur-deficient brittle hair and ichthyosis (scaling of the skin) in combination with mental and physical retardation is referred to as trichothiodystrophy (TTD), emphasizing sulfur-deficiency of the hairs as the hallmark of the heterogeneous clinical entity to include TTD patients without ichthyosis (Price et al. 1980, Boer et al 2000). UV-sensitivity is associated with approximately 50% of TTD cases (Stefanini et al. 1986).

Table 1-1 Evolutionary Conservation of Eukaryotic NER genes. Adapted from Francis, M. A., PhD Thesis, McMaster University, 2000.

human disorder	CHO group	s. cerevisiae	s. pombe	other[a]
XPA		Rad 14 (27% id. / 54% sim.)	rhp 14	haywire[DM] (66% / 76%)
XPB	ERCC 3	Rad25 / SSL 2 (54% id.)	Ercc3	ERCC3[MM] (96% / 98%)
XPC		Rad 4 (23 % id.)		XPC[DM] (50% / 68%)
XPD	ERCC 2	Rad 3 (52% id. / 73% sim.)	rhp 3 (rad 15) (56% id. / 75% sim.)	XPD[MM] (97% / 99%)
XPE	(p125)			XPE[DD] (44% id.)
XPE	(p48)			
XPF	ERCC 4	Rad 1 (28% id. / 51% sim.)	rad 16 (33% id. 57% sim.)	MEI-9[DM] (40% / 61%)
XPG	ERCC 5	Rad 2 (24% id. / 39% sim.)	rad 13 (33% id.)	XPG[XL] (51% / 60%)
CSA	ERCC 8	Rad 28 (23% id. 36% sim.)		
CSB	ERCC 6	Rad 26 (44% id. / 64% sim.)		
HHR23B		Rad 23 (33% id. / 41% sim.)		MHR23B[MM] (88% / 95%)
	ERCC 1	Rad 10 (35% id.)	Swi 10 (39% id.)	C-terminal region of UvrC[EC] (38% sim.)
RPA1	(p70)	33% id. / 45% sim.	39% id. / 50% sim.	
RPA2	(p34)	30% id. / 38% sim.	34% id. / 44% sim.	
RPA3	(p14)	28% id. / 35% sim.	26% id. / 37% sim.	

[a] superscripts indicate species: DM = *D. melanogaster* (fruit fly), MM = *M. musculus* (mouse), DD = *D. discoideum* (slime mold), XL = *X. laevis* (frog) and EC = *E. coli.*

Table 1-2: Roles of NER proteins and residual repair capacity in NER deficient cells. Adapted from Francis, M. A., PhD Thesis, McMaster University, 2000.

	NER			BER		Function
	6-4	CPD				
		TCR	GGR	TCR	GGR	
XP-A	-	-	-	+	+	damage recognition
XP-B	-	-	-	+	+	3'-5' helicase promoter clearance
XP-C	-	+	-	+	+	damage recognition (GGR only)
XP-D	-	-	-	+	+	5'-3' helicase promoter clearance
XP-E	slow	+	+/-	+	+	damage recognition
XP-F	-	-	+	+	+	5'-3' incision (damage recognition?)
XP-G	-	-	-	+	+	3' incision enhance BER
CS-A	+	-	+	-	+	TCR/ recovery of RNA synthesis
CS-B	+	-	+	-	+	TCR/ recovery of RNA synthesis transcript elongation
XPB/CS	-	-	-	(-)	+	same as XP-B
XPD/CS	-	-	-	(-)	+	same as XP-D
XPG/CS	-	-	-	-	-	same as XP-G
TTD-I	+	+	+	+	+	unknown
TTD-II	+	-	-	?	+	same as XPB or XPD
TTD-III	+/-	-	-	?	+	same as XPB or XPD
RPA	-	+	-	+	+	damage recognition

+   = no evidence of repair defect
-    = significantly impaired repair capacity
+/-   = inconclusive results
?   = has not been examined
(-)   = proposed (but unsubstantiated) defect

Inducible NER

There are several lines of evidence that suggest mammalian NER is an inducible process. The major approaches used in studying inducible DNA responses include: spilt-dose experiments using UV light 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. It has shown that exposure of human fibroblasts cells to an initial dose of UV-C light enhanced their ability to repair potentially lethal damage from the subsequent challenging UV stress (Tyrrell 1984). However, results with repair deficient XPA cells exhibit an enhanced recovery. This has made it difficult to determine whether the enhanced recovery is a result of enhanced repair per se or due to other cellular responses.

Host cell reactivation (HCR) of UV-damaged reporter gene activity has been used in order to more directly examine repair of active genes. Our laboratory have reported enhanced host cell reactivation (HCR) of a UV-damaged reporter gene in pre-treated compared to non-treated human cells (Francis and Rainbow, 1999; McKay et al., 1997; Li and Ho, 1998; Boszko and Rainbow, 2002; McKay and Rainbow, 1996). Prior UV-irradiation of normal human fibroblasts with low UV fluences resulted in an enhancement of host cell reactivation (HCR) for expression of the UV-damaged reporter gene (Francis and Rainbow, 1999). Using a quantitative PCR technique we have reported that similar prior low UV fluences to normal fibroblasts results in an enhanced rate of removal of photoproducts from the UV-damaged reporter gene indicating the enhanced

HCR for reporter gene expression results from UV-induced DNA repair (Boszko and Rainbow, 2002).

There is evidence that some of the p53 regulated gene products are involved in inducible NER, including the p53-mediated and DNA damaged induced GADD45 gene (Smith et al., 1995, Smith et al., 1996), the p48XPE gene (Hwang et al., 1998; Hwang et al., 1999) and the XPC gene (Amundson et al., 2000; Amundson et al., 2002).It has been reported that GGR is inducible through p53 dependent upregulation of XPC and p48 gene (Hwang et al. 1999, Amundson et al. 2000).

Role of p53 in NER

The p53 tumor suppressor gene is the most commonly altered gene in cancer (Hollstein et al. 1991) and germline transmission of a single mutant p53 allele is frequently associated with Li-Fraumeni syndrome (LFS), a disorder characterized by a predisposition to a variety of cancers (Malkin 1994, Smith and Seo 2002). The antineoplastic effect of p53 is conferred, at least in part, by inhibiting propagation of cells with unrepaired DNA damage by enhancing DNA repair, promoting cell cycle arrest and/or facilitating apoptosis (Levine 1997; Smith and Seo 2002). Cells lacking functional p53 exhibited defective repair of UV damage (Smith et al., 1995; Ford and Hanawalt, 1995) and are more sensitive to UV irradiation than their wild-type p53 counterparts (Smith et al. 1995; Havre et al. 1995; Ford et al. 1998; McKay et al. 2000). Over the past few years it has become clear that p53 and/or p53-regulated gene products contribute to the repair of UV-induced DNA damage in both human and mouse cells (Smith et al. 1995;

Mckay et al. 1997a; Mckay et al 1997;Li et al. 1996; Li and Ho 1998). Whereas it is generally accepted that p53 contributes to GGR (Ford and Hanawalt 1995; Ford et al. 1998; Smith and Fornace 1997), the involvement of p53 in TCR is more controversial. Ford and Hanawalt have reporter that GGR, but not TCR, is disrupted in two LFS strains and two primary fibroblast strains expressing human papilloma virus 16 E6 (HPV-E6) (Ford and Hanawalt 1997; Ford and Hanawalt 1995; Ford et al. 1998). However there is also evidence for a role of p53 in TCR using host cell reactivation (HCR) of UV-damaged reporter gene (McKay and Rainbow 1996; Francis and Rainbow 1999). Additionally Zhu et al.2000 have shown that p53 may contribute to TCR and not just GGR (Zhu et al.2000).

At least three p53-regulated genes have been found to contribute to NER. Two gene products associated with the cancer-prone genetic disease xeroderma pigmentosum, p48XPE and XPC, are specifically p53-regulated (Hwang et al. 1999; Amundson et al. 2000). Both are DNA binding proteins (DDBs) involved in the recognition of DNA damage. A third p53-regulated protein is Gadd45, which binds to UV-damaged DNA in vitro (Carrier et al. 1999) and may, therefore, also play a role in damage recognition. Gadd45-null mice have been generated and they exhibit an NER defect (Smith et al. 2000; Hollander et al. 2001).

Role of pRb proteins in NER

The retinoblastoma (Rb) protein is a member of a family of three closely related mammalian proteins that includes p107 and p130. These proteins are referred to as the

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'pocket proteins' because their main sequence similarity resides in a domain (the pocket domain) that mediates interactions with the viral oncoproteins. The Rb protein has been implication in many cellular processes, such as regulation of the cell cycle, DNA-damage responses, DNA repair, DNA replication, protection against apoptosis, and differentiation, all of which could contribute to its function as a tumor suppressor (Classon and Harlow 2002). Billecke et al. 2002 have shown that pRb-null human breast cancer cells are deficient in their ability to repair UV radiation-induced DNA damage compared to wild type pRb proficient cells. Data are consistent with a model in which pRb is invovlved in the repair of UV radiation-induced lesions. Inactivation of pRb may contribute to an increased sensitivity to UV radiation by attenuating repair of DNA lesions and recovery of mRNA synthesis following UV radiation (Billecke et al. 2002).

Although a role for pRb in DNA repair is just beginning to be elucidated, pRb has already been implicated in GGR (Therrien et al.1999). Lung fibroblasts expressing the HPV E7 gene product (which inactivates the retinoblastoma tumor-suppressor protein (pRb)) exhibited reduced CPD removal, but only along the nontranscribed strand. These results provide striking evidence that the observed DNA-repair defect in HPV E7-expressing cells reveals a function for the pRb oncoprotein in HPV-mediated carcinogenesis, and may suggest a role for pRb in global nucleotide excision repair. Furthermore, pRb family members have been shown to co-localize with chromatin assembly factor-1 (CAF-1) (Kennedy et al. 2000), a protein important in NER of UV-induced lesions. Hence it appears likely that pRb plays a role in NER.

Base Excision Repair

Base excision repair is an essential process that repairs many types of damaged bases, including methylated, deaminated (e.g., U resulting from deamination of C), and oxidized bases, and abasic (AP) sites (Cadet et al. 1997). The initial step is removal of a single damaged base from the DNA backbone by a glycosylase that cuts the N-glycosyl bond between the sugar and the base. This step does not break the sugar-phosphate backbone of the DNA (Figure1-6, Mitra et al 2001). It leaves an abasic deoxyribose in the backbone, and AP site, that must be removed. Two different activities are required to remove this sugar: an AP endonuclease that cleaves the phosphodiester bond at the 5' side but leave the sugar still attached to the next nucleotide, and an AP lyase or AP endonuclease that cut 3' to the AP site to remove the sugar. The resulting single nucleotide gap has a free 3'- Hydroxyl. The gap is filled and ligated by a DNA polymerase and DNA ligase, respectively (Mitra et al. 1997).

There are many different glycosylases, at least 10 different glycosylases in humans, each of which recognizes certain types of damaged bases (Krokan et al. 2000). DNA glycosylases are generally small, monomeric proteins. Removal of the damaged base is the only catalytic function of monofunctional DNA glycosylases, such as uracil DNA glycosylases (UNG), the mismatch-specific thymine/ uracil DNA glycosylase (TDG) and the methylpurine DNA glycosylases (MPG). Whereas UNG and TDG have narrow substrate specificities, MPG removed a large array of damaged bases which have a weakened glycosylic bond as their only common feature (Krokan et al 1997). Several

DNA glycosylases have associated lyase activities that cleave at the 3' side of the abasic site (Krokan et al. 2000).

After removal of the damaged base by a DNA glycosylase and incision by AP endonuclease, in mammalian cells completion BER following DNA backbone cleavage at an AP site can occur by either short patch BER, in which 1 nucleotide is replaced, or by long patch BER, in which 2-13 nuclotides are replaced. In the former pathway, the 5'-deoxyribose phosphate (dRP) terminus created by AP endonuclease can be removed by the dRPase activity of mammalian polymerase β(Pol β) and the 3'-abasic terminus left by AP lyase can be removed by the 3'-diesterase activity associated with AP endonucleases (Figure 1-7, Memisoglu and Samson, 2000). In both case, the resulting gap can be filled in by Pol βand the remaining DNA strand break sealed by either DNA ligase I or DNA ligase III.

Cockayne syndrome may serve as a human model for defective BER. CS cells exhibit an impaired ability to remove oxidative damage from active genes, but not from inactive DNA (Leador and Cooper 1993; Cooper et al. 1997). This suggests the existence of a mechanism coupling BER to transcription, and allowing for the preferential removal of damage in active genes, as has been demonstrated for NER (Venema et al. 1990a; van Hoffen et al. 1993). Indeed, CS cells appear to be defective in the transcription-coupled repair components of both NER and BER. Cooper et al. 1997 have shown that XPG is involved in BER (Cooper et al. 1997).
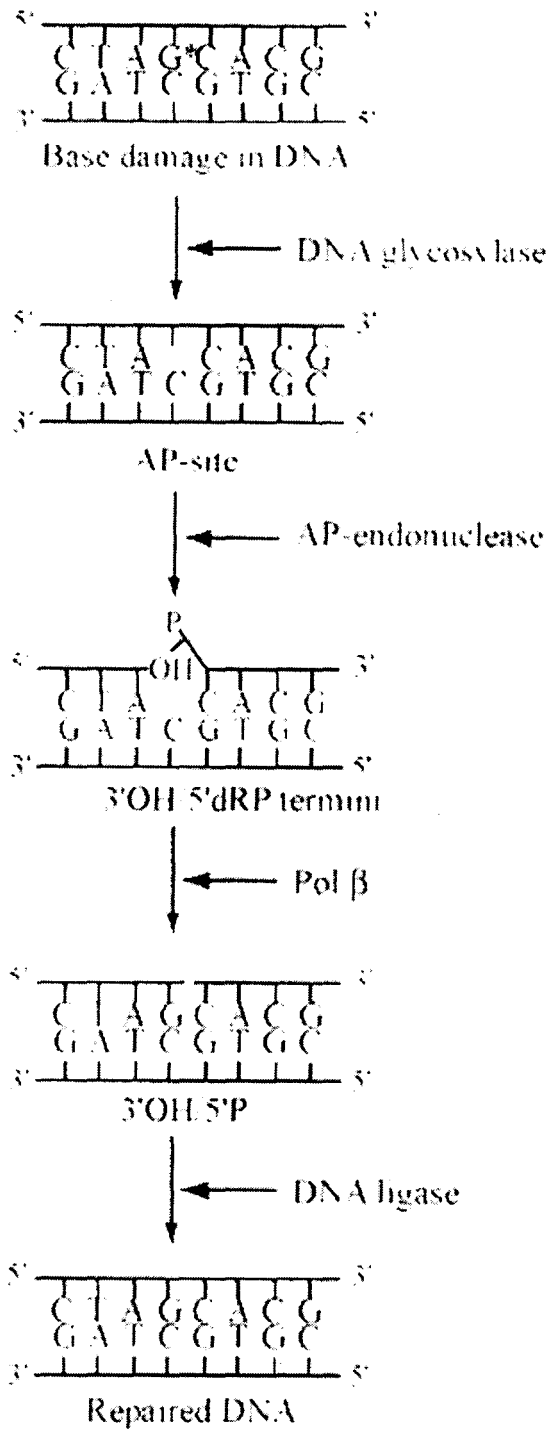
Figure 1-6 Schematic outline of the basic steps in DNA base excision repair in mammalian cells. The damaged base is represented by G* in duplex DNA, and the AP site by the missing base. Adapted from Mitra et al.2001.
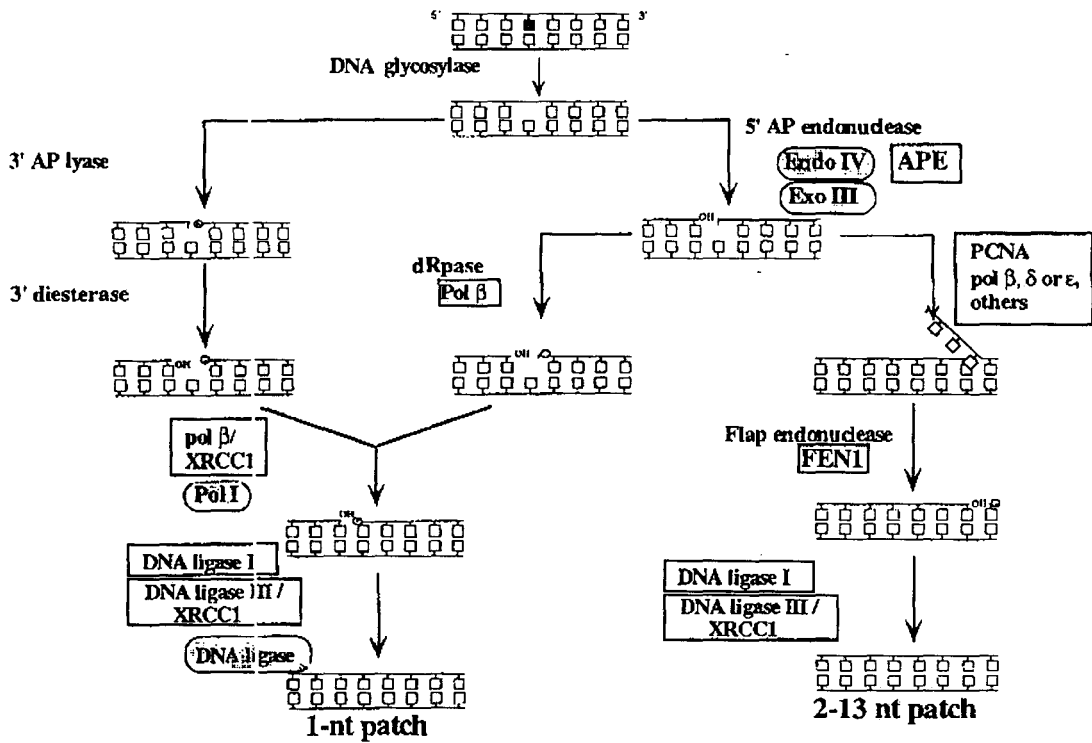
Figure 1-7 Diagram of base excision repair pathways. Shown above are the steps and components of BER. Specific E. coli and mammalian proteins for a given enzymatic activity are indicated within shaded ellipses or within boxes, respectively. Adapted from Memisoglu and Samson 2000.

