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THE RELATIONSHIP BETWEEN THE REPAIR OF ULTRAVIOLET LIGHT INDUCED DNA DAMAGE IN HUMAN CELLS AND THE p53 TUMOUR SUPPRESSOR

By

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p53 AND TRANSCRIPTION COUPLED REPAIR

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Abstract

UV-induced dimers and bulky chemical adducts in DNA are repaired by nucleotide excision repair (NER). Several lesions repaired by NER including UV dimers pose a persistent block to transcription which in turn promotes apoptosis. A specialization of NER, termed transcription coupled repair (TCR), facilitates removal of transcription-blocking DNA damage by a process which is coupled to transcription by RNA polymerase II (RNAPII). It has been proposed that the presence of RNAPII stalled at a lesion facilitates NER by increasing the affinity of repair enzymes for the site of DNA. However, previous results from our laboratory have demonstrated that host cell reactivation of an ultraviolet (UV)-damaged RNAPII transcribed reporter gene is reduced in TCR proficient but global NER deficient fibroblasts. These results indicate that the RNAPII blocked at the site of a UV-induced lesion is not sufficient to promote the preferential repair of that lesion. It was further demonstrated that UV treatment of these cells increased repair of the reporter gene, strongly suggesting that TCR is inducible.

In the present thesis, primary human fibroblast strains with well characterized DNA repair phenotypes were used to demonstrate that heat shock enhanced reactivation of reporter gene activity (HSER) and the capacity of UV-irradiated cells to support adenovirus DNA synthesis (viral capacity), reflect TCR of UV-induced DNA damage. HSER requires repair of the transcribed strand of a reporter gene whereas viral capacity requires repair of genomic DNA. HSER and UV enhanced reactivation (UVER) were: dependent on wildtype p53, disrupted in most tumour cell lines examined and deficient in SV40 transformed fibroblasts. These results were confirmed using the viral capacity assay, indicating that TCR of genomic

DNA is inducible through a p53 dependent mechanism.

The primary activity ascribed to the p53 tumour suppressor gene is transactivation of genes involved in maintaining genome stability. A key downstream target of p53 is the cyclin dependent kinase inhibitor, $p21^{waf1}$. This gene product plays a role in mediating p53 dependent G_1 arrest and it has been suggested to play a role in NER. We hypothesized that UV induced activation of p53 is unlikely to result in efficient transactivation of p53 responsive genes because UV induced DNA damage blocks transcription. We report here that UV inhibits $p21^{waf1}$ expression immediately following irradiation. The efficient recovery of $p21^{waf1}$ expression and subsequent induction of $p21^{waf1}$ are dependent on TCR of UV induced DNA damage. The delayed induction of $p21^{waf1}$ expression following UV irradiation is not consistent with the proposed role of $p21^{waf1}$ transactivation in NER.

The results presented here predict a dose dependent reduction in the efficiency of G_1 arrest following UV. We addressed the consequences of attenuated G_1 arrest on UV sensitivity by transiently expressing SV40 large T antigen, polyoma virus large T antigen, E2F-1 and E2F-4 from recombinant adenovirus (Ad) constructs. Expression of these gene products conferred UV sensitivity on normal diploid fibroblasts. In contrast, stimulation of the p53 dependent G_1 checkpoint pathway with a similar Ad construct expressing $p21^{waf1}$ led to increased clonogenic survival following UV irradiation. We propose a model in which UV induced lesions lead to increased p53 activity and p53 stimulated DNA repair but persistent DNA damage prevents expression of p53 responsive gene products in a dose dependent manner. In this way, cells with irreparable DNA damage are likely to be eliminated by apoptosis as a consequence of the inability to express anti-apoptotic genes.

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Preface

This thesis is comprised of a series of chapters in the form of journal articles Three of these articles are published in *Mutation Research*, *Carcinogenesis* and *Photochemistry and Photobiology*. Written permission from Elsevier Science, Oxford University Press and the American Association of Photobiology was acquired to reprint these articles as a part of this thesis. Two additional papers are submitted for publication and a sixth paper is expected to be submitted. Unpublished results relevent to chapters 7 and 8 have been included as an addendum to each chapter. In chapter 8, a model is presented which incorporates results from all previous chapters. Lastly, an appendix which reports preliminary work using a polymerase chain reaction based method to assess DNA repair in a UV-damaged adenovirus has been included.

I wrote all manuscripts presented in this thesis. However, the papers in chapters 3 (*Methods in Molecular Biology, submitted*), 5 (*Carcinogenesis*, 18, 245-249) and 6 (*Photochem Photobiol, 66, 659-664*) are multi-authored. My contribution to each is briefly described below.

I was invited to contribute a paper to *Methods in Molecular Biology, Transgene Delivery and Expression in Mammalian Cells* (Ed RE Aubin). This article has been submitted. Three authors are included on that method paper. My contribution included the writing of the manuscript as well as the data presented in figures 1A and 3.

Three authors are also included in the *Carcinogenesis* paper. My contribution to that paper included writing the manuscript , all heat shock enhanced reactivation experiments, all heat shock survival experiments and all UV survival experiments.

My contribution to the *Photochemistry and Photobiology* paper included the writing of the manuscript and all data presented. Christopher Winrow's authorship was based on preliminary unpublished experiments which contributed to the rationale for the experiments.