Involvement of NER and BER in cell survival following UVA, UVB and UVC.

The frequently measured cellular response to radiation is cell death, usually assayed by colony-forming ability. This method reflects the ability of a single cell to restore the integrity of its DNA to the extent necessary for cell division. The DNA damage induced by UVB and UVC form two major classes of pyrimidine photoproducts: cyclobutane pyrimidine dimmers (CPDs) and (6-4) pyrimidine-pyrimidone photoproducts (6-4 PP) (Lippke et al. 1981), which can only be repaired by nucleotide excision repair (NER). UVB can also produce 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG) which is repair by the BER pathway (Beehler et al. 1992). UVA can produce oxidative DNA via the production of reactive oxygen species (ROS), which are primarily corrected by base excision repair (BER) (Taylor and Lehmann 1998).

The use of recombinant adenovirus vectors to study DNA repair

Recombinant adenovirus vectors

Adenovirus (Ad) is a non-enveloped double stranded DNA virus of approximately 36kbp. Ad is capable of efficient infection in a wide variety of replicating and quiescent cells (review in (Horvitz 1990; Hitt et al. 1999)). Adenovirus (Ad) vectors are a very efficient method for delivering foreign genes into mammalian cells (Hitt et al. 1997).

Generally 4 regions of the viral genome are considered to be expressed prior to the onset of DNA replication (E1 to E4). The E3 region is dispensable for viral

replication (Horwitz et al. 1990). Replacement of the E3 region with foreign genes permits transgenes to be delivered into a variety of cell type (Hitt et al. 1997). These constructs replicate in primary cells and allow high expression of transgenes. The E1 region contains the first Ad sequences to be expressed during viral infection, and acts to trans-activate all early regions of the viral genome (Horwitz 1990). Deletion of the E1 region renders the vector incapable of replication (except in cell lines expressing E1 in trans). Consequently, replacement of the E1 region with a foreign gene allows the transgene to be delivered into a variety of cell types without significant viral replication (Hitt et al. 1997; Hitt et al. 1999).

Our lab has constructed or obtained from other labs a number of replication-proficient and replication-deficient adnoviral vectors expressing DNA repair genes (Colicos et al. 1991; Rainbow et al. 1992; Valerie and Singhal 1995), tumour antigens (Gluzman and Ahrens 1982; Massie et al. 1986), cellular genes proposed to affect cellular responses to UV (Bacchetti and Graham 1993; Schwaz et al. 1995; DeGregori et al. 1997) and reporter genes (Morsy et al. 1993; Addison et al. 1997). These vectors can be used to infect a variety of mammalian cell types.

Host cell reactivation of adenovirus

Reactivation of UV-irradiated virus has been used as measure of excision repair capacity in both bacterial and mammalian cells (Defais et al. 1983; Rainbow 1981). Host cell reactivation (HCR) is a term used to describe the capacity of a given cell line to reactivation damaged virus. Recently, recombinant non-replication Ad constructs have

been used to introduce a UV-damaged reporter gene into non-treated human and rodent cells in order to assess the repair of damaged DNA in the absence of cellular stress using HCR of reporter gene activity as an endpoint (McKay and Rainbow 1996; McKay et al. 1997; Francis and Rainbow 1999; Valerie and Singhal 1995). UV-induced DNA lesions are removed from recombinant adenovirus reporter genes when introduced into repair proficient human cells and the removal is reduced when the same reporter genes are introduced into NER deficient cells (Boszko and Rainbow 2000; Rainbow et al. 2000). Recently, HCR assays for UV-damaged adenovirus reporter gene expression has been applied in the study of enhanced reactivation. Our laboratory was reported that pretreatment of normal and XPC human fibroblasts with low UV fluences (Francis and Rainbow 1999, McKay et al. 1997a) as well as heat shock (McKay and Rainbow 1996, McKay et al. 1997a) results in enhanced HCR of the UV-damaged reporter gene, but not in TCR deficient XP and CS strains and Li-Fraumeni syndrome cells which harbor a mutation is the p53 tumor suppressor gene. These results suggested the existence of an inducible repair response for UV damaged DNA in human cells that is dependent on the TCR pathway of NER and the wild type tumor suppressor.

Research project

My research project examines some aspects of the effects of UV irradiation in rodent cells. In particular I have examined the response to UVC-induced DNA damage and repair of UVC-induced DNA damage in Chinese hamster ovary (CHO) cells and mouse embryonic fibroblasts (MEFs). A non-replicating recombinant adenovirus,

Ad5MCMVlacZ, which expresses the β-galactosidase (β-gal) reporter gene, was used to examine both constitutive and inducible repair of UV-damaged DNA in CHO cells and MEFs. I have also examined some aspects of the response of mammalian cells to UVA, which produces predominantly single base damage that is repaired through the BER pathway) compared to UVC exposure (which produces CPDs and 6-4PP which are repaired through the NER pathway). A conventional cell colony assay was used to measure cell sensitivity to UVC and UVA exposure.

The results presented in this thesis can be divided into three general sections:

1.   UV-enhanced reactivation of a UV-damaged reporter gene in CHO AA8 but not in mutant UV61 cells suggests transcription coupled repair is UV-inducible in hamster cells. This is a manuscript recently submitted for publication.

2.   UV-enhanced reactivation of a UV-damaged reporter gene in mouse embryonic fibroblast cells: The role of pRb and p53.

3.   Dependence of cell sensitivity on UV wavelength in NER proficient and NER deficient mammalian cells.

Chapter 2

UV enhanced reactivation of a UV-damaged reporter gene in CHO-AA8 but not in

mutant CHO-UV61 cells suggests transcription coupled repair is UV-inducible in

hamster cells

UV enhanced reactivation of a UV-damaged reporter gene in CHO-AA8 but not in mutant CHO-UV61 cells suggests transcription coupled repair is UV-inducible in hamster cells

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Abstract

We have used a non-replicating recombinant adenovirus, Ad5MCMVlacZ, which expresses the β-galactosidase (β-gal) reporter gene, to examine both constitutive and inducible repair of UV-damaged DNA in Chinese hamster ovary (CHO) cells. Host cell reactivation (HCR) of β-gal activity for UV-irradiated Ad5MCMVlacZ was examined in non-irradiated and UV-irradiated nucleotide excision repair (NER) proficient parental CHO-AA8 and in mutant CHO-UV61 cells which are deficient in the transcription-coupled repair (TCR) pathway of NER. Cells were infected with either UV-irradiated or non-irradiated Ad5MCMVlacZ and scored for β-gal activity 24 h later. HCR of β-gal activity for UV-irradiated Ad5MCMVlacZ was significantly reduced in non-irradiated CHO-UV61 cells compared to that in non-irradiated CHO-AA8 cells suggesting that repair in the transcribed strand of the UV-damaged reporter gene in untreated CHO-AA8 cells utilizes TCR. Prior UV-irradiation of cells with low UV fluences resulted in a transient enhancement of HCR for expression of the UV-damaged reporter gene in CHO-AA8 cells but not in TCR deficient CHO-UV61 cells. Pre-UV-treatment of cells resulted also in an enhanced expression of β-gal for unirradiated Ad5MCMVlacZ in both CHO-AA8 and CHO-UV61 cells. However, compared to CHO-AA8 cells, the CHO-UV61 cells exhibited comparable levels of enhanced β-gal activity following significantly lower UV exposures to cells suggesting that persistent damage in active genes plays a direct role in enhancing β-gal activity driven by the MCMV promoter in CHO cells. These results suggest that prior UV treatment results in a transient enhancement in repair of

UV-damage DNA in the transcribed strand of the active reporter gene in CHO-AA8 cells through an enhancement of TCR or a mechanism that involves the TCR pathway and that the upregulation of reporter gene expression alone is not sufficient for enhanced repair of the reporter gene in CHO-UV61 cells.

Introduction

The primary DNA lesions induced by far UV radiation are repaired by the nucleotide excision repair (NER) pathway. NER can be divided into two interrelated, but separate sub-pathways: (1) transcription-coupled repair (TCR) pathway which preferentially removes DNA damage at a faster rate from the transcribed strand of actively transcribing genes, and (2) global genomic repair (GGR) which removes damage from throughout the entire genome (bulk repair) and from the non-transcribed strand of active genes (Mellon, 1986; 1987).

The genetic disorders xeroderma pigmentosum (XP) and Cockayne syndrome (CS) are each characterized by deficiencies in NER. XP is composed of at least 7 complementation groups (A to G), each displaying a general deficiency in NER which compromises at least GGR and usually TCR as well. The exception is XP-C, which retains viable TCR in spite of severely compromised GGR (Venema et al., 1990b; 1991). Two complementation groups of CS (A and B) have been identified, each of which exhibits a defect in TCR, while GGR appears to function normally (Venema et al., 1990a). Many of the XP and CS genes were identified and cloned by virtue of their

ability to correct the UV sensitivity of several different laboratory isolated Chinese hamster ovary (CHO) cell DNA repair mutants (Collins and Johnson 1987; Collins, 1993; Jones, 1994). In particular, the CSB (earlier know as ERCC6) gene, mutations in which have been shown in a CSB patient (Orren et al., 1996), was cloned by virtue of its ability to correct the UV sensitivity of UV61 cells (Troelstra et al.,1990). UV61 cells were isolated from NER proficient CHOAA8 parental cells and are the hamster homologue of human CSB cells. Unlike CHOAA8 cells, UV61 cells are deficient in the TCR and transfection with CSB cDNA fully restores their ability to survive after UV irradiation (Balajee et al., 2000; Orren et al., 1996).

Examination of cellular repair generally requires that the cells be damaged in some manner, which makes it difficult to determine if the repair pathways are constitutively active or induced by the damaging agent. However, pretreatment of a variety of mammalian cells with various physical and chemical DNA damaging agents results in an increased survival (or enhanced reactivation) for several nuclear replicating virus damaged by UV or ionizing radiation. It has been suggested that enhanced reactivation of DNA-damaged virus results from an induced DNA repair pathway (Bennett and Rainbow, 1988; Jeeves and Rainbow, 1983). We and others have reported enhanced host cell reactivation (HCR) of a UV-damaged reporter gene in pre-treated compared to non-treated human cells (Francis and Rainbow, 1999; McKay et al., 1997; Li and Ho, 1998; Boszko and Rainbow, 2002; McKay and Rainbow, 1996). Prior UV-irradiation of normal human fibroblasts with low UV fluences resulted in an enhancement of host cell reactivation (HCR) for expression of the UV-damaged reporter

gene (Francis and Rainbow, 1999). Using a quantitative PCR technique we have reported that similar prior low UV fluences to normal fibroblasts results in an enhanced rate of removal of photoproducts from the UV-damaged reporter gene indicating the enhanced HCR for reporter gene expression results from UV-induced DNA repair (Boszko and Rainbow, 2002).

Prior exposure of cells to low UV fluences resulted in enhanced HCR for expression of the UV-damaged reported gene in normal and XP-C fibroblast strains, but the enhanced HCR was absent or delayed in TCR deficient XP and CS strains and Li-Fraumeni syndrome cells which harbor a mutation is the p53 tumor suppressor gene (Francis and Rainbow, 1999; McKay et al., 1997). These results suggested the existence of an inducible repair response for UV damaged DNA in human cells that is dependent on the TCR pathway of NER and the wild type p53 tumor suppressor. A p53 dependent upregulation of HCR for a UV-damaged reporter gene has also been reported for pre-UV-treated mouse embryo fibroblasts (MEFs) (Li and Ho, 1998; Huang et al.,1998). However, there are no previous reports on enhanced HCR of a UV-damaged reporter gene in CHO cells.

A recent report has demonstrated that TCR is inducible in CHO-K1 cells (Germanier et al., 2000). Since CHO-K1 cells express a mutant p53 protein (Lee et al., 1997), it has been suggested that the inducible TCR in CHO cells is p53 independent. We were therefore interested to examine HCR of a UV-damaged reporter gene in pre-UV-irradiated CHO cells. Ad5MCMVlacZ is a non-replicating recombinant adenovirus that contains the lacZ gene under control of the mouse cytomegalovirus (MCMV) immediate

early promoter. Here we have examined HCR of β-gal activity for UV-irradiated Ad5MCMVlacZ in unirradiated and pre-UV-irradiated NER proficient CHO-AA8 cells and TCR deficient CHO-UV61 cells. We also examined the expression of β-gal following infection of pre-UV-irradiated CHO-AA8 and CHO-UV61 cells with unirradiated Ad5MCMVlacZ.

Materials and Methods

Cells and virus

Chinese hamster ovary (CHO) parental CHO-AA8 (Thompson *et al.*, 1980a) and CHO-UV61 cells (Thompson *et al.,* 1980b) were provided by Dr. Larry Thomson (Lawrence Livermore National Laboratory, CA) with the help of Dr. Gordon Whitmore, Ontario Cancer Institute, Toronto, Ontario. The increased UVC sensitivity of UV61 cells compared to CHO AA8 was confirmed using a clonagenic assay (data not shown). All cell cultures were maintained in Eagle's α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum together with antibiotic-antimycotic (100μg/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml amphotercinB; Gibco BRL) in an atmosphere of 5% CO_2 at 37oC and 90-100% humidity.

Ad5MCMV*lacZ* is a recombinant adenovirus that contains the *lacZ* gene under control of the mouse cytomegalovirus (MCMV) immediate early promoter inserted in the deleted E1 region of the virus. Ad5MCMV*lacZ* is unable to replicate in human cells, but can efficiently infect and express the β-galactosidase reporter gene in many mammalian

cell types including CHO cells. Viral stocks were prepared as described previously (Graham and Prevec, 1991).

UV irradiation of virus

UV irradiation of the virus has been described previously (Bennett and Rainbow, 1988). Viral suspensions in 1.8ml phosphate buffered saline (PBS) were irradiated in 35mm dishes on ice with continuous stirring using a General Electric germicidal lamp (model G8T5) emitting predominantly at 254nm at an incident fluence rate of 2 J/m2/sec as measured using a J-255 shortwave UV meter (Ultraviolet Products, San Gabriel, CA). Aliquots of 200 µl were removed for each exposure to the virus and diluted appropriately with unsupplemented medium.

HCR and UV enhanced HCR of the reporter gene

Cells were seeded at a density of 4×10^4 cells/well in 96-well microtitre plates (Falcon, Lincoln Park, NJ). Between 20 and 24hr after seeding, medium was aspirated from the microtitre plates and replaced with 40 µl of warm PBS per well. Cells were then UV irradiated at an incident fluence rate of 1 $J/m^2/sec$, or left unirradiated and subsequently re-fed with warm supplemented growth medium. Between 0-25 h after UV irradiation, cells were infected with either UV-irradiated or non-irradiated Ad5MCMV*lacZ* in a volume of 40 µl at a multiplicity of infection (MOI) of 10 - 80 plaque forming units/cell. Following viral adsorption for 60 min at 37^oC the cells were re-fed with warmed supplemented growth medium. Infected cells were harvested at 24 h

after infection. The optical density (OD) was determined at several times following addition of CPRG using a 96 well plate reader (Labsystems multiscan MCC/340).

Results

HCR of reporter gene expression for UV-irradiated Ad5MCMVlacZ in untreated CHOAA8 and UV61 cells.

CHO-AA8 and CHO-UV61 cells were infected with UV-irradiated and non-irradiated Ad5MCMVlacZ. Typical results for the $\circledR-\gamma\alpha\lambda$ activity of UV-irradiated Ad5MCMVlacZ in non-irradiated cells are presented in Figure 2-1. The UV fluence required to reduce $\circledR-\gamma\alpha\lambda$ activity to 37% of that for non-irradiated virus (D_{37}) was extrapolated from the curve and the relative HCR value was determined by normalizing the D_{37} for CHO-UV61 cells to that of CHO-AA8 cells in each experiment. The mean D_{37} values from 7 independent experiments were 37.06 $\pm$ 3.58 and 60.64 $\pm$ 4.85 J/m^2 for CHO-UV61 and CHOAA8 cells respectively and the relative HCR value for CHO-UV61 cells was 0.63 $\pm$ 0.067. It can be seen that the CHO-UV61 cells exhibited a significant deficiency in the ability to reactivate the UV damaged reporter gene following infection of non-irradiated cells (P < 0.005 by one-tailed *t*-test).

HCR of reporter gene expression for UV-irradiated Ad5MCMVlacZ in pre-UV-treated

CHOAA8 and UV61 cells

Prior UV irradiation of cells resulted in an enhanced HCR of the UV-damaged

reported construct in CHO-AA8 but not in CHO-UV61 cells. UV-treated (9 J/m^2) and

untreated cells were infected with either untreated or UV-treated (80 J/m^2) virus. The

relative ®–γαλ expression of the UV-treated compared to the untreated virus was then

examined in the pre-UV-treated compared to the untreated cells as a function of time

between UV treatment of cells and virus infection. It can be seen that pre-UV-treatment

of CHO-AA8 cells resulted in an enhanced HCR of the UV-damaged reporter gene that

was transient reaching a maximum when cells were infected between 19 and 25 h after 9

J/m^2 UV and disappearing when cells were infected at greater than 48 h after UV (Figure

2-2). In contrast, no enhanced HCR was detected in CHO-UV61 cells for infection of

cells from 0 h to 48 h after UV-treatment (Figure 2-2). Typical results for β-gal activity

of UV-irradiated Ad5MCMV*lacZ* in pre-UV-irradiated (9 J/m^2) compared to non-

irradiated cells are presented in Figure 3. It can be seen that UV-treatment of CHO-AA8

cells 25 h prior to infection resulted in a significantly enhanced HCR of β-gal activity for

UV-irradiated Ad5MCMV*lacZ* (Figure 2-3), whereas when infection was carried out

immediately after UV exposure of cells no significant enhancement of HCR was detected

(Figure 2-3A). Significantly enhanced HCR of β-gal activity for UV-irradiated

Ad5MCMV*lacZ* was also detected in CHO-AA8 cells when cells were UV-irradiated at

other times between 19 and 25 h prior to infection (data not shown). In contrast, no

enhanced HCR was detected in CHO-UV61 cells for infection of cells immediately or 25 h after UV-treatment (Figures 2-3C and 3D).

D37 values for the survival of β-gal activity for UV-irradiated Ad5MCMVlacZ were extrapolated from the survival curves and used to quantitate the relative HCR of the UV-damaged reporter gene in pre-UV-treated compared to that in untreated cells within individual experiments. Mean D37 and relative HCR values for CHO-AA8 and CHO-UV61 cells are presented in (Tables 2-1 and 2-2). It can be seen that UV-enhanced HCR was not detected when CHO-AA8 or CHO-UV61 cells were infected immediately after UV and scored for β-gal 24 h after infection. In contrast, enhanced HCR was consistently detected in CHO-AA8 cells when cells were infected at between 19 to 25 h after UV and scored for β-gal 24 h after infection, but not detected in UV61 cells under similar conditions. We also examined whether the detection of UV-enhanced HCR of β-gal activity for UV-irradiated Ad5MCMVlacZ in CHO-AA8 and CHO-UV61 was dependent on the amount of virus used to infect the cells. Experiments were carried out at four different infecting MOIs for cells infected 25 h after UV treatment with 9 J/m2 (Table 2-2). UVER was consistently detected at MOIs ranging from 10-80 pfu/cell in CHO-AA8 but not CHO-UV61 cells. The relative HCR values in pre-UV-treated compared to untreated CHO-AA8 and CHO-UV61 exhibited no significant differences between the different MOI examined.

Prior UV irradiation of cells resulted in an enhanced HCR for the UV-damaged reported construct in CHO-AA8 cells over the UV fluence range to cells employed (Figure 2-4). Results were consistent with a fluence dependent increase over the range of

exposures given to the cells. In contrast, HCR of the UV-damaged reported gene exhibited a decrease following a similar range of UV exposure to CHO-UV61 cells (Figure 2-4).

These results suggest that prior UV treatment results in an induced repair of UV-damage DNA in the transcribed strand of the active reporter gene in CHO cells through an enhancement of TCR or a mechanism that involves the TCR pathway.

Pre-UV-exposure of CHO cells enhances the expression of unirradiated Ad5MCMVlacZ.

We have reported previously that pre-UV-treatment of human fibroblasts results in an enhanced β-gal expression of an undamaged reporter gene. UV-enhanced expression of the undamaged reporter gene is induced at lower UV fluences to cells and at higher levels in TCR deficient but not GGR deficient fibroblasts compared to normal fibroblasts indicating that persistent damage in active cellular genes triggers increased activity from the CMV driven reporter construct (Francis and Rainbow, 2000). In the present report we examined the effects of prior UV exposure to CHO cells on the expression of β-gal following infection with unirradiated Ad5MCMVlacZ (Figure 2-5). Cells were infected with unirradiated Ad5MCMVlacZ at various times after UV to cells and scored for β-gal 24 h after infection. It can be seen that pre-UV-treatment of cells resulted in an enhanced expression of β-gal for unirradiated Ad5MCMVlacZ for all the times between UV to cells and infection examined for both CHO-AA8 and CHO-UV61 cells. For CHO AA8 cells the increase in expression of β-gal increased with UV fluence

to cells reaching a maximum at the highest exposure to cells used of 18 J/m^2. In contrast, the TCR deficient UV61 cells exhibited comparable levels of enhanced β-gal activity following significantly lower UV exposures to cells (2-6 J/m^2). These results suggest that persistent damage in active cellular genes plays a direct role in enhancing β-gal activity driven by the MCMV promoter in CHO cells as has been reported previously for human fibroblasts (Francis and Rainbow 2000). Our results show that enhanced HCR of β-gal activity for UV-irradiated Ad5MCMVlacZ was not detected in CHO-UV61 (Figures 2-2, 2-3 and 2-4) under conditions that resulted in an enhanced expression of β-gal for unirradiated Ad5MCMVlacZ (Figure 2-5). This suggests that the upregulation of reporter gene expression alone is not sufficient for enhanced repair of the reporter gene in CHO-UV61 cells.

Discussion

UV irradiation of cultured mammalian cells results in a decrease in RNA synthesis. In normal cells RNA synthesis recovers quickly. The importance of efficient repair of active cellular genes is indicated by the correlation between the ability to recover RNA synthesis and cell survival after UV irradiation (Mayne and Lehmann, 1982). A single UV-induced lesion in a reporter construct is enough to block RNA synthesis of a reporter gene (Protic-Sabljic and Kraemer, 1985) and in mammalian cells TCR is responsible for the repair of the majority of transcription-blocking lesion. RNA Polymerase II stalled at a lesion doesn't dissociate from the template (Donahue et al.,

1994) and presumably functions to direct repair enzymes to the lesion. Previous reports from our laboratory suggest that this recruitment of repair enzymes to the transcribed strand of active genes is an active process and can be induced in normal human cells by cellular stresses such as UV (Francis and Rainbow, 1999; McKay et al., 1997) or heat shock (McKay and Rainbow, 1996; McKay et al., 1997).

The relative β-gal activity for UV-irradiated compared to untreated Ad5MCMVlacZ was significantly reduced in non-irradiated cells for CHO-UV61 compared to CHO-AA8 (Figure 2-1). The relative HCR value for CHO-UV61 compared to CHO-AA8 cells was 0.63 + 0.067 (P<0.005). Similar relatively high levels (close to normal levels) of basal HCR have also been observed in untreated CSA and CSB human fibroblast cells (Francis and Rainbow, 1999). Both CS and CHO-UV61 cells have been shown to be deficient in the repair of the transcribed strand of active genes following UV irradiation of cells, but retain proficient NER of inactive (or bulk) DNA (Orren et al.,1996). Relative HCR values for non-irradiated XPC cells, which are proficient in TCR, but deficient in GGR, are significantly less than HCR values for CS (Francis and Rainbow, 1999) and CHO-UV61 cells (this work). This suggest that in the absence of sufficient UV exposure to the cell, damage in the transcribe strand of the reporter gene is repaired in normal human and repair proficient CHO cells to a large extent by the bulk DNA sub-pathway of NER, although a small amount of TCR must also occur.

Irradiation of cells with low UV fluence at 19-25 hr prior to infection resulted in a significant enhancement of HCR for CHO-AA8, but not for CHO-UV61 cells (Figures 2-3, 2-4 and Table 2-1). The most likely explanation of our data is that prior UV treatment

results in an induced repair of UV-damage DNA in the transcribed strand of the active reporter gene in CHO cells through an enhancement of TCR or a mechanism that involves the TCR pathway. The potential contribution of p53 to NER is controversial (McKay et al., 1999). A recent report has demonstrated that TCR is inducible in CHO-K1 cells. Since CHO-K1 cells express a mutant p53 protein, it has been suggested that the inducible TCR in CHO cells is p53 independent (Germanier et al., 2000). The p53 gene in CHO-K1 cells contains a single missense mutation at codon 211, which changes Thr211 to Lys211 (Lee et al., 1997), which may play a role in DNA binding (Walker et al., 1999). Lee et al. reported that although the p53 mutation in CHO-K1 cells abrogates the ability to arrest in G1 it has no effect on the G2 or S phase checkpoints or the acute down-regulation of DNA replication after a radiation challenge (Lee et al., 1997). In addition, Tzang et al. reported that CHO-K1 cells transfected with and expressing a wildtype human p53 gene fail to arrest at G1 phase following X-rays, suggesting that the failure to arrest in G1 was not caused by dysfunction of its p53, but by some other deficiency in CHO-K1 cells (Tzang et al., 1999). Tzang et al. showed also that the p53 gene in CHO-K1 cells is induced by UV irradiation and is capable of binding to DNA containing p53-binding sequences and transactivating p53 responsive promoters and upregulating p53 responsive genes such as Gadd45 (Tzang et al., 1999). Thus although CHO-K1 cells have a mutation at codon 211, the mutation apparently has no effect on several functional properties of the protein. It is therefore possible that upregulation of TCR in CHO-K1 cells is p53 dependent and that that the mutation in CHO-K1 doesn't alter the p53 function required for this. Although we are not aware of reports specifically

describing the p53 status of CHO-AA8 cells, since CHO-K1 and CHO-AA8 cells were derived from the same stock of CHO cells, it appears likely that they would carry the same p53 mutation as that in CHO-K1 cells.

We and others have shown that repair in the transcribed strand of a UV-damaged reporter gene under the control of the HCMV immediate early promoter is inducible by UV in human fibroblast cells (Francis and Rainbow, 1999) and mouse embryo fibroblast (MEF) cells (Li and Ho, 1998; Huang et al., 1998), and that a induced repair is dependent on wild type p53 (McKay et al., 1997; Li and Ho, 1998; Huang et al., 1998). In addition, Smith et al. 2000 report a p53 dependent upregulation of GGR and not TCR in MEF cells. Although MEF cells and CHO cells are both rodent cells, it is possible that CHO cells show a different dependency of NER on p53 compared to MEF cells.

There is evidence that some of the p53 regulated gene products are involved in inducible NER, including the p53-mediated and DNA damaged induced GADD45 gene (Smith et al., 1995, Smith et al., 1996), the p48XPE gene (Hwang et al., 1998; Hwang et al., 1999) and the XPC gene (Amundson et al., 2000; Amundson et al., 2002). In particluar, transcription from the p48 gene, which is mutated in GGR-deficient, damage-specific DNA binding (DDB) protein deficient, XP-E cells (Hwang et al., 1998), is up regulated (in a p53-dependent manner) in response to UV treatment in human cells. This is consistent with a p53 dependent upregulation of GGR in cellular DNA reported for human cells (Hwang et al., 1999).

UV induced DNA lesions efficiently block transcript elongation and induce the p53 response (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996; Ljungman et

al., 1999). Although p53 contributes to the transcriptional activation of many p53 responsive genes (reviewed in Ko and Prives, 1996), accumulation of the protein products from these p53 responsive genes requires that these genes are free of UV induced pyrimidine dimers (McKay et al., 1998). For example, accumulation of the p53 regulated p21 protein following UV exposure is delayed in TCR deficient cells (McKay et al., 1998). Thus, TCR deficient fibroblasts do not recover RNA synthesis following UV as efficiently as repair proficient cells (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996; Mayne and Lehman, 1982) and do not accumulate the protein products of p53 responsive genes as efficiently as repair proficient cells (McKay et al., 1998). The UV-induced increase in p48 transcription which leads to enhanced GGR would require removal of any UV-induced lesions from the p48 gene and therefore be delayed in TCR deficient cells compared to repair proficient cells. We have reported recently that UV-enhanced reactivation of the UV-damaged reporter gene is delayed and not absent in CSB cells (Rainbow et al., 2003) suggesting a p53 and TCR dependent upregulation of GGR in the transcribed strand of the reporter gene in human fibroblasts. Since the enhanced reactivation of the UV-damaged reporter gene in UV61 cells appears to be absent and not delayed it appears more likely that UV enhanced reactivation in CHO-AA8 cells occurs through an enhancement of TCR and not an enhancement of GGR, consistent with the report of Germanier et al., 2000.

Pre-UV treatment of cells also resulted an enhanced expression of ®−γαλ activity for unirradiated Ad5MCMVlacZ in both CHO-AA8 and CHO-UV61 cells (Figure 2-5). However, pre-UV treatment did not result in enhanced HCR of ®−γαλ activity for UV-

irradiated Ad5MCMV*lacZ* in CHO-UV61 cells. This suggests that the upregulation of reporter gene expression alone is not sufficient for enhanced repair of the reporter gene in CHO-UV61 cells.

In summary, the evidence presented in this paper suggests that prior UV treatment of CHO-AA8 cells but not CHO-UV61 cells results in an induced repair of UV-damage DNA in the transcribed strand of the active reporter gene through an enhancement of TCR or a mechanism that involves the TCR pathway.

Table 2-1 HCR of β-gal activity for UV-irradiated Ad5MCMV*lacZ* in UV-irradiated and non- irradiated cells.

Cell	Time (h)	D_{37} (J/m^2)		Relative HCR (a)
		0 J/m^2 to Cells	9 J/m^2 to Cells	
	0	50.09±2.82	51.14±3.13	1.03±0.06 (5)
CHOAA8	19-25	49.04±3.18	68.92±4.39	**1.45±0.09 (23) (b)**
	0	36.04±1.61	32.40±1.44	0.90±0.03 (5)
UV61	19-25	39.91±1.46	30.55±1.30	0.78±0.04 (16)

(a) D_{37} values for survival of UV-irradiated reporter activity in UV-irradiated cells normalized to values obtained in non-irradiated cells. Shows mean +/- standard error for a number of experiments as indicated in the brackets.
(b) Significantly greater than 1 (P<0.005)

Table 2-2 HCR of β-gal activity for UV-irradiated Ad5MCMV*lacZ* in UV-irradiated and non-irradiated cells at different MOI.