Abbreviations

6-4PP, pyrimidine (6-4) pyrimidone dimer

Ad, adenovirus

β-gal, β-galactosidase

CPD, cyclobutane pyrimidine dimer

CS, Cockayne syndrome

CS-A, CS group A

CSA, CS-A complementing protein

ERCC-1, excision repair cross complementing group 1

GGR, global genome repair

HCR, host cell reactivation

HS, heat shock

HSV, herpes simplex virus

LFS, Li-Fraumeni syndrome

MOI, multiplicity of infection

NER, nucleotide excision repair

ONPG, O-nitrophenol β-D-galactopyranoside

PCNA, proliferating cell nuclear antigen

PCR, polymerase chain reaction

pfu, plaque forming unit

Py, polyomavirus

PyLT, Py large tumour antigen

RNAPII, RNA polymerase II

RP-A, replication protein A

S-phase, DNA synthesis phase

SV40, simian virus 40

SV40LT, SV40 large tumour antigen

TCR, transcription coupled repair

TFIIH, transcription factor IIH

UV, ultraviolet light

UVER, UV enhanced reactivation

XP, xeroderma pigmentosum

XP-A, XP group A

XPA, XP-A complementing protein.

Chapter 1

Literature Review

UV and skin cancer

The incidence of skin cancers is increasing primarily in countries with a large proportion of people of northern European descent (reviewed in Armstrong and Kricker, 1995). Basal cell carcinoma (BCC) is the most common form of cancer in North America. These cancers are relatively non-invasive and rarely metastasize. Squamous cell carcinomas (SCC) are less frequent, more invasive and have a higher frequency of metastasis. Cutaneous malignant melanoma (CMM) are the least common of these cancers but they are highly invasive and readily metastasize. Of the cutaneous cancers, CMM has the worst prognosis. The etiology of each of these cancers has been related to UV exposure. Lifelong exposure to UV has been linked to SCC and BCC whereas sunburn in childhood correlates with the occurrence melanoma (reviewed in Armstrong and Kricker, 1995).

Ultraviolet light is the primary environmental agent that contributes to dermatological neoplasia (reviewed in Armstrong and Kricker, 1995). Replication of DNA with persistent UV induced DNA damage is mutagenic as a result of replicative bypass of persistent UV induced dimers (Konze-Thomas et al, 1982; Spivak and Hanawalt, 1992). For this reason, the induction of point mutations in critical genes involved in carcinogenesis has been examined extensively. UV signature mutations, C to T and CC to TT transitions, form opposite UV lesions induced in adjacent pyrimidines (reviewed in Freidberg *et al*, 1995). BCC and SCC frequently have UV signature mutations in one or both alleles of the p53 tumour suppressor gene (Rady *et al*, 1992; Brash *et al*, 1991; Moles *et al*, 1993; Ziegler *et al*, 1994). Similar mutations have been reported in mouse models of SCC (Burns *et al*, 1991; Ruggeri *et al*, 1991; Kress *et al*, 1992; van Kranen *et al*, 1995; Dumaz *et al*, 1997).

Mutant p53 has also been detected in unaffected skin adjacent to BCC (Urano *et al*, 1995), in actinic keratosis (Ziegler *et al*, 1994) and in unaffected sun exposed skin cells (Jonason *et al*, 1996) suggesting that p53 mutations arise as an early stage in development of non-melanoma skin cancers (NMSC). Consistent with this interpretation, transgenic mice with mutant p53 have elevated levels of UV induced SCC (Li *et al*, 1995).

Induction of CMM, by contrast, does not correlate with lifetime exposure to UV but rather to sunburns in childhood. In addition to dimers induced by UV, wavelengths greater than 320 nm including visible light may play a role in CMM (Setlow *et al*, 1993). Mutations in p53 are infrequent in CMM (reviewed in Basset-Seguin *et al*, 1994) whereas alterations in the tumour suppressor gene, $p16^{ink4a}$ are more common (Reed *et al*, 1995; Pollock *et al*, 1995). The $p16^{ink4a}$ locus has been identified as a familial melanoma susceptibility locus (Hussussian *et al*, 1994; Kamb *et al*, 1994). UV signature mutations in the $p16^{ink4a}$ gene have been reported suggesting that UV can be involved in the inactivation of this locus in sporadic CMM (Pollock *et al*, 1995).

The importance of UV in skin carcinogenesis is evident in the elevated incidence of skin cancers in individuals clinically affected by xeroderma pigmentosum. The incidence of skin cancers is elevated as much as 2000 fold compared to unaffected individuals of similar ethnic background. BCC, SCC and CMM rates are all elevated in individuals affected with xeroderma pigmentosum compared to unaffected individuals (reviewed in Kraemer, 1994). Fibroblasts derived from individuals with XP are both hypersensitive and hypermutable following UV exposure due to a defect in DNA repair (reviewed in Freidberg *et al*, 1995). Murine XP and Cockayne syndrome models of carcinogenesis provide additional support for

both UV in the pathogenesis of skin cancers and DNA repair as an antineoplastic mechanism (Nakane *et al*, 1995; DeVries *et al*, 1995; Sands *et al*, 1995; Cheo *et al*, 1996; Cheo *et al*, 1997; van der Horst *et al*, 1997). Both topical liposome mediated transfer of a prokaryotic DNA repair gene (Bito *et al*, 1995) or topical application of sunscreens (Anamthaswamy *et al*, 1997) reduce the carcinogenic effect of UV exposure in murine models of carcinogenesis. Although the cellular targets of UV induced DNA damage which lead to skin carcinogenesis may vary with tumour type, UV exposure contributes to the pathogenesis of cutaneous neoplasms and DNA repair is protective against UV induced cancers.