Cell	MOI	D_{37} (J/m^2)		Relative HCR (a)
		0 J/m^2 to Cells	9 J/m^2 to Cells	
CHO-AA8	80	54.35±13.92	63.64±12.52	1.23±0.13 (3)
	40	59.48±16.39	68.03±7.77	1.25±0.19 (3)
	20	38.49±2.79	64.45±4.94	1.71±0.26 (3)
	10	47.24±3.89	60.59±0.83	1.30±0.10 (3)
CHO-UV61	80	35.87±2.35	34.42±1.85	0.97±0.07 (3)
	40	36.95±2.14	29.37±2.46	0.80±0.10 (3)
	20	42.16±4.67	34.21±4.14	0.81±0.02 (3)
	10	42.51±6.52	26.05±1.28	0.64±0.13 (2)

(a) D_{37} values for survival of UV-irradiated reporter activity in UV-irradiated cells normalized to values obtained in non-irradiated cells. Shows mean +/- standard error for a number of experiments as indicated in the brackets.
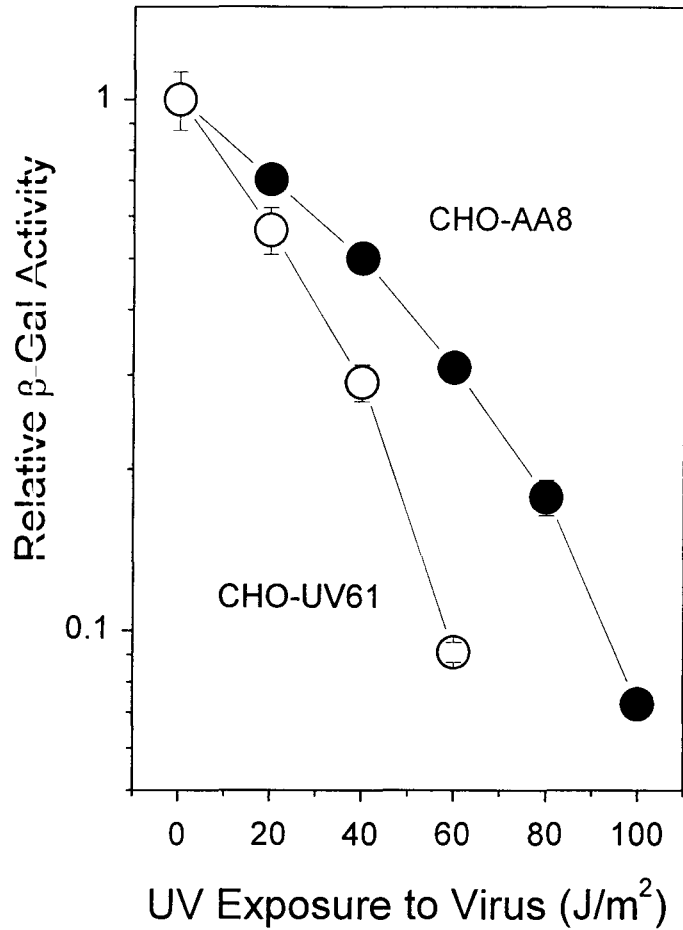
Figure 2-1 Relative β-gal activity for UV-irradiated Ad5MCMV*lacZ* in non-irradiated cells. Cells were infected with UV-irradiated or untreated Ad5MCMV*lacZ* and scored for β-gal activity 24 h later. Shows representative results for a single experiment using CHO-AA8 (●) and CHO-UV61 (○) cells. Each datum point is the mean ± SE of four replicates.
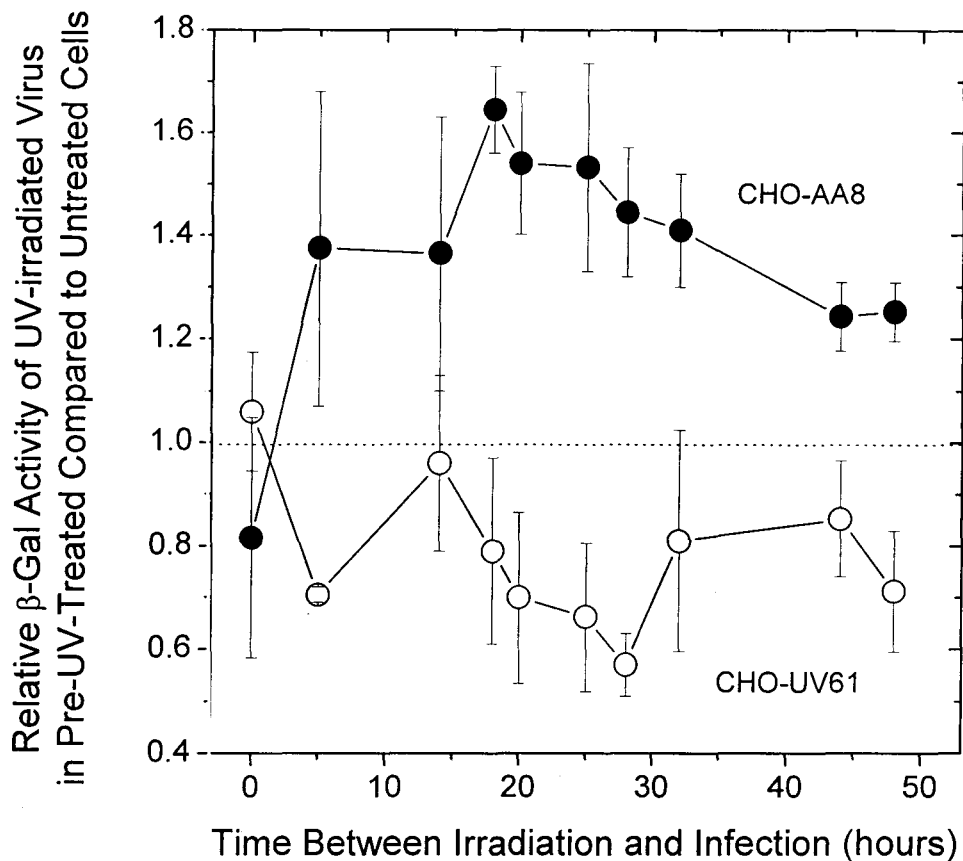
Figure 2-2: Relative β-gal activity for UV-irradiated Ad5MCMV*lac*Z in pre-UV treated compared to untreated cells as a function of time between irradiation and infection. Shows the ratio of the relative β-gal activity for UV-treated ($80 \ \text{J/m}^{2)}$ compared to untreated Ad5MCMV*lac*Z, in UV treated ($9 \ \text{J/m}^2$) compared to untreated cells. Shows pooled results for CHO-AA8 (●) and CHO-UV61 (○) cells. Each datum point is the mean ± SE of multiple experiments. Each experiment has 3 replicates.

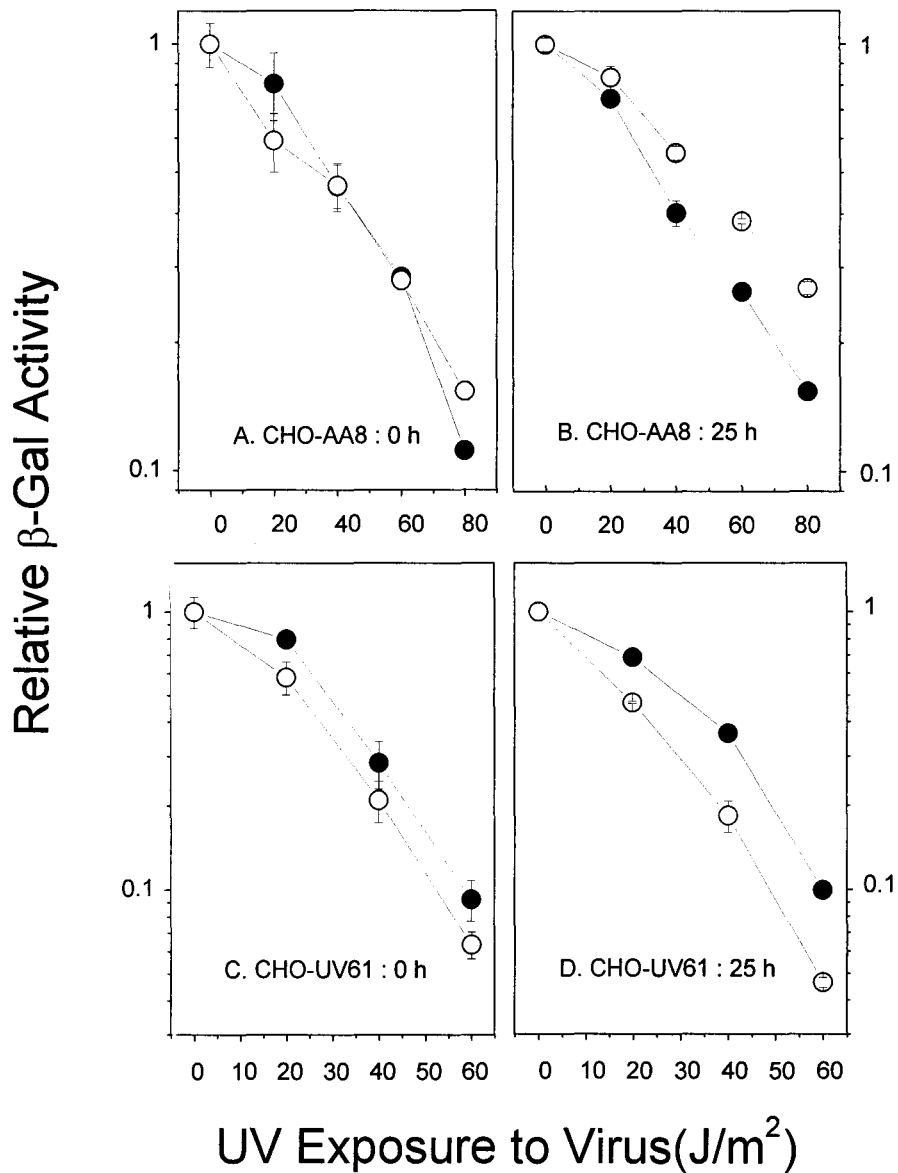**Figure 2-3 UV irradiation of cells results in enhanced HCR of UV-irradiated reporter activity in CHO-AA8 but not in CHO-UV61 cells.** Representative results of a single experiment for non-irradiated CHO-AA8 and CHO-UV61 (●); UV-irradiated (9 J/m^2) CHO-AA8 and CHO-UV61 (○) cells. Cell type and time between UV-irradiation of cells and infection are indicated on the figure. Each datum point is the mean $\pm$ SE of four replicates.
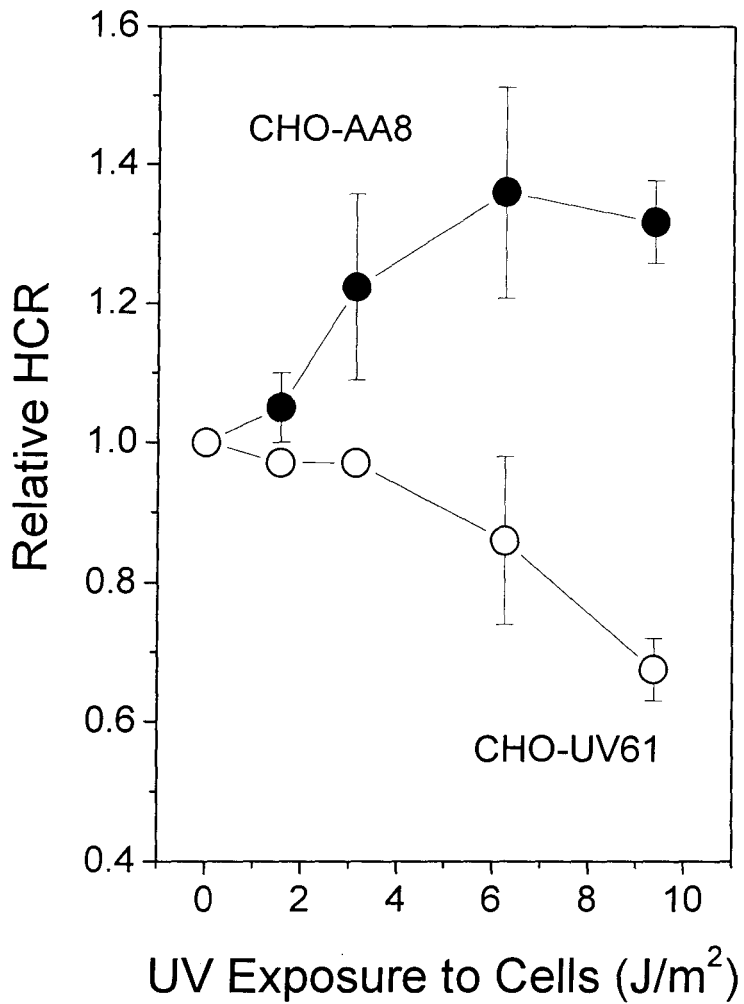
Figure 2-4 Relative HCR of UV-irradiated reporter activity in UV-irradiated CHO-AA8 (●) and CHO-UV61 (○) cells. D_{37} values for survival of UV-irradiated reporter activity in UV-irradiated cells were normalized to values obtained for non-irradiated cells. Each point is the mean ± SE of multiple independent experiments. Each experiment has 3 replicates.
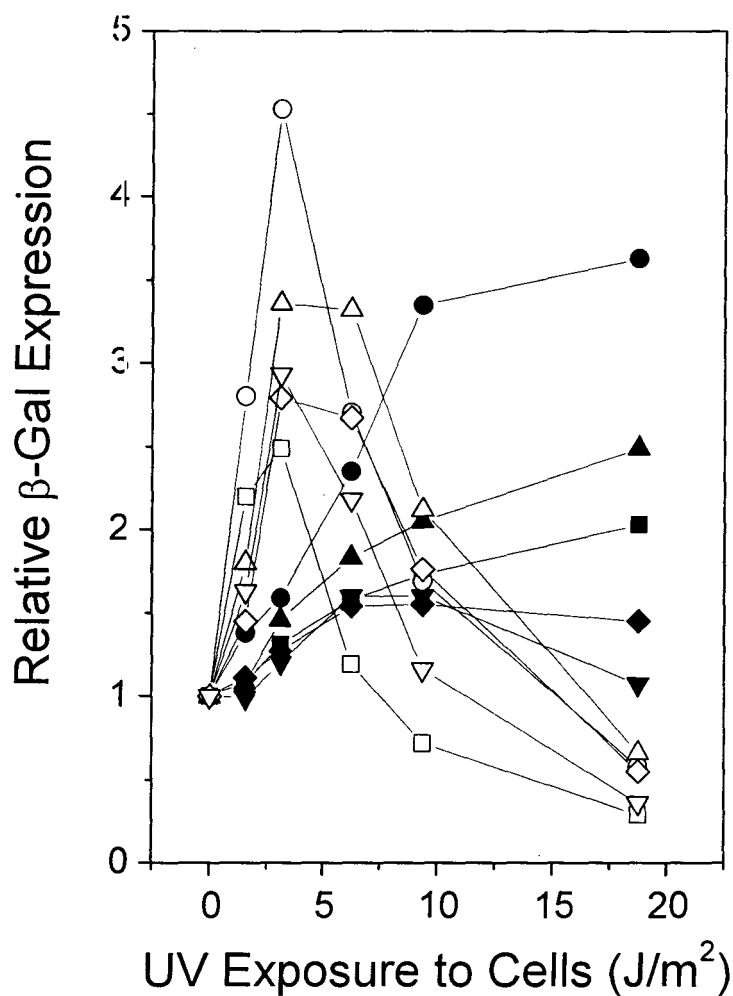
Figure 2-5 Relative expression of β-gal following infection of unirradiated Ad5MCMVlacZ in UV-irradiated CHO-AA8 (closed symbols) and CHO-UV61 cells (open symbols). Expression of unirradiated Ad5MCMVlacZ in UV-irradiated cells was normalized to values obtained in their non-irradiated counterparts. Time between UV-irradiation of cells and infection are 0h (■,□); 3h (●,○); 14h (▲,△); 19h (◆,◇); 25h (▼,▽). Shows representative results of a single experiment. Each point is the mean ± SE of four replicates.

Chapter 3

UV enhanced reactivation of a UV-damaged reporter gene in mouse embryonic fibroblast cells: The role of pRb and p53

Abstract

We have used a non-replication recombinant adenovirus, Ad5MCMVlacZ, which express the β-galactosidase (β-gal) reporter gene, to examine both constitutive and inducible repair of UV-damaged DNA in Mouse embryonic fibroblasts (MEF) cells. Host cell reactivation (HCR) of β-gal activity for UV-irradiated Ad5MCMVlacZ was examined in non-irradiated and UV-irradiated nucleotide excision repair (NER) proficient parental wild type MEF cells and in MEF cells with specific knockouts in the p53 (p53-/-), pRb (pRb-/-), and p107 (p107-/-) genes. Cells were infected with either UV-irradiated or non-irradiated Ad5MCMVlacZ and scored for β-gal activity 24 h later. HCR of β-gal activity for UV-irradiated Ad5MCMVlacZ did not show a significant difference in non-irradiated cells for any of the MEF knockouts cells compared to the parental strain suggesting that p53, pRb and p107 may not play a role in NER in untreated MEF cells. Prior UV-irradiation of cells with low UV fluences resulted in an enhancement of HCR for expression of the UV-damaged reporter gene in MEF wild type cells, low passage pRb-/- and p107-/- MEF cells but not in p53-/- MEF cells or in high passage pRb-/-and p107-/- MEF cells. These results suggest that prior UV treatment MEF cells results in an induced repair of UV-damaged DNA that is dependent on p53. The presence of an enhancement of HCR for the UV-damaged reporter gene in pre-UV-treated cells in low passage, but not in high passage, pRb-/- and p107-/- cells suggests that the lack of pRb or p107 expression per se does not result in a deficiency in inducible DNA repair. However,

these results suggest that the lack of pRb or p107 expression results in alteration in MEF cells at high passage number which abrogate inducible repair of UV-damaged DNA.

Introduction

The primary DNA lesions induced by far UV radiation are repaired by the nucleotide excision repair (NER) pathway. NER can be divided into two sub-pathways: (1) transcription-coupled repair (TCR) pathway which preferentially removes DNA damage at a faster rate from the transcribed strand of actively transcribing genes, and (2) global genomic repair (GGR) which removes damage from throughout the entire genome (bulk repair) and from the non-transcribed strand of active genes (Mellon, 1986; 1987).

The p53 tumor suppressor gene is the most commonly altered gene in cancer (Hollstein et al. 1991) and germline transmission of a single mutant p53 allele is frequently associated with Li-Fraumeni syndrome (LFS), a disorder characterized by a predisposition to a variety of cancers (Malkin 1994; Smith and Seo 2002). The antineoplastic effect of p53 is conferred, at least in part, by inhibiting propagation of cells with unrepaired DNA damage by enhancing DNA repair, promoting cell cycle arrest and/or facilitating apoptosis (Levine 1997; Smith and Seo 2002). Cells lacking functional p53 exhibited defective repair of UV damage (Smith et al., 1995; Ford and Hanawalt, 1995) and are more sensitive to UV irradiation than their wild-type p53 counterparts (Smith et al. 1995; Havre et al. 1995; Ford et al. 1998; McKay et al. 2000). It has been suggest that p53 plays a role in the DNA repair of UV damage by the nucleotide excision

repair (NER) pathway (Levine, 1997; Smith and Seo 2002). It has been reported that repair in the transcribed strand of a UV-damaged reporter gene under the control of the HCMV immediate early promoter is inducible by UV in human fibroblast cells (Francis and Rainbow 1999) and mouse embryo fibroblasts (MEF) cells (Li et al. 1997; Huang et al. 1998), and that the inducible repair is dependent on wild type p53 (McKay et al. 1997, Li et al. 1997; Huang et al. 1998; Li and Ho 1997). In addition, a recent report has demonstrated that the GGR subpathway of NER, but not TCR is mainly affected by p53 loss or inactivation in mouse embryo fibroblasts (MEF) (Smith et al. 2000). We were therefore interested to examine HCR of β-gal activity for UV-irradiated Ad5MCMV*lacZ* in unirradiated and pre-UV-irradiated wild type proficient MEF cells and p53 knock out MEF cells (p53-/-). Ad5MCMV*lacZ* is a non-replicating recombinant adenovirus that contains the *lacZ* gene under control of the mouse cytomegalovirus (MCMV) immediate early promoter.

The retinoblastoma (Rb) protein is a member of a family of three closely related mammalian proteins that includes p107 and p130. These proteins are referred to as the 'pocket proteins' because their main sequence similarity resides in a domain (the pocket domain) that mediates interactions with the viral oncoproteins (Mulligan and Jack 1998). The Rb protein has been implicated in many cellular processes, including regulation of the cell cycle, DNA-damage responses, DNA repair, DNA replication, protection against apoptosis, and differentiation, all of which could contribute to its function as a tumor suppressor (Classon and Harlow 2002). Billecke et al. 2002 have shown that pRb-null human breast cancer cells are deficient in their ability to repair UV radiation-induced

DNA damage compared to wild type pRb proficient cells. Data are consistent with a model in which pRb is invovlved in the repair of UV radiation-induced lesions (Billecke et al. 2002). Therrier et al. have shown that human cells compromised for p53 function exhibit defective global and transcription-coupled nucleotide excision repair, whereas cells compromised for pRb function are defective only in global repair which suggests GGR is deficient in cells abrogated in pRb (Therrien et al. 1999). We were therefore interested to examine if pRb plays a role in HCR and/or UV-enhanced HCR of a UV-damaged reporter gene driven by the MCMV promoter in MEF cells. Here we have examined HCR of β-gal activity for UV-irradiated Ad5MCMV*lacZ* in unirradiated and pre-UV-irradiated wild type pRb proficient MEF cells and pRb knock out (pRb-/-), p107 knock out (p107-/-), p53 knock out (p53-/-) MEF cells.

Material and Methods

Cells and virus

Mouse embryonic fibroblasts with a specific knockout in the p53 (p53-/-) (Originally obtained from Jackson Laboratories, Bar Harbor, Maine), pRb (pRb-/-), or p107 (p107-/-) (Leccuter et al., 1998) genes, as well as a parental strain (MEFwt) expressing the wild type counterparts of all these genes, were obtained from Dr. M.A. Rudnicki (Currently at the Ottawa Regional Cancer Centre, Ottawa, Ontario). All cell cultures were maintained in Eagle's α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum together with antibiotic-antimycotic (100µg/ml penicillin, 100

μg/ml streptomycin and 250 μg/ml amphotercin B; Gibco BRL) in an atmosphere of 5% CO_2 at 37°C and 90-100% humidity.

Ad5MCMVlacZ is a recombinant adenovirus that contains the *lacZ* gene under control of the mouse cytomegalovirus (MCMV) immediate early promoter inserted in the deleted E1 region of the virus. Ad5MCMVlacZ is unable to replicate in human cells, but can efficiently infect and express the β-galactosidase reporter gene in many mammalian cell types including MEF cells. Viral stocks were prepared as described previously (Graham and Prevec 1991).

UV irradiation of virus

UV irradiation of the virus has been described previously (Bennett and Rainbow 1988). Viral suspensions in 1.8ml phosphate buffered saline (PBS) were irradiated in 35mm dishes on ice with continuous stirring using a General Electric germicidal lamp (model G8T5) emitting predominantly at 254nm at an incident fluence rate of 2 J/m2/sec as measured using a J-255 shortwave UV meter (Ultraviolet Products, San Gabriel, CA). Aliquots of 200 μl were removed for each exposure to the virus and diluted appropriately with unsupplemented medium.

HCR and UV enhanced HCR of the reporter gene

Cells were seeded at a density of $4x10^4$ cells/well in 96-well microtitre plates (Falcon, Lincoln Park, NJ). Between 20 and 24hr after seeding, medium was aspirated from the microtitre plates and replacde with 40 μl of warm PBS per well. Cells were then

UV irradiated at an incident fluence rate of 1 J/m^2/sec, or left unirradiated and subsequently re-fed with warm supplemented growth medium. Between 24 h after UV irradiation, cells were infected with either UV-irradiated or non-irradiated Ad5MCMVlacZ in a volume of 20 μl at a multiplicity of infection of 20 plaque forming units/cell. Following viral adsorption for 60 min at 37°C the cells were re-fed with warmed supplemented growth medium. Infected cells were harvested at 24 h after infection. The optical density (OD) was determined at several times following addition of CPRG using a 96 well plate reader (Labsystems multiscan MCC/340).

Results

(1) HCR of reporter gene expression for UV-irradiated Ad5MCMVlacZ in untreated MEF cells.

MEF cells were infected with UV-irradiated and non-irradiated Ad5MCMV*lacZ*. The relative expression of β-gal activity for UV-irradiated Ad5MCMV*lacZ* in non-irradiated MEF cells was determined in the various MEF cells (Figure 3-1 to 3-3). The UV fluence required to reduce β-gal activity to 37% of that for non-irradiated virus (D$_{37}$) was extrapolated from the survival curves and the mean D$_{37}$ values for the four cell lines (MEFwt, p53-/-, pRb-/- and p107-/-) in several independent experiments are presented in Table 3-1.

It can't be seen that there was no significant difference in HCR for β-gal activity of UV-damaged AdMCMVlacZ for untreated cells in any of the MEF knockouts

compared to the parental strain. These results suggest that p53, pRb and p107 are not involved in HCR of the UV-damaged reporter gene in untreated MEF cells.

(2) HCR of reporter gene expression for UV-irradiated Ad5MCMVlacZ in pre-UV-treated MEF cells

Typical curves for the relative expression of β-gal activity for UV-irradiated Ad5MCMV*lacZ* in pre-UV-irradiated (6 J/m^2) compared to non-irradiated cells are presented in (Figure 3-1, Figure 3-2, Figure 3-3). It can be seen that UV-treatment of MEFwt cells at 24hr prior to infection resulted in a significantly enhanced HCR of β-gal activity for UV-irradiated Ad5MCMV*lacZ*. In contrast, a significantly decrease HCR was detected in p53-/- cells under the same conditions (Figure 3-1). It also can be seen that a significantly enhanced HCR was detected in pRb-/- cells at the passage 10-12 (Figure 3-2), whereas when pRb-/- cells were at the passage 18-31 a significantly decrease in HCR was detected (Figure 3-2). It also can be seen that an enhanced HCR was detected in p107-/- cells at the passage 4, whereas when P107-/- cells were at passage 7-12 they showed a significantly decrease HCR (Figure 3-3).

D_{37} values for the survival of β-gal activity for UV-irradiated Ad5MCMV*lacZ* were extrapolated from the survival curves and used to quantitate the relative HCR of the UV-damaged reporter gene in pre-UV-treated compared to that in untreated cells within individual experiments. Mean D_{37} and relative HCR values for the four cell lines (MEFwt, p53-/-, pRb-/- and p107-/-) are presented in (Tables 3-2). It can be seen that UV- enhanced HCR was detected in MEFwt, pRb-/- (Passage 10-12) and p107-/-

(Passage 4) MEF cells. In contrast, significant decrease in HCR was detected in p53-/-.

pRb-/-(Passage 18-3 .) and p107- (Passage 7-12) MEF cells (Table 3-2). In addition,

these results show that the relative D_{37} values in pre-UV-treated compared to untreated

cells do not correlate with cell passage number in MEFwt and p53-/- MEF. In contrast,

in pRb-/- MEF cells the relative D_{37} values show a significant correlate with cell passage

number and in p107-/- MEF cells relative D_{37} values also correlate with cell passage

(p=0.06) (Figure 3-4) although this is not significant.

These results suggest that prior UV treatment results in an induced repair of UV-

damaged DNA that is dependent on wild-type p53. The presence of an enhancement of

HCR for the UV-damaged reporter gene in pre-UV-treated cells in low passage, but not

in high passage, pRb-/- and p107-/- cells suggests that the lack of pRb or p107 expression

per se does not result in a deficiency in inducible DNA repair. However, these results

suggest that the lack pRb or p107 expression results in alteration in MEF cells at high

passage number which abrogates inducible repair of UV-damaged DNA.

Discussion

To determine the role of the p53, pRb and p107 in the cellular response to UV-

induced DNA damage, we investigated the DNA repair characteristics of a UV-damaged

reporter gene in mouse embryonic fibroblasts with specific knockout in the p53 (p53-/-),

pRb (pRb-/-), and p107 (p107-/-) genes, as well as in a parental strain (MEFwt) which

have wild type expression of these genes. We found no significant difference in HCR for

β-gal activity in untreated cells for any of the MEF knockouts compared to the parental strain. These results suggest that p53, pRb and p107 are not involved in HCR of the UV-damaged reporter gene in untreated MEF cells. However, UV-treatment of MEFwt cells at 24hr prior to infection resulted in a significantly enhanced HCR of β-gal activity for UV-irradiated Ad5MCMV*lacZ*. In contrast, a significantly decreased HCR was detected in p53-/- MEF cells under the same conditions (Figure 3-1). These results suggest that prior UV treatment results in an induced repair of UV-damaged DNA that is dependent on wild-type p53. This is consistent with the report of Li and Ho 1997 who also report a p53 dependent upregulation HCR of a UV-damaged reporter gene in MEF cells (Li and Ho, 1997). Smith et al. also report a p53 dependent GGR but not TCR, in MEF cells (Smith et al. 2000). In addition, results from our laboratory have shown that repair of a UV-damaged reporter gene under the control of the HCMV immediate early promoter is inducible by UV in human fibroblast cells (Francis and Rainbow 1999), and that the inducible repair is dependent on wild type p53 (McKay et al. 1997). It is possible that p53 or p53-dependent gene products may directly interact with or affect the activity of NER proteins and cells lacking p53 exhibit defective repair of damaged DNA.

A significant decrease in HCR was detected in pRb-/- and p107-/- MEF cells at the higher passage, whereas an enhanced HCR was detected in the low passage number pRb-/- and p107-/-cells (Figure 3-2, Figure3-3, Figure 3-4). These results suggests prior UV treatment MEF cells results in an induced repair of UV-damaged DNA which is not dependent on pRb and p107 per se,. However, the detection of a reduced repair of the UV-damaged reporter gene in pre-UV-treated cells at high passage number for pRb-/-

and p107-/- cells suggests that the lack of pRb or p107 expression in MEF cells produces cellular alterations at high passage number which result in a deficiency in inducible DNA repair.

A recent report has shown that pRb-null human breast cancer cells are deficient in their ability to repair UV radiation-induced DNA damage compared to wild type pRb proficient cells (Billecke et al. 2002). Therrien et al. have shown that human cells compromised for p53 function exhibit defective global and transcription-coupled nucleotide excision repair, whereas cells compromised for pRb function are defective only in global repair which suggests GGR is deficient in cells abrogated in pRb (Therrien et al. 1999). This is consistent with the current results which show that pRb, p107 may result in cellular change affecting the repair of UV radiation-induced lesions. Since pRb-/- and p107-/- both showed an enhancement HCR in the lower passage, it is possible that pRb or p107 may indirectly result in alteration affecting cells at higher passage number.

pRb and p107 are checkpoint participants downstream of p53 (Levine 1997). The current results show that the relative D_{37} values do not correlate with cell passage number in MEFwt and p53-/- MEF, whereas in pRb-/- MEF cells the relative D_{37} values do correlate with cell passage number and in p107-/- MEF cells the relative D_{37} values may correlate with cell passage (p=0.06) (Figure 3-4). The loss of cell cycle checkpoints in pRb-/- and p107-/- cells may lead to increased mutations and deletions in these cells at high passage number which result in a deficiency of inducible DNA repair.

Table 3-1 Mean D_{37} values for HCR of β-gal activity in non-irradiated MEF cells.

Cell strain	Number of Expts.	Mean $D_{37} \pm$ SE (a)
MEF wt	9	64.9 ± 22.30
pRb-/-	10	69.08 ± 4.70
p53-/-	4	71.47 ± 2.26
p107-/-	6	73.76 ± 3.78

(a) Shows mean +/- standard error for a number of independent experiments.

Table 3-2. HCR of β-gal activity for UV-irradiated Ad5MCMV*lacZ* in UV-irradiated and non- irradiated cells

Cell strain	Passage	D_{37} $(J/m^2) \pm SE$		Relative HCR (a)
		0 J/m^2 to cells	6 J/m^2 to cells	
MEF wt	P9-P25	64.92 ± 2.30	80.50 ± 3.03	1.25 ± 0.05 (9) (b)
pRb-/-	P10-P12	60.61 ± 5.22	75.98 ± 5.14	1.29 ± 0.23 (5) (c)
	P18-P31	77.54 ± 6.02	66.99 ± 3.99	0.87 ± 0.05 (5) (d)
p53-/-	P8-P18	71.47 ± 2.26	58.83 ± 7.79	0.82 ± 0.05 (4) (d)
p107-/-	P4	68.20 ± 2.45	76.34 ± 5.90	1.11 ± 0.03 (2)
	P7-P12	76.54 ± 5.18	69.59 ± 5.00	0.90 ± 0.01 (4) (d)

(a) D_{37} values for survival of UV-irradiated reporter activity in UV-irradiated cells normalized to values obtained in non-irradiated cells. Shows mean $\pm$ /- standard error for a number of experiments as indicated in the brackets.