Repair of UV induced DNA damage.

Nucleotide excision repair

All organisms are continuously exposed to both endogenous and exogenous DNA damaging agents. Several highly conserved DNA repair mechanisms have evolved to reduce the consequences of exposure to these agents. Nucleotide excision repair (NER) is a plastic repair mechanism which has broad substrate specificity (Reviewed in Freidberg *et al*, 1995). UV induces primarily 2 intrastrand dimers, the cyclobutane pyrimidine dimer (CPD) and the pyrimidine (6-4) pyrimidone dimer (6-4PP). Dimers induced by UV light as well as bulky adducts induced by a variety of chemical agents are repaired by this pathway. Removal of these and other lesions by NER requires 4 general steps. 1. The lesion must be recognized. 2. Incisions are made in the damaged DNA strand on each side of the lesion. 3. The oligonucleotide containing the dimer or bulky adduct is released. 4. The new DNA strand is synthesized and ligated. Thus the undamaged strand is used as a template for repair of the

damaged strand (Reviewed in Freidberg *et al*, 1995).

Although NER repairs UV induced DNA damage, it has been recognized for some time that repair is heterogeneously distributed throughout the genome. Rapid repair of transcribed genes was initially reported in Chinese hamster ovary cells (Bohr *et al*, 1985) and subsequently demonstrated in human cells (Mellon *et al*, 1987), *Escherichia coli* (Mellon *et al*, 1989) and *Saccharomyces cerevisiae* (Sweder and Hanawalt, 1992; Leadon and Lawrence, 1992). This rapid repair results from strand specific repair of the template strand of transcribed genes, termed transcription coupled repair (TCR) with the global genome repair (GGR) component of NER responsible for removal of lesions from both the non template strand of active genes and inactive chromatin (Bohr *et al*, 1985; Mellon *et al*, 1987). The ubiquitous nature of TCR suggests that rapid repair of transcriptionally active genes is of central importance to all organisms. Since UV lesions inhibit transcription (Mayne and Lehman, 1982), it has been proposed that removal of transcription blocking DNA damage from essential genes confers resistance to UV irradiation (Bohr *et al*, 1985). Furthermore, TCR may be protective against UV induced cancers (Madhani *et al*, 1986). Cells derived from individuals with XP and CS have been instrumental in studying the mechanism of TCR and GGR.

XP is a heterogeneous disorder in which affected individuals are typically hypersensitive to sunlight exposure, have atypical pigmentation and develop cutaneous tumours at high frequency (for review see Kraemer *et al*, 1994). Cells derived from XP probands are reduced in their capacity to repair UV induced DNA damage (for review see Kraemer *et al*, 1994). Complementation studies initially revealed at least 7 loci to be involved

Table 1. DNA repair phenotypes of xeroderma pigmentosum and Cockayne syndrome fibroblasts.

stain	UV sensitivity[a]	GGR[a]	TCR[a]	protein function
NDF[b]	NS [c]	+[d]	+[d]	not applicable
XP-A	HS	-	-	lesion recognition
XP-B	HS	-	-	TFIIH (transcription/repair factor)
XP-C	MS	-	+	unknown
XP-D	HS	-	-	TFIIH (transcription/repair factor)
XP-E	MS	-	-	unknown, dispensible *in vitro*
XP-F	HS	-	-	5' incision (with ERCC1)
XP-G	HS	-	-	3' incision
XP-V	MS	+	+	unknown
CS-A	MS	+	-	unknown
CS-B	MS	+	-	unknown

a See text for references.
b normal diploid fibroblasts (NDF).
c normal sensitivity (NS), moderate sensitivity (MS) and highly sensitive (HS).
c normal or near normal repair (+) and reduced repair compared to NDF (-).

in NER: XPA through XPG (for review see Freidberg *et al*, 1995). Most of the XP proteins have been cloned and biochemical activities have been ascribed for most of these (table 1). In addition, several other human DNA repair genes have been cloned based on their ability to complement UV sensitive Chinese hamster ovary cell lines. These gene products are referred to as excision repair cross complementing gene products (ERCC 1 through 11).

CS is a clinically distinct autosomal recessive disorder characterized by UV sensitivity, short stature and progressive neurological dysfunction (for review see Kraemer *et al*, 1994). Despite UV hypersensitivity, CS is not characterized by a predisposition to UV induced cancers. CS individuals fall within one of two complementation groups (CS-A and CS-B), however a third group has been proposed based on clinical overlap between XP and CS. Several XP individuals have CS-like symptoms. These individuals fall into the XP-B, XP-D and XP-G groups (For review see Kraemer *et al*, 1994). More recently, a single CS-B individual reportedly has characteristics of XP (Itoh *et al*, 1996). The NER defect in CS cells is the result of a specific deficiency in TCR indicating that the CSA and CSB proteins are required for repair of active genes.