(b) Significantly greater than 1 (P<0.0001)

(c) Significantly greater than 1 (P<0.1)

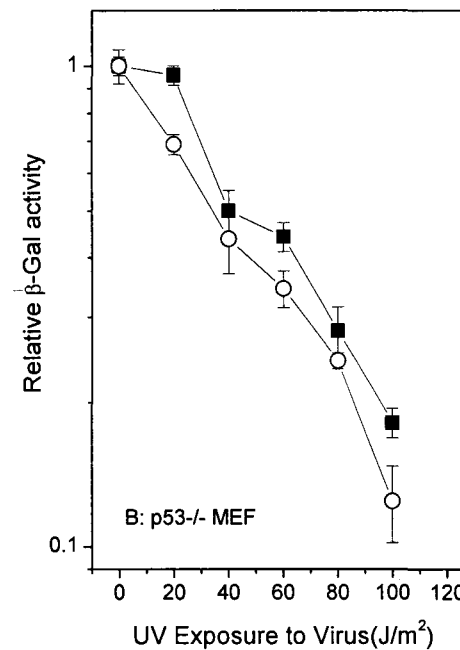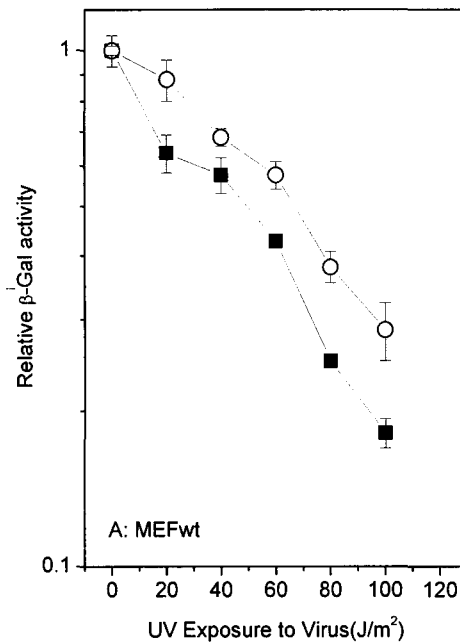(d) Significantly less than 1 (P<0.05)

Figure 3-1 Relative expression of β-gal activty for UV-irradiated Ad5MCMVlacZ in unirradiated and pre UV-irradiated MEFwt and p53-/- MEF cells. Representative results of a single experiment for non-irradiated MEFwt and p53-/- MEF cells (■) and UV-irradiated (6J/m²) MEFwt and p53-/- (○) MEF cells. Cell type is indicated on the figure. Each datum point is the mean ± SE of three replicates.
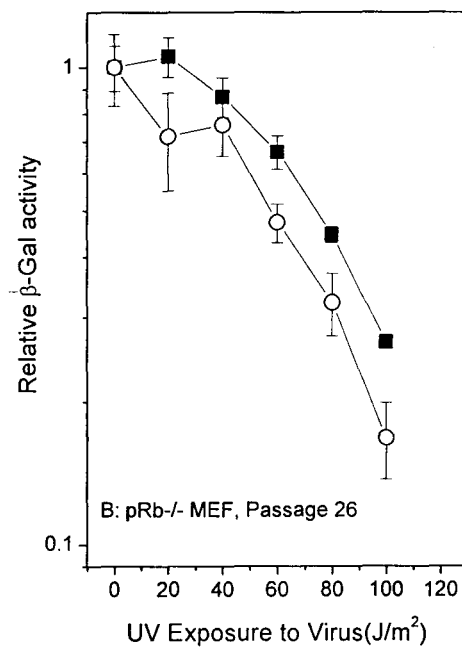
Figure 3-2 Relative expression of β-gal activity for UV-irradiated Ad5MCMVlacZ in unirradiated and pre-UV-irradiated low passage and high passage pRb-/- MEF cells. Representative results of a single experiment for non-irradiated pRb-/- MEF cells (■) and UV-irradiated (6J/m²) pRb-/- (○) MEF cells. Cell type and different passage number are indicated on the figure. Each datum point is the mean ± SE of three replicates.
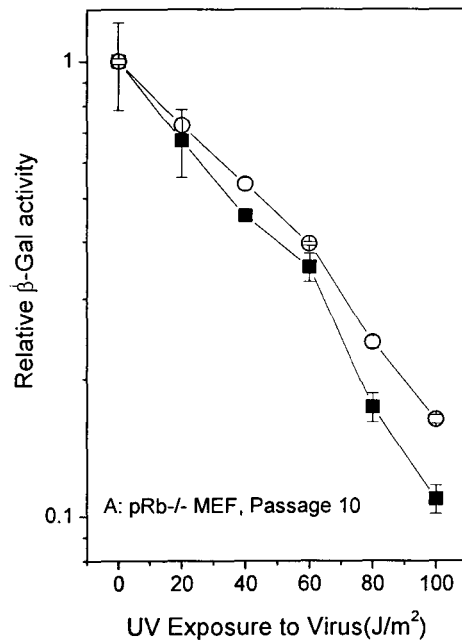
Figure 3-3 Relative expression of β-gal activty for UV-irradiated Ad5MCMVlacZ in unirradiated and pre-UV-irradiated low passage and high passage p107-/- MEF cells. Representative results of a single experiment for non-irradiated p107-/- MEF cells (■) and UV-irradiated (6J/m^2) p107-/- (○) MEF cells. Cell type and different passage number are indicated on the figure. Each datum point is the mean ± SE of three replicates.
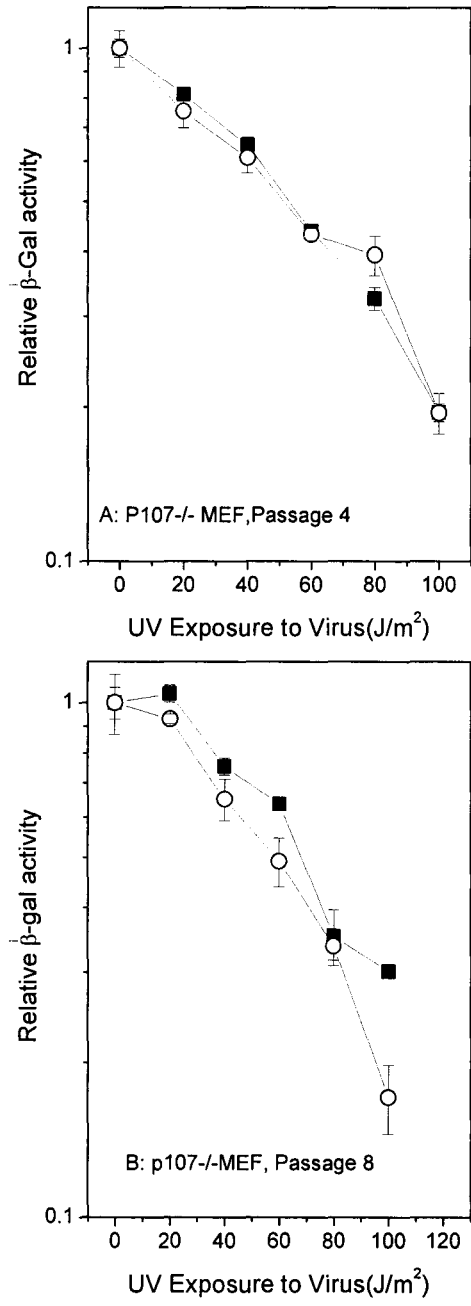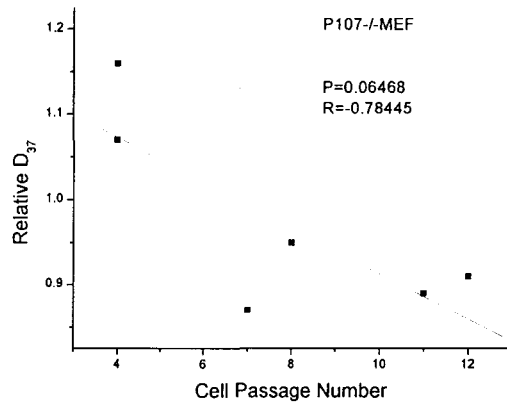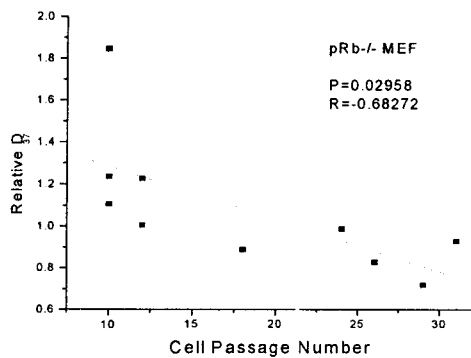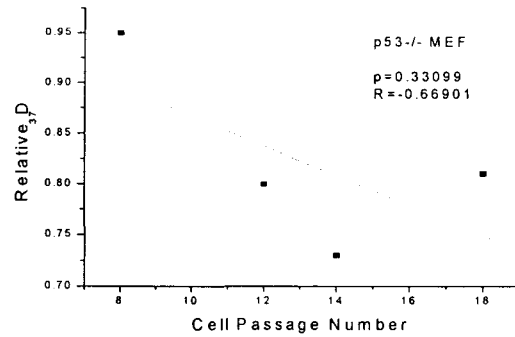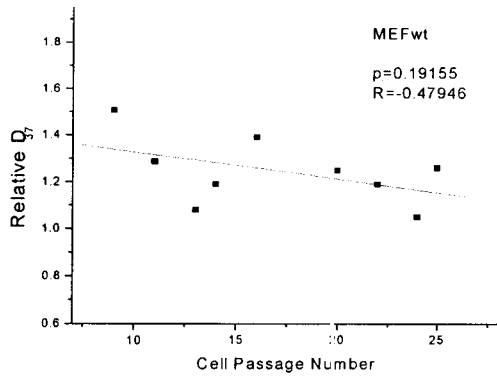
Figure 3-4 Relative D_{37} values of UV-irradiated reporter activity in UV-irradiated compare to unirradiated MEF cells as a function of cell passage number. D_{37} values for survival of UV-irradiated reporter activity in UV-irradiated cells were normalized to values obtained for non-irradiated cells. Data points were fitted with a linear regression line. Each point is a single experiment . Each experiment has 3 replicates.

Chapter 4

Dependence of Colony Survival on UV Wavelength: Repair Proficient and NER

Deficient Cells

Abstract

UVA produces predominantly single base damage that is repaired through base excision repair (BER), whereas UVC and UVB produce predominantly cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PP) that are repaired through nucleotide excision repair (NER). We have examined the colony survival following exposure to various UV sources of cells proficient and deficient in (NER). The UV sources were a UVC source from a germicidal lamp emitting predominantly at 254 nm; and a UVA source from a 1KW Hg-Xe arc lamp using either a Band pass filter (BPF) or a 335 Cut-off-filter (335COF). NER deficient CHO-UV5 and CHO-UV61 cells were more sensitive to UVC exposure compared to NER proficient CHO-AA8 cells, consistent with the production of UVC-induced DNA damage predominantly in the form of CPDs and 6-4PPs which are repaired through the NER pathway. NER deficient xeroderma pigmentosum cells from complementation group D (XPD) were more sensitive compared to NER proficient normal human cells following exposure to the UVA-BPF source. In addition XPDdenV cells, which express the denV gene from bacteriophage T4, were more resistant than XPD cells following exposure to the UVA-BPF source. Since the denV protein is specific for excision of CPDs these results indicate a substantial proportion of the induced DNA damage resulting from the UVA-BPF is in the form of CPDs, presumably due to a significant UVB component in the beam. In contrast, the NER deficient CHO-UV5 and CHO-UV61 cells showed a similar sensitivity compared to the NER proficient CHO-AA8 cell line following UVA-335COF exposures up to 60

KJ/m^2. However, for UVA-335COF exposures greater than 60 KJ/m^2 the NER deficient cells were more sensitive compared to the NER proficient CHO-AA8 cells, although the difference in sensitivity between NER deficient and NER proficient cells was less than that detected following UVA-BPF exposure. These results suggest that the UVA-335COF exposure produces predominantly DNA damage of the single base type for exposures less 60 KJ/m^2. This is consistent with the calculated spectral distribution, which showed a 5.62% UVB component for the UVA-BPF, but only 0.14% UVB component for the UVA-335COF.

Introduction

The biological effects and relative occurrence of different UV wavelengths varies considerably across the UV spectrum. This has led to the division of the spectrum into three ranges on the basis of wavelength: UVA (320-400nm), UV-B (280-320nm) and UV-C (200-280nm). UVA produces predominantly single base damage that is repaired through the base excision repair (BER) pathway, whereas UVC and UVB exposure produces predominantly CPDs and 6-4PP which are repaired through the nucleotide excision repair (NER) pathway (Lippke et al. 1981, Cooke et al. 20000). UVB can also produce 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG) which is repaired by the BER pathway (Beehler et al.1992). T4 endonuclease V (DenV protein) has the capacity to recognize specific CPD lesions and incise CPD damage (Boiteux et al. 1992). To assess the importance of CPDs in UV-induced photodamage, their formation, measured as

denV-sensitive sites, has been investigated after irradiation of plasmid DNA with UVC, UVB and UVA. The yield of CPD per kbp per (J/m2) was calculated and corresponds to $(1.5\pm0.3)*10-2$ for UVC, $(1.5\pm0.4)*10-4$ for UVB and $(1.4\pm0.2)*10-7$ for UVA (Kuluncsics et al. 1999).

XP is composed of a minimum of seven complementation groups (XP-A-G), each displaying a general deficiency in NER (Wood 1991). Runger et al. 1995 showed that most of the XP complementation groups studied (XP-A, XP-D, XP-E) were not different from normal cells in their ability to repair singlet oxygen-induced DNA damage, suggesting proficient BER in these cells (Runger et al. 1995). Stary et al. 1997 examined the sensitivity to UVA, UVB or UVC of human XPD cells which are deficient on NER and proficient in BER (Wood 1991, Runger er al. 1995), and human normal cells 293 embryonic kidney lines which have proficient NER and BER. The normal cells exhibited a much higher resistance to UVB and UVC compared to XPD cells, whereas after UVA exposure, XPD and normal cells showed a similar sensitivity (Stary et al.1997). Otto et al. 1999 examined the survival of the normal and XPD cells in keratinocytes and fibroblasts after UVA and UVB radiation. Normal and XPD fibroblasts showed a similar survival following UVA exposures up to 100 KJ/m2, whereas XPD fibroblasts were more sensitive compared normal fibroblasts after UVB radiation (Otto et al. 1999). In order to produce DNA damage that is predominantly single base changes and is repaired by the BER pathway we require a UVA source emitting predominantly in the 320-400 nm wavelength region of the UV spectrum, with little, if any, UVB in the 300-320 nm wavelength range. Contamination with UVB wavelengths produces CPDs and 6-4PP that

are repaired through the NER pathway. In order to examine the type of DNA damage induced by our UVA source under various conditions of filtration, we examined the colony survival of cells proficient and deficient in NER.

Materials and Methods

Cells

CHO-AA8 cells are NER proficient and BER proficient; CHO-UV5 cells have a mutation in the ERCC2 (XPD) gene and are NER deficient, but BER proficient; CHO-UV61 have a mutation in the ERCC6 (CSB) gene and are NER deficient. In addition the ERCC6 gene, which is deficient in CSB cells, has been reported to be involved in BER of oxidative damage in human cells (Cooper et al. 1997). It is therefore possible that UV61 cells are also deficient in BER.

GM637 cells are SV40-transformed normal human fibroblasts which are NER proficient; XPDSV40 are SV40-transformed human XPD fibroblasts and are NER deficient, but BER proficient; XPDSV40denV are XPDSV40 cells which express the bacterophage T4 endonuclease gene which can make an incision at CPDs and partially correct the NER deficiency in XPD cells. All these 3 cell strains were obtained from NIGMS repository, Camden, NJ.

All cell cultures were maintained in Eagle's α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum together with antibiotic-antimycotic (100μg/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml amphotercinB; Gibco BRL)

in an atmosphere of 5% CO2 at 37OC and 90-100% humidity. In order to passage cells, monolayers were rinsed with phosphate buffered saline (PBS) and then treated with 0.1% trypsin (GibcoBRL) in PBS. Cells were suspended in an appropriate volume of growth medium and aliquots were placed in culture flasks.

UV radiation sources

UVC: The UVC radiation source was from a General Electric germicidal lamp (model G8T5) emitting predominantly at 254nm at an incident fluence rate of 1 J/m^2/sec as measured using a Black-Ray-J-255 shortwave UV meter.

UVA: The UVA source with "Band-pass filter" (UVA-BPF) utilized a Sciencetech Model 200-1K light source housing a Sciencetech 100-1KMX. IKW Hg-Xe lamp together with a "Band-pass filter" which blocks some of UVB, at a fluence rate of 25 J/m^2/s (as measured by a Black-Ray J-221 long wave UV meter). The UVA source with "335 Cut-off filter" (UVA-335COF) utilized the same light source and a "WG335" filter which also cuts off UVB. The calculated spectrum of the UVA source under various conditions of filtration is shown in Table 4-1.

Clonogenic survival

Cells were seeded in 6-well plates at a density of 200 cells/well. After incubation for 6 hr, the growth media in each well was replaced with 0.5 ml PBS for UVC irradiation and 1 ml PBS for UVA irradiation. Cells were irradiated for varying times with UVC (254nm) or UVA. After irradiation, growth media was added to each well (2

ml for UVC, 4 ml for UVA, and the cells were further incubated for 6 days to allow surviving cells to form colonies, dishes were stained with crystal violet (0.5% in 70% ethanol and 10% methanol), and briefly submerged in tap water to destain. Only Colonies of greater than 32 cells were scored. Clonogenic survival is expressed as the ratio of plating efficiencies for treated cells compared to their mock-treated counterparts.

Calculation of UV Spectra

The published spectral distribution of the Mercury-Xenon arc lamp is shown in Figure 4-6 and the spectral distribution of lamp over the range 300-400 nm is shown in Curve 1: Input (Figure 4-5). Curve 2: No filter (Figure 4-5) was obtained by multiplying the spectral output (Curve 1: Input) by the published percent reflection of the dichroic mirror (Figure 4-7). Further multiplication of curve 2 by the published transmission curve for either the 335CCF (Figure 4-8) or the BPF (Figure 4-9) resulted in the spectral distribution of the UVA-355COF (Curve 5:Filter 335) or the UVA-BPF (Curve 3:Band Pass Filter) beam. The spectral distribution for a 320 cut off filter was also calculated (Curve 4: Filter 320), although this filter was not used in any of the experiments. The relative fluence of the beam over a particular wavelength range was determined from the area under the appropriate spectrum (Table 4-1).

Results

(1) *Colony survival curve in CHO cells following UVC exposure*

A typical colony survival curve for NER proficient and NER deficient CHO cells following UVC exposure is presented in Figure 4-1. It can be seen that both CHO-UV5 and CHO-UV61 cells were more sensitive to UVC compared to CHO-AA8 cells. This is expected since CHO-UV5 and CHO-UV61 are both NER deficient and UVC produces predominantly CPDs and 6-4PPs that are repaired through the NER pathway.

(2) *Colony survival curve in CHO cells using UVA source and the "Band-pass filter"*

A typical colony survival curve for NER proficient and NER deficient CHO cells following UVA exposure with the "Band-pass filter" (UVA-BPF) is presented in Figure 4-2. It can be seen that following exposure of cells using the UVA-BPF, both CHO-UV5 and CHO-UV61 cells are more sensitive compared to CHO-AA8 cells. Since CHO-UV5 and CHO-UV61 cells are both NER deficient this result suggests that there are still a substantial number of CPDs and 6-4PP produced by the UVA-BPF source under these conditions.

(3) *Colony survival curve in human cells using UVA source and "Band-pass filter"*

A typical colony survival curve for NER proficient and NER deficient human cells following UVA-BPF exposure is presented in Figure 4-3. It can be seen that XPD cells were more sensitive compared to normal cells and that the XPDdenV cells showed

80

an increased survival compared to XPD cells. These results also indicate that the under these conditions of filtration the UVA source still produces a substantial number of CPDs and 6-4PPs.

(4) Colony survival curve in CHO cells using UVA source and "335 cut-off" filter

Following UVA exposure using the "335 cut-off" filter (UVA-335COF) the NER deficient CHO-UV61 and CHO-UV5 cell lines show a similar sensitivity to the NER proficient CHO-AA8 cell line up to an exposure of 60 KJ/m^2 (Figure 4-4). For UVA-335COF exposures greater than 60 KJ/m^2 the NER deficient cells were more sensitive compared to the NER proficient CHO-AA8 cells, although the difference in sensitivity between NER deficient and NER proficient cells was less than that detected following UVA-BPF exposure. This is consistent with a reduced proportion of DNA damage in the form of CPDs and 6-4PP produced by UVA-335COF compared to UVA-BPF.

(5) Comparison of UVA spectra

A comparison of the expected spectra for various filter combinations is shown in Table 4-1. It can be seen that Filter 335 can cut out more UVB compared to the other filter combinations examined and still retains 89.58% of the unfiltered UVA intensity (Table 4-1). This is consistent with other reports that use a Filter 335 to cut off UVB when a pure UVA radiation source is needed (Robert et al. 1996).

The calculated spectra together with the colony survival results suggest that the "335 cut-off" filter results in a UVA beam producing predominantly DNA damage of the single base type for exposures less than 60 KJ/m^2 (that is repaired by the BER pathway).

Discussion

The frequently measured cellular response to radiation is cell death and is usually assayed by colony-forming ability. Colony formation reflects the ability of a single cell to restore the integrity of its DNA to the extent necessary for cell division. A typical colony survival curve for NER proficient and NER deficient CHO cells following UVC exposure is presented in Figure 4-1.CHO-UV5 and CHO-UV61 cells are more sensitive to UVC exposure compared to CHO-AA8 cells. This is expected since CHO-UV5 and CHO-UV61 are both NER deficient and UVC produces predominantly CPDs and 6-4PP, which are repaired through the NER pathway.

Colony survival curves for human cells using the UVA-BPF source showed that XPD cells were more sensitive compared to normal cells and that the XPDdenV cells showed increased survival compared to XPD cells. These results indicate that the under these conditions of filtration the UVA source produces a substantial number of CPDs and 6-4PP suggesting the UVA-BPF beam still has a substantial UVB component. This is consistent with the calculated spectral distribution for this filter combination (Table 4-1), which shows the UVA-BPF beam still has a 5.62% UVB component.

Following UVA-335COF exposure the NER deficient CHO-UV5 and CHO-UV61 cell lines show a similar sensitivity compared to the CHO-AA8 cell line at least up

to an exposure of 60 KJ/m2 (Figure 4-4). CHO-UV5 and CHO-UV61 cells are more sensitive compared to CHO-AA8 cells when exposures were greater than 60KJ/m2 of UVA-335COF, although the difference in sensitivity is less than following exposure to UVA-BPF. It therefore appears that the UVA-335COF beam still has a UVB component although the UVB component is less than that for the UVA-BPF beam. This is consistent with the calculated spectral distribution, which showed a 5.62% UVB component for UVA-BPF, but only a 0.14% UVB component for UVA-335COF. There are few reports on the survival of NER deficient cells after UVA radiation (Stary et al.1997; Otto et al.1999). Stary et al. 1997 reported a similar sensitivity to UVA for NER deficient XPD cells compared to an NER proficient human embryonic kidney cell line and Otto et al. 1999 reported that the survival of normal and XPD fibroblasts did not differ from one another after UVA exposures up to 100 KJ/m^2. (Otto et al. 1999).

The calculated spectra together with the colony survival results suggest that the "335 cut-off" filter results in a UVA beam producing predominantly DNA damage of the single base type for exposures less than 60 KJ/m2. Even if it were possible to completely remove the UVB component of the spectrum from the UVA source this will not necessarily result in no induced CPDs since low levels of CPD induction have been reported after UVA exposure of plasmid DNA (Kuluncsics et al. 1999).
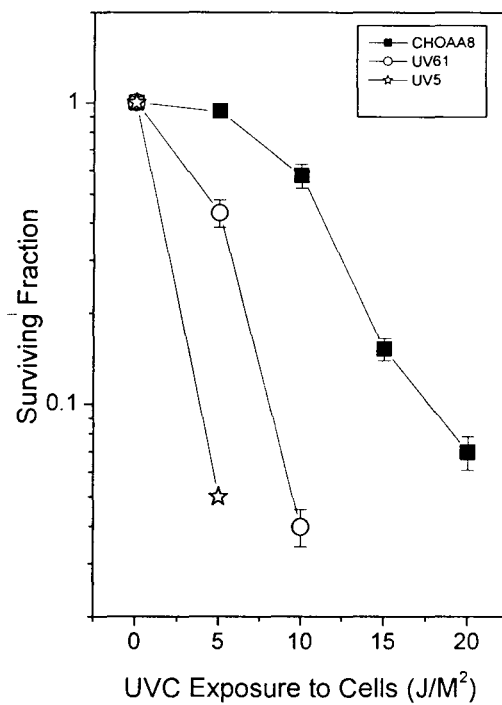
Figure 4-1 Colony survival curve for CHO-AA8, CHO-UV61 and CHO-UV5 after UVC exposure. Shows results for CHO-AA8 and CHO-UV61 in a single experiment. CHO-UV5 results adapted from Thompson et al. 1990. Cell type and symbol are indicated on the figure. Each data point is the mean ±SE of 2 replicates.

Figure 4-2 Colony survival curve for CHO-AA8, CHO-UV5 and CHO-UV61 cells using the UVA source with the "Band-pass filter". Shows pooled results from at least 2 experiments with each cell line. Each data point is the average ± standard error. Cell type and symbol are indicated on the figure.
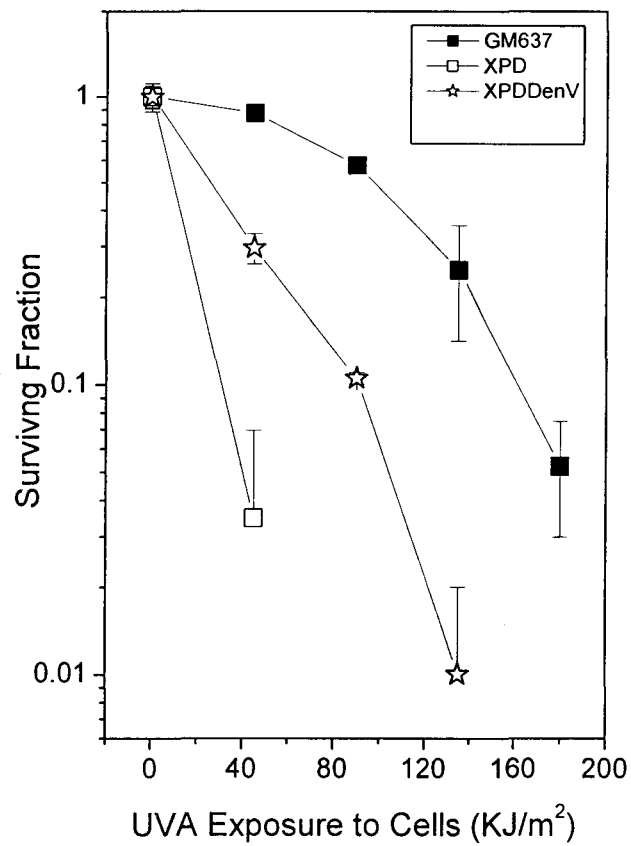
Figure 4-3 Colony survival curves for GM637, XPD and XPDDenV cells using the UVA source with the "Band-pass filter". Shows pooled results from at least 2 experiments with each cell line. Each data point is the average $\pm$ standard error. Cell type and symbol are indicated on the figure.
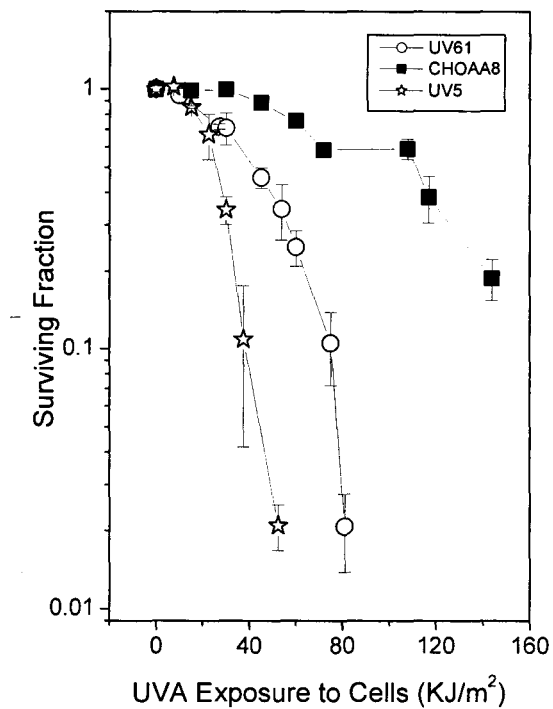
Figure 4-4 Colony survival curves for CHO-AA8, CHO-UV5 and CHO-UV61 cells using the UVA source with the "335 cut-off filter". Pooled results from at least 2 experiments with the CHO-AA8 cell line. Results from a single experiment with CHO-UV5 and CHO-UV61 cells. Each data point is the average ± standard error. Cell type and symbol are indicated on the figure.
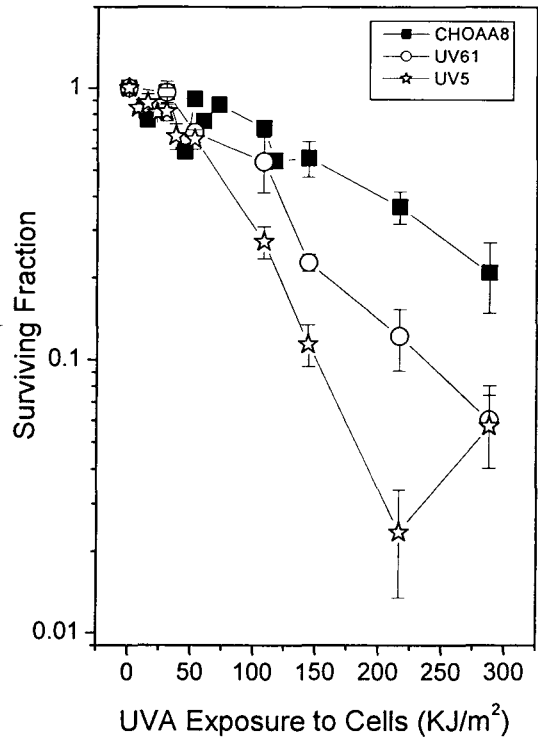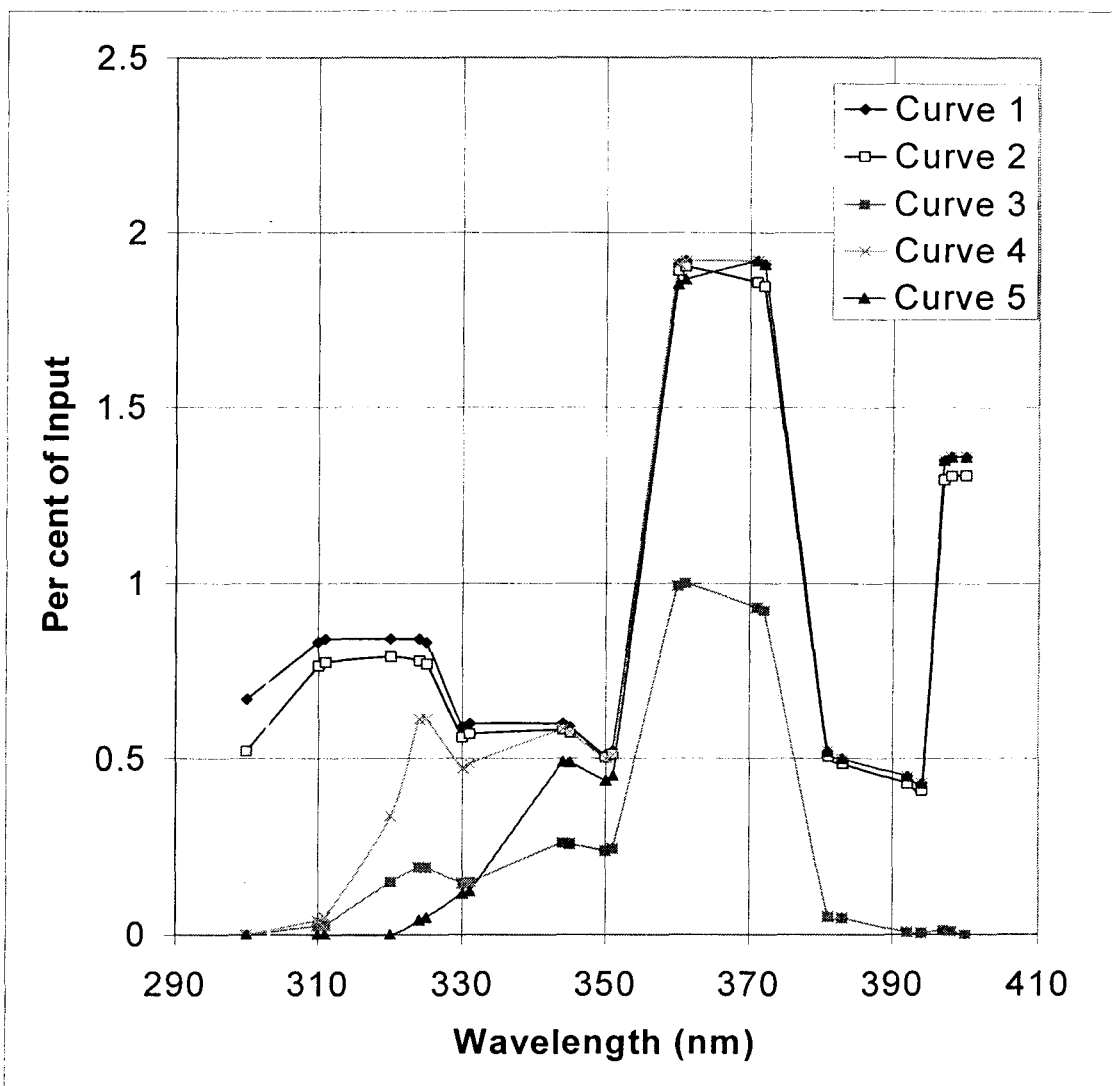
Figure 4-5: **Spectral Distribution of UVA Machine (Sciencetech).**

Curve1: Input
Curve2: NOFilter
Curve3: Our Filter (Band-Pass Filter)
Curve4: Filter 320
Curve5: Filter 335

Table 4-1. A comparison of the UV spectra for various filter combinations.