Clinically, CS is more severe than XP despite the lack of UV induced skin cancers. Clear developmental abnormalities in CS and XP/CS individuals are prevalent. Since the repair phenotype of CS cells is less pronounced than that of XP, it has been proposed that the clinical CS phenotype is not simply the result of a TCR deficiency but may involve a transcription defect. Although deficiencies in transcription have been reported in CS cells (Balajee *et al*, 1997), there is no conclusive evidence of a transcriptional defect (Tu *et al*, 1997). Decreased repair of oxidative base damage in CS and CS/XPG cells (Leadon and

Cooper, 1993; Cooper *et al*, 1997) may contribute to the observed transcriptional defect. Endogenous oxidative damage is always present in cells (reviewed in Ames *et al*, 1995) and thus a transcriptional defect may reflect persistent transcription blocking DNA damage (Leadon and Cooper, 1993; Cooper *et al*, 1997).

Several mouse lines have been established in which DNA repair genes have been inactivated or replaced with mutant alleles to model the human XP and CS disorders (reviewed in Freidberg *et al*, 1997). XPA and XPC null mice were sensitive to UV induced skin and occular cancers (Nakane *et al*, 1995; de Vries *et al*, 1995; Sands *et al*, 1995). The crossing of XPC mice with mice which were either homozygous or heterozygous for p53 null alleles greatly enhanced the extent of skin cancers induced by chronic UV exposure (Cheo *et al*, 1996). Although this result demonstrate that p53 is protective against UV induced neoplasia, individuals with inherited p53 mutations are not prone to the development of non-melanoma skin cancers. In any case, the phenotype of these mouse strains is clearly consistent with the clinical phenotype of individuals affected with XP.

A transgenic mouse strain expressing a CSB truncation mutant which mimics an allele from an individual affected with CS has also been generated (van der Horst *et al*, 1997). Mouse embryonic fibroblasts from the CSB mouse (mCS-B) do not exhibit strand specific repair of the p53 gene indicating that mCS-B fibroblasts are deficient in TCR (van der Horst *et al*, 1997). Thus the repair phenotype of mCS-B fibroblasts mirrors that of CS-B cells. In contrast to clinically affected individuals, CSB mice were sensitive to the induction of UV induced skin cancers (van der Horst *et al*, 1997). TCR accounts for a greater proportion of the total repair in rodent cells compared to human cells following UV-irradiation (reviewed

in Freidberg *et al*, 1995). The difference in the cancer predisposition between CSB individuals and CSB mice may reflect a greater dependence on TCR in murine cells (van der Horst *et al*, 1997). Together these results indicate that both TCR and GGR can be protective against UV induced neoplasia.

Proteins required for global genome repair

In vitro reconstitution of the NER incision reaction requires only ERCC-1, XPA, XPC, XPF, XPG and TFIIH (Bessho *et al*, 1997) although replication protein A (RPA) reportedly facilitates *in vitro* NER (Matsunaga *et al*, 1996). XPA contains a zinc finger motif characteristic of several DNA binding proteins (Tanaka *et al*, 1990). XPA preferentially binds to UV irradiated DNA suggesting that it may be involved in lesion recognition (Robins *et al*, 1991). Several other proteins interact with XPA including RPA (He *et al*, 1995; Matsuda *et al*, 1995; Li *et al*, 1995), ERCC-1 (Li *et al*, 1994; Park and Sancar, 1994), XP-F (Li *et al*, 1994; Park and Sancar, 1994) and the basal transcription factor TFIIH (Park *et al*, 1995). RPA participates prior to incision (Coverly *et al*, 1992) and may interact with XPA to facilitate lesion recognition. XP-A fibroblasts are considered to be almost completely repair deficient (For review see Kraemer *et al*, 1994).

XP-G fibroblasts are also considered to be virtually repair deficient. The XPG protein interacts with RPA (He *et al*, 1995), TFIIH (Iyer *et al*, 1996) and CSB (Iyer *et al*, 1996). XPG is the structure specific endonuclease which makes the 3' incision between 5 and 7 nt from the damaged bases (Scherly *et al*, 1993; Huang *et al*, 1992). This incision is facilitated by an interaction with RPA (Matsunaga *et al*, 1996). A function of XPG other than its

structure specific endonuclease activity is required for the 5' incision induced by ERCC-1/XPF (Wakasugi *et al*, 1997). The XPG protein may be required for recruitment of ERCC-1/XPF into the recognition and incision complex (Wakasugi *et al*, 1997). XP-G fibroblasts have similar UV sensitivity to XP-A fibroblasts (For review see Kraemer *et al*, 1994) presumably because the XPG protein is required prior to full assembly of the recognition and incision complex thus inactivation of XPG disrupts subsequent 5' incision as well (Wakasugi *et al*, 1997).

ERCC-1 and XPF form a tight complex (Mu *et al*, 1995; Park *et al*, 1995) which binds to XPA (Li *et al*, 1994; Park and Sancar, 1994). The ERCC-1/XPF proteins make the incision 22-24 nt to the 5' side of the damaged nucleotides (Huang *et al*, 1992; Mu *et al*, 1995; Matsunaga *et al*, 1995). RPA is required for efficient incision by the ERCC-1/XPF incision nuclease (Matsunaga *et al*, 1996). This complex also participates in recombinational repair of interstrand crosslinks (Harrington and Lieber, 1994; Weeda *et al*, 1997), indicating that distinct repair pathways can share common protein components.

In addition to its requirement for promoter clearance in RNA pol II dependant transcription (Goodrich *et al*, 1994; Maxon *et al*, 1994), the basal transcription factor TFIIH is also required *in vitro* for the incision reaction at the site of UV induced DNA damage (Bessho *et al*, 1997). TFIIH is a multiprotein complex made up of several proteins including the XPB and XPD proteins (Van Vuuren *et al*, 1994; Drapkin *et al*, 1994). The XPB gene was cloned by virtue of the ability of a human cDNA to complement the UV sensitive phenotype of a Chinese hamster ovary (CHO) cell line from complementation group 3 (Weeda *et al*, 1990). The XPD gene was isolated by complementation of UV sensitive CHO

cells from complementation group 2 (Weber *et al*, 1988). Both XPB and XPD are helicases and exhibit 3' to 5' and 5' to 3' activity respectively (Weeda *et al*, 1990; Drapkin *et al*, 1994). These proteins may be involved in unwinding DNA around the lesion during NER.

The XPC protein is required for *in vitro* incision (Bessho *et al*, 1997). The requirement for XPC appears to be specific for GGR as TCR is operative in XP-C fibroblasts (Venema *et al*, 1990; Kantor *et al*, 1990). XPC interacts strongly with the human homologs of the *S.cerevisiae* protein Rad23, HHR23A and HHR23B (Masutani *et al*, 1994; Li *et al*, 1997). The requirement for XPC in *in vitro* NER can be circumvented by creating a 10 nt mismatch adjacent to the CPD (Mu and Sancar, 1997). The "bubble" formed in this synthetic substrate reportedly mimics the conformation of DNA associated with a stalled RNA polymerase at the site of a dimer, suggesting that XPC may be required to stabilize a specific DNA secondary structure during incision which is stabilized by alternate means during NER.

Transcription coupled repair proteins

Attempts to develop an *in vitro* TCR assay for either mammalian cells or yeast have been unsuccessful despite the ability to perform both transcription and repair simultaneously in yeast extracts (Wang *et al*, 1996). The requirement of specific proteins is less well characterized for this reason and depends primarily on the repair phenotypes of NER deficient fibroblast strains. Of the proteins required for NER, only the XPC protein appears to be dispensable (Venema *et al*, 1990). CS cells by contrast are specifically deficient in TCR with normal levels of GGR, implicating the CS-A and CS-B proteins in TCR but not GGR.

The CSA gene product encodes a protein with WD repeats (tryptophan-aspartate

repeat motif)(Henning *et al*, 1995). No member of the WD repeat family of proteins has been demonstrated to have catalytic function (reviewed in Neer *et al*, 1994). These proteins are thought to mediate protein-protein interactions critical to a number of cellular processes including cell signaling and cell cycle regulation (reviewed in Neer *et al*, 1994). CSA is likely involved in mediating protein-protein interactions required for TCR. CSA binds to the p44 subunit of TFIIH (Henning *et al*, 1995) although the function of this interaction is not known.

The CSB protein contains putative helicase domains (Troelstra *et al*, 1992), however helicase activity has not been demonstrated in CSB (Selby and Sancar, 1997). The CSB protein has been postulated to be a transcription repair coupling factor which facilitates the association between TFIIH and the incision complex. CSB interacts with XPG (Iyer *et al*, 1996) and CSA which in turn binds TFIIH (Henning *et al*, 1995), raising the possibility that the CS proteins mediate an interaction between TFIIH and incision proteins during TCR. However, no *in vitro* TCR system exists to directly demonstrate the requirement of CS gene products in the repair of UV induced DNA damage. It remains to be determined whether the CS gene products play direct or indirect roles in TCR.

Use of Adenovirus to study DNA repair

Recombinant Ad constructs expressing DNA repair genes

Adenovirus (Ad) is a nonenveloped double stranded DNA virus of approximately 36 kbp (reviewed in Horwitz, 1990). Ad is capable of efficient infection of a variety of cell types including primary fibroblasts. Generally 4 regions of the viral genome are considered to be expressed prior to the onset of DNA replication (E1 to E4) (Reviewed in Horwitz, 1990).

The E3 region is dispensable for viral replication (Reviewed in Horwitz *et al*, 1990). Replacement of the E3 region with foreign genes permits transgenes to be delivered into a variety of cell types (Reviewed in Hitt *et al*, 1996). These constructs replicate in primary cells and allow high levels of expression of transgenes.

Our lab has taken advantage of available Ad vectors to make recombinant constructs expressing DNA repair proteins (Colicos *et al*, 1991; Castillo *et al*, unpublished results). Replicating constructs expressing either endonuclease V (*denV*) of T4 phage or ERCC-1 have been made. The *denV* gene product incises DNA at the site of CPD. Since XP fibroblasts are deficient in the preincision and incision steps of NER, *denV* partially complements the repair deficiency in XP fibroblasts (Valerie *et al*, 1987). The *denV* expressing construct was capable of partial complementation of the repair defects in XP fibroblasts from complementation groups A, C and E (Colicos *et al*, 1991). The ERCC-1 construct complements the repair defect in CHO group 1 cells by complementing the deficiency in the ERCC-1 gene product (Castillo *et al*, unpublished results).

There is a packaging constraint limiting the size of DNA inserted into recombinant Ad vectors (Reviewed in Hitt *et al*, 1997). Deletion of the E1 and E3 regions of Ad permits insertion of DNA greater than 6 kbp and allows viral replication only in cell lines expressing E1 in *trans* (Reviewed in Hitt *et al*, 1997). These vectors are used extensively to deliver transgenes into primary cells without significant viral replication (Reviewed in Hitt *et al*, 1997). A non replicating recombinant Ad construct expressing ERCC-1 under control of the human cytomegalovirus immediate early promoter (HCMV) was capable of complementing the UV sensitive phenotype of CHO group 1 cells (Gu *et al*, unpublished results). Together

these reports demonstrate the feasibility of using recombinant Ad constructs to express repair proteins in *trans*. In the present work (chapters 8), recombinant Ad constructs expressing cell cycle regulatory proteins or viral oncoproteins were used to assess the role of cell cycle regulation in sensitivity to UV irradiation.

Host cell reactivation of Adenovirus

Restoration of Ad function following infection of unirradiated cells with a UV damaged virus has been used to assess DNA repair for many years (reviewed in Rainbow, 1981; Defais *et al*, 1983). This assay is referred to as host cell reactivation (HCR). A number of outcomes of Ad infection have been assessed as endpoints for HCR including plaque forming ability (Lytle, 1975; Rainbow and Mak, 1972), viral structural antigen formation (Rainbow, 1978) and viral DNA synthesis (Arnold and Rainbow, 1996). XP-A, -B, -C, -D, -E and -F fibroblasts are all reduced in their capacity to repair Ad as assessed by HCR of viral structural antigen formation (reviewed in Rainbow, 1981). Similarly, CS fibroblasts are reduced in their capacity to reactivate UV damaged Ad (Rainbow and Howes, 1982). Recently, HCR of viral DNA synthesis was reduced in CHO cells from complementation groups 1 through 6 (Arnold and Rainbow, 1996). These results implicate the XPA through XPG, CSA, CSB and ERCC-1 gene products in repair of UV irradiated Ad DNA. HCR of Ad structural antigen formation has been used to suggest that both SV40-transformed fibroblasts and tumour cell lines are reduced in their ability to repair UV-damaged Ad DNA compared to primary fibroblasts (Rainbow, 1989). Similarly, several tumour cell lines are deficient in HCR of Ad DNA synthesis for cisplatin treatment (Bulmer

et al, 1997). NER was found to be critical to reactivation of cisplatin treated Ad suggesting that NER efficiency could affect the resistance of tumour cells to chemotherapy (Bulmer *et al*, 1997).

Viral capacity

The capacity of cisplatin treated cells to support Ad DNA synthesis, has been used to assess repair of cisplatin induced lesions in cellular DNA (Davis *et al*, 1996). Cisplatin induces both interstrand and intrastrand adducts which are removed by recombinational repair and NER, respectively (reviewed in Freidberg *et al*, 1995). Reduced repair by either repair pathway, in Fanconi's anemia and XP-A, XP-D and XP-G fibroblasts, is reflected in a reduced capacity to support Ad DNA synthesis (Davis *et al*, 1996). Since cisplatin treated XP fibroblasts are reduced in their capacity to support Ad infection and since cisplatin induces DNA damage repaired by NER, this assay reflects the NER capacity of the cells (Davis *et al*, 1996). Viral capacity is more likely to reflect differences in cellular responses to DNA damage because cells are treated with the DNA damaging agent whereas HCR does not require treatment of cells. In the present work (McKay *et al*, 1997b; chapter 7), the capacity of human fibroblast strains, transformed fibroblasts as well tumour derived cell lines to support Ad DNA synthesis was assessed. The capacity of UV irradiated CS and XP-A fibroblasts to support Ad DNA synthesis was reduced, suggesting that repair of cellular genes by TCR was critical to permit viral replication. Furthermore, transformed cell types were reduced in their capacity to support Ad DNA synthesis indicating that transformed cells have reduced DNA repair capacity.

Enhanced reactivation of UV-irradiated virus

Treatment of cells with UV, ionizing radiation, chemical carcinogens or heat shock increases HCR of UV damaged double stranded DNA viruses including Ad, SV40 and HSV (Bockstahler and Lytle, 1970; Bockstahler and Lytle, 1977; Jeeves and Rainbow, 1979; Sarasin and Hanawalt, 1978; Sarasin and Benoit, 1980; Defais *et al*, 1983; Piperakis and McLennan, 1984). The SV40 genome is relatively small and circular making it easy to manipulate. Restriction fragments representing defined portions of the SV40 genome can be irradiated separately, ligated and used to assess HCR (Brown and Cerutti, 1987). Using this system, it was demonstrated that UV induced DNA damage in early genes is more lethal to SV40 (Brown and Cerutti, 1987). Furthermore, UV-enhanced reactivation (UVER) of SV40 plaque formation was only observed in constructs with UV induced DNA damage in the early region of SV40 implicating enhanced recovery of early gene expression in UVER (Brown and Cerutti, 1989). Since early gene function is similarly required in other DNA tumour viruses prior to onset of DNA replication, enhanced recovery of early gene expression may play a role in UVER of these viruses. UV treatment of CV-1 monkey cells prior to infection of UV irradiated HSV-1 also leads to UVER of plaque forming ability and this process was sensitive to caffeine treatment (Hellman *et al*, 1976). Recently, caffeine has been demonstrated to disrupt TCR in human cells (Link *et al*, 1996). As TCR would permit rapid recovery of early gene expression from UV damaged viruses, these observations are consistent with UVER arising, at least in part, from stimulation of TCR.

UVER is frequently associated with enhanced mutagenesis suggesting that functions other than repair, such as enhanced lesion bypass, may contribute to UVER (reviewed in

Defais *et al*, 1983). Replication of genomic DNA in mammalian cells is inhibited by UV in a fluence dependent manner, however the extent of this inhibition is less than expected in the absence of lesion bypass (Spivak and Hanawalt, 1992). In normal human fibroblasts, the extent of global repair prior to the onset of DNA replication following UV determines the mutagenic effect of UV irradiation (Konze-Thomas *et al*, 1982). Enhanced recovery of early gene expression with persistent DNA damage in the remainder of the Ad genome would thus be expected to permit lesion bypass. However, it is unlikely that UVER and enhanced mutagenesis (EM) result exclusively from inducible DNA repair processes since UVER and EM for UV irradiated Ad has been observed in UV irradiated XP fibroblasts (Jeeves and Rainbow, 1983; Ryan and Rainbow, 1986). More than one mechanism is likely responsible for these cellular processes.

HCR of reporter gene activity

A more recent approach has been to assess restoration of reporter gene activity from a recombinant, replication-defective Ad (Valerie and Singhal, 1995; Francis and Rainbow, 1995; Francis and Rainbow, unpublished results; McKay and Rainbow, 1996; McKay *et al*, 1997a; McKay and Rainbow, unpublished results). HCR of secreted alkaline phosphatase activity reflected the repair phenotype of primary normal, XP-A and XP-G fibroblasts (Valerie and Singhal, 1995) and HCR of β-gal activity was similarly found to be reduced in XP-A, XP-B, XP-C, XP-D, XP-F and XP-G strains (Francis and Rainbow, 1995; Francis and Rainbow, unpublished results). Since expression of UV damaged reporter genes requires repair of the template strand of the reporter gene and since CS-A and CS-B fibroblasts deficient in TCR

are reduced in their capacity to repair Ad DNA (Rainbow and Howes, 1982), one would expect that the rate of repair of the reporter gene would reflect TCR. In apparent contrast to this prediction, XP-C fibroblasts were severely compromised in their ability to reactivate the UV damaged *lacZ* gene, indicating that HCR of this reporter construct does not reflect the ability of cells to support TCR (Francis and Rainbow, 1995; Francis and Rainbow, unpublished results).

UVER of β-gal activity was assessed in these cell types. An advantage of this system over UVER of viral replication is that UVER of β-gal activity is independent of replicative bypass and thus EM (Francis and Rainbow, 1995). As indicated above, HCR of β-gal activity was found to be decreased in XP-A, XP-B, XP-C, XP-D, XP-F and XP-G (Francis and Rainbow, 1995; unpublished results). In addition, CS-A and CS-B fibroblasts were found to be reduced in their capacity to reactivate reporter gene activity from a UV damaged *lacZ* gene. Since HCR of β-gal activity was significantly reduced in XP-C cells compared to NDF, TCR was not operative under the conditions of the assay (Francis and Rainbow, 1995; Francis and Rainbow, unpublished results). However, UV pretreatment of normal and XP-C but not CS or other XP fibroblasts prior to infection with the UV damaged reporter construct led to a significant increase in reporter gene activity from the UV damaged reporter gene. The XPC gene product was thus required for HCR but not UVER of β-gal activity. In contrast, UVER was dependent on the CSA and CSB gene products which are known to be required for TCR (Francis and Rainbow, unpublished results). These results suggest that treatment of cells with UV can stimulate repair of the transcribed strand of a reporter gene by a CSA and CSB dependent and XPC independent process, strongly suggesting that TCR

is inducible by UV treatment (Francis and Rainbow, 1995; Francis and Rainbow, unpublished results).

Assessment of UVER of reporter gene activity was made in several tumour and transformed cell lines (Hill and Rainbow, unpublished results). Transformed cells were not capable of supporting this inducible process at UV fluences which elicit inducible repair in normal fibroblasts (Hill and Rainbow, unpublished results). Transformation of human cells appears to disrupt inducible repair of a UV damaged reporter gene. As the Ad5HCMVsp1*lacZ* vector lacks the E1 region of Ad, this construct does not replicate in the absence of exogenous E1 gene expression. This process is thus separable from both enhanced replicative bypass and EM and likely depends on gene products that are altered in transformed cells. We have also reported that HS can stimulate repair of a UV damaged reporter construct. This response, termed heat shock enhanced reactivation (HSER), was dependent on the CSA, XPG, XPA and XPD gene products but not the XPC gene product indicating that this response, as reported for UV treatment of cells, relies on TCR (McKay and Rainbow, 1996). Furthermore, HSER was absent in p53 deficient fibroblasts as well as a wide variety of tumour cell lines, demonstrating that inducible DNA repair is disrupted in both p53 deficient primary fibroblasts and transformed cells (McKay and Rainbow, 1996; McKay *et al*, 1997a; McKay and Rainbow, unpublished results).

DNA damage response

Cellular responses to UV exposure

Persistent UV induced DNA damage is mutagenic in normal and repair defective

fibroblasts (Konze-Thomas *et al*, 1982). The efficiency of repair prior to the onset of DNA synthesis is critical for the prevention of point mutations (Konze-Thomas *et al*, 1982) since UV induced DNA damage does not pose a complete block to replication (Spivak and Hanawalt, 1992). Not surprisingly, tumour suppressor genes such as p53 play a role in preventing mutagenesis in response to exogenous and endogenous DNA damaging agents (Lane, 1992). Prevention of replicative bypass of UV damaged nucleotides is achieved in at least 3 ways.

Firstly, UV induced dimers can be removed by NER by a combination of TCR and GGR as discussed above. Mammalian cells appear to be capable of increasing their DNA repair capacity in response to cellular stresses (Smith *et al*, 1995; Francis and Rainbow, 1995; Francis and Rainbow, unpublished results; McKay and Rainbow, 1996; McKay *et al*, 1997a; McKay *et al*, 1997b). The capacity to repair UV induced DNA damage, in part, reflects the status of the p53 tumour suppressor gene product (Smith *et al*, 1995; Wang *et al*, 1995; Ford and Hanawalt, 1995; McKay *et al*, 1997a; McKay *et al*, 1997b). Mutations in one allele is sufficient to affect NER in human and mouse cells (Wang *et al*, 1995; Ford and Hanawalt, 1995; Mirzayans *et al*, 1996; McKay *et al*, 1997a and 1997b; Li *et al*, 1996 and 1997).

Cells are also capable of arresting cell cycle progression prior to the onset of DNA synthesis in response to DNA damage (Kastan *et al*, 1991). Cell cycle arrest has been proposed to allow time for DNA repair prior to entry into S phase (Lane, 1992). Cell cycle dependent changes in the rate of removal of UV induced DNA damage has been reported (Gupta *et al*, 1981), however these results have not been confirmed (Kaufman *et al*, 1990). The duration of time between UV irradiation and S phase entry may be a critical mutagenic

determinant (Konze-Thomas *et al*, 1982). G_1 arrest is mediated in large part through the p53 tumour suppressor gene (reviewed in Ko and Prives, 1996).

Extensive DNA damage can result in elimination of cells by programmed cell death, alternatively referred to as apoptosis (Lowe *et al*, 1993). This process is also mediated in large part by the p53 protein (Lowe *et al*, 1993). Thus DNA repair (Smith *et al*, 1995; Wang *et al*, 1995; Ford and Hanawalt, 1995), cell cycle arrest (Kuerbitz *et al*, 1992) and apoptosis (Lowe *et al*, 1993; Ljungman and Zhang, 1996) in response to UV induced DNA damage can be mediated by the antioncogene, p53 (figure 1).

DNA damage response and p53

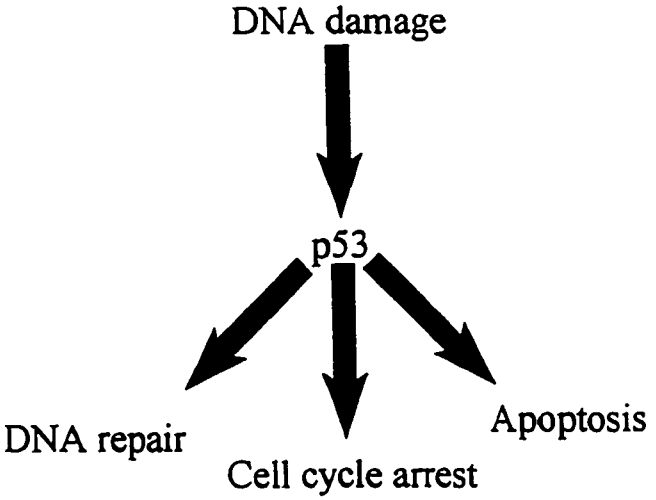
In response to UV (Maltzman and Czyzyk, 1984), IR (Kastan *et al*, 1991) as well as other DNA damaging agents, p53 is post translationally stabilized (for review see Ko and Prives, 1996). The half life of p53 is approximately 30 min in unstimulated cells but increases several fold following exposure to genotoxic agents (for review see Ko and Prives, 1996). Stabilization of p53 can occur in response to either DNA strand breaks (Nelson and Kastan, 1994) or inhibition of transcription (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996). Strand breaks do not occur in response to UV irradiation directly, but only as repair intermediates (reviewed in Freidberg *et al*, 1995). As incision deficient XP-A fibroblasts are hypersensitive to UV induced accumulation of p53 (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996), inhibition of transcription is likely the signal for induction of p53 dependent apoptosis in response to UV exposure (Ljungman and Zhang, 1996). Other cellular stresses such as hypoxia and heat shock (HS) which do not induce DNA damage can

also stimulate accumulation of p53 (Graeber *et al*, 1994; Matsumoto *et al*, 1994; Matsumoto *et al*, 1995; Nitta *et al*, 1997). The regulation of p53 stability is likely controlled by several signaling pathways.

Several protein kinases can phosphorylate residues on p53 (reviewed in Ko and Prives, 1996). These include DNA dependent protein kinase (DNA-PK), several cyclin dependent kinases (cdk), casein kinase I and II, mitogen activated protein kinase, Jun amino-terminal kinase (JNK) and Raf kinase (reviewed in Ko and Prives, 1996). Although the phosphorylation sites for each of these have been mapped, the role of each in regulation of p53 activity is unclear at this time.

The p53 protein in unstimulated cells is in a latent from which can be activated by DNA damage (Hupp *et al*, 1993), binding of small oligonucleotides (Jayaraman *et al*, 1995) or interaction with a monoclonal antibody, mAb421 (Hupp *et al*, 1993) through a mechanism requiring