	NO Filter	Our Filter	Filter 320	Filter 335
300-400nm (a)	89.701	29.25	76.213	64.885
300-320nm (b)	17.369	1.645	3.891	0.089
320-400nm (c)	72.332	27.605	72.322	64.796
Filter Transmission Efficiency (d)	100%	38%	99.98%	89.58%
Percent UVB (e)	19.36%	5.62%	5.10%	0.14%

(a) Relative fluence in the 300-400 nm. range for various filter combinations (Figure 4-5).

(b) Relative fluence in the 300-320 nm. range for various filter combinations (Figure 4-5).

(c) Relative fluence in the 320-400 nm. range for various filter combination (Figure 4-5).

(d) Filter transmission efficiency was the relative fluence in the 320-400nm range for the various filters (Our Filter, Filter 320, Filter 335) expressed as percentage of the relative fluence of this range obtained for No Filter.

(e) Percent UVB was the relative fluence in the 300-320nm range for the various filters (Our Filter, Filter 320, Filter 335) expressed as percentage of the relative fluence in the 300-400 nm range.

Mercury-Xenon verses Xenon Short Arc Lamps

Spectral Distribution energy of HgXe and Xe short arc lamps.
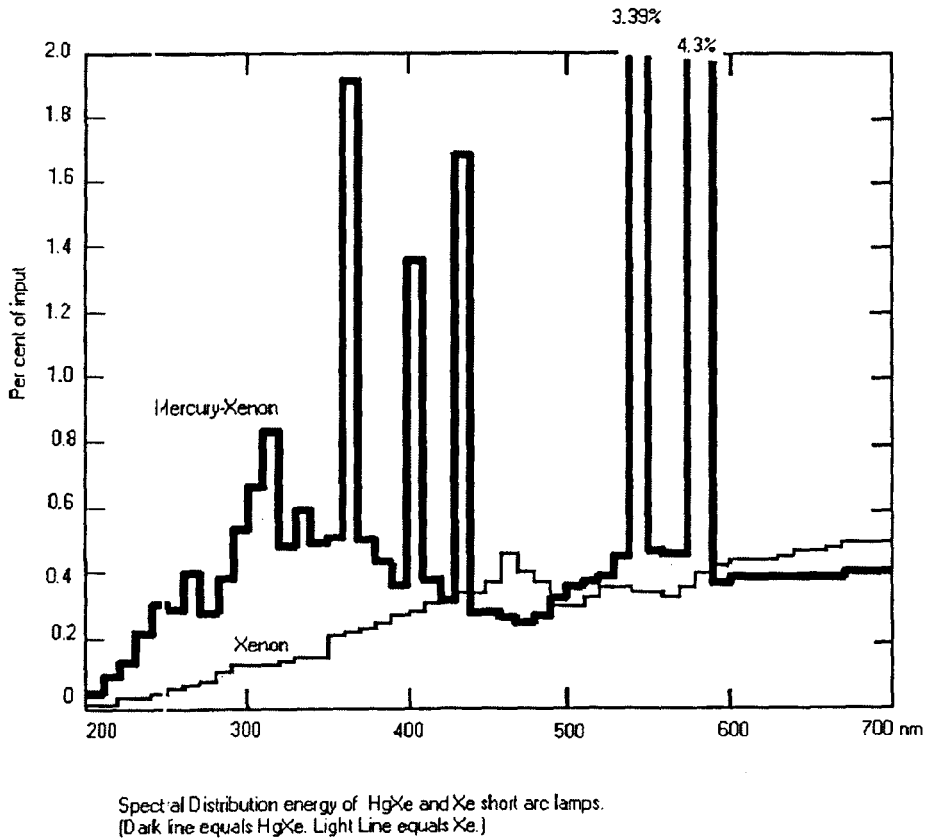(Dark line equals HgXe. Light Line equals Xe.)

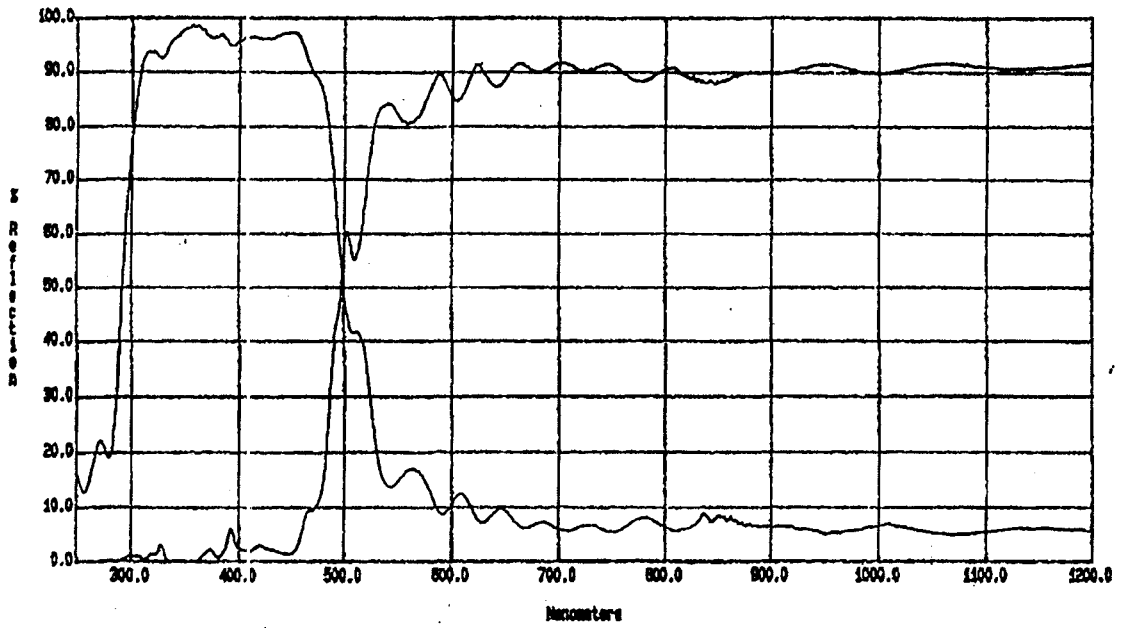Figure 4-6 Spectral Distribution energy of UVA lamp (Adapted from Sciencetech company).

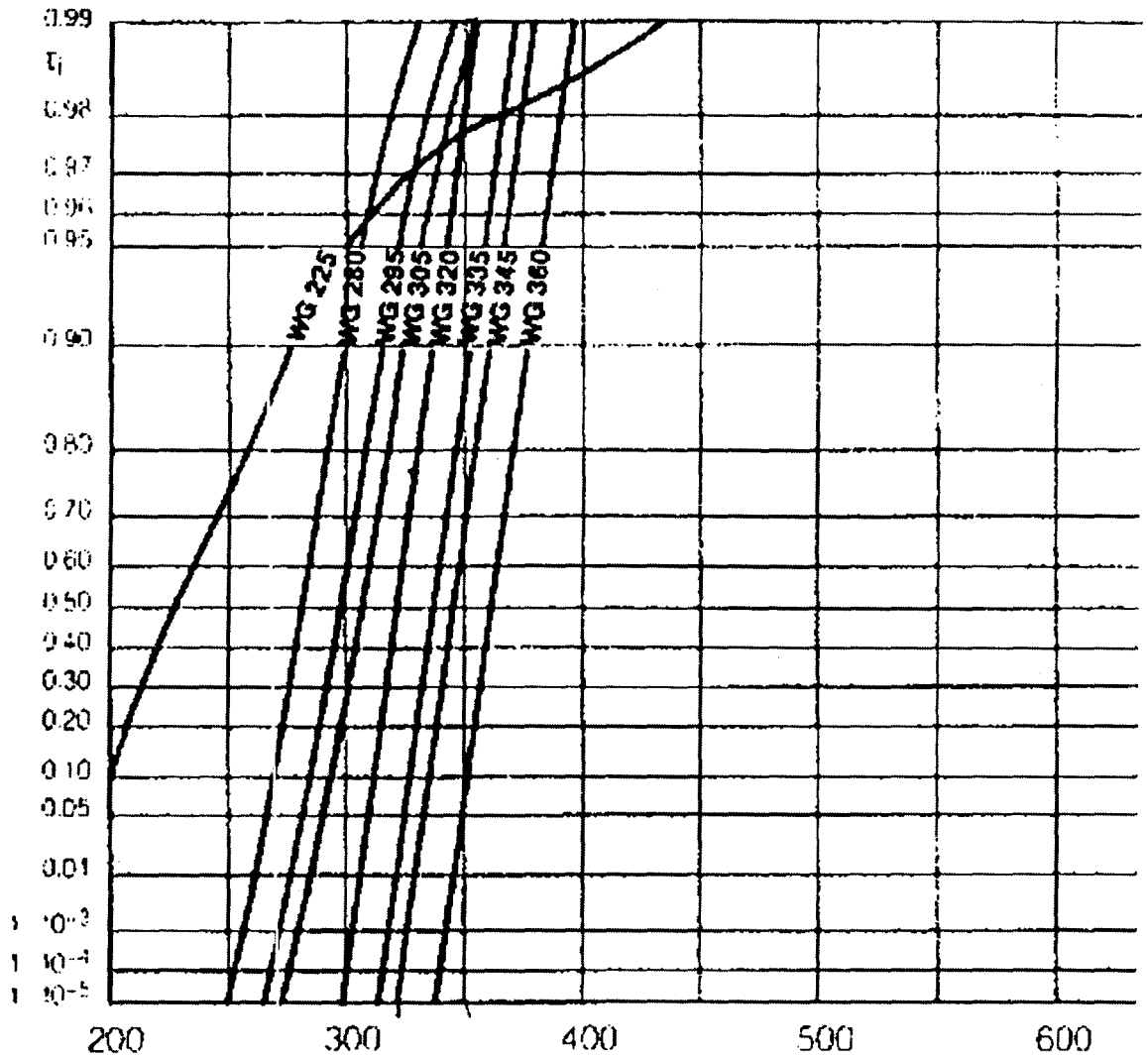**Figure 4-7 Percent reflection of the dichroic mirror.**

Figure 4-8 Filter transmission curve (Adapted from Harvard Apparatus Canada).
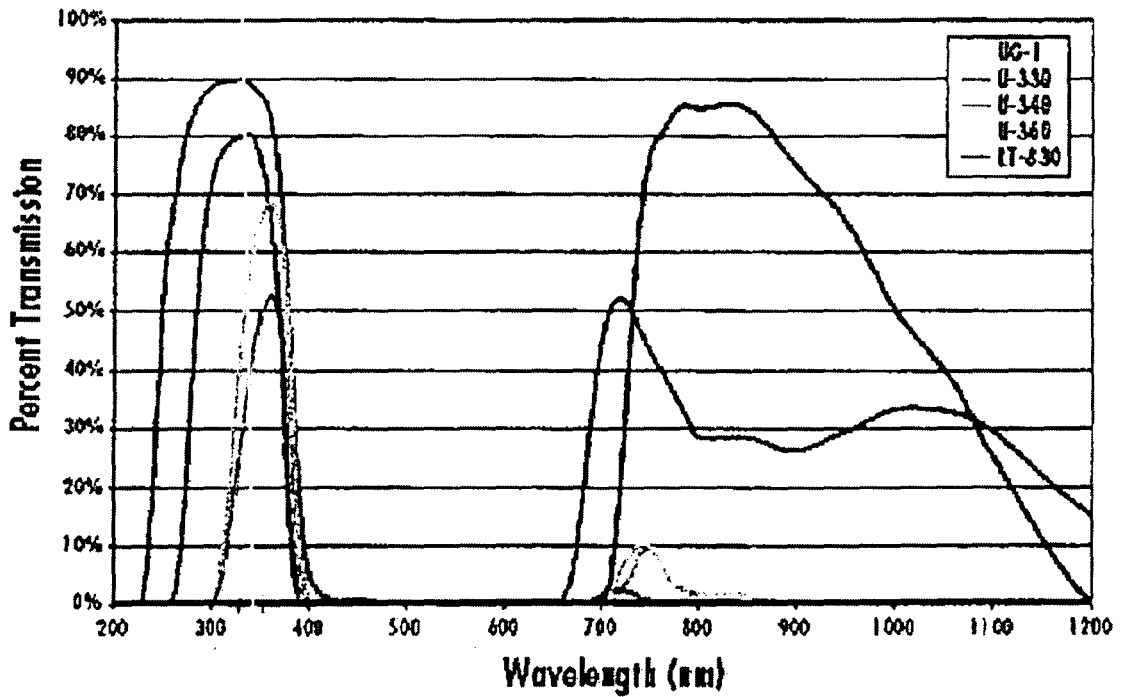
92

Figure 4-9 "Bandpass Filter" transmission curve (Adapted from Harvard Apparatus Canada)

Chapter 5

Summary

Host cell reactivation (HCR) of a UV-damaged reporter gene was used to examine constitutive and inducible DNA repair in rodent cells. Chapter two and three of this thesis describe the application of the HCR assay in Chinese hamster ovary (CHO) cells and mouse embryo fibroblasts (MEFs) respectively.

In chapter two, HCR of β-gal activity for UVC-irradiated Ad5MCMVlacZ was examined in non-irradiated and UVC-irradiated NER proficient parental CHO-AA8 and in mutant CHO-UV61 cells that are deficient in the TCR pathway of NER. HCR of β-gal activity for UVC-irradiated Ad5MCMVlacZ was significantly reduced in non-irradiated CHO-UV61 cells compared to that in non-irradiated CHO-AA8 cells suggesting that repair in the transcribed strand of the UVC-damaged reporter gene in untreated CHO-AA8 cells utilizes TCR. Prior UVC-irradiation of cells with low UV fluences resulted in a transient enhancement of HCR for expression of the UVC-damaged reporter gene in CHO-AA8 cells but not in TCR deficient CHO-UV61 cells. Pre-UVC-treatment of cells resulted also in an enhanced expression of β-gal for unirradiated Ad5MCMVlacZ in both CHO-AA8 and CHO-UV61 cells. These results suggest that prior UVC treatment results in a transient enhancement in repair of UVC-damage DNA in the transcribed strand of the active reporter gene in CHO-AA8 cells through an enhancement of TCR or a mechanism that involves the TCR pathway and that the upregulation of reporter gene expression alone is not sufficient for enhanced repair of the reporter gene in CHO-UV61 cells.

In chapter three, HCR of β-gal activity for UVC-irradiated Ad5MCMVlacZ was examined in non-irradiated and UVC-irradiated NER proficient parental wild type MEF

cells and in MEF cells with specific knockouts in the p53 (p53-/-), pRb (pRb-/-), and p107 (p107-/-) genes. HCR of β-gal activity for UVC-irradiated Ad5MCMVlacZ did not show a significant difference in non-irradiated cells for any of the MEF knockouts cells compared to the parental strain suggesting that p53, pRb and p107 do not play a role in NER of the UV-damaged reporter gene in untreated MEF cells. Prior UVC-irradiation of cells with low UVC fluences resulted in an enhancement of HCR for expression of the UVC-damaged reporter gene in MEF wild type cells, low passage pRb-/- and p107-/- MEF cells but not in p53-/- MEF cells or in high passage pRb-/-and p107-/- MEF cells. These results suggest that prior UVC treatment MEF cells results in an induced repair of UVC-damaged DNA that is dependent on p53. The presence of an enhancement of HCR for the UVC-damaged reporter gene in pre-UVC treated cells in low passage, but not in high passage, pRb-/- and p107-/- cells suggests that the lack of pRb or p107 expression per se does not result in a deficiency in inducible DNA repair. However, these results suggest that the lack of pRb or p107 expression results in alterations in MEF cells at high passage number that abrogate inducible repair of UVC-damaged DNA.

In Chapter 4, the colony survival following exposure to various UV sources was examined for cells proficient and deficient in (NER). The UV sources were a UVC source from a germicidal lamp emitting predominantly at 254 nm. and a UVA source from a 1KW Hg-Xe arc lamp using either a Band pass filter (BPF) or a 335 Cut-off-filter (335COF). Results suggest that the UVA-335COF exposure produces predominantly DNA damage of the single base type for exposures less 60 KJ/m^2. Since single base

changes are repaired by the BER pathway, the UVA source could be used in future experiments to examine the capacity of mammalian cells for BER.

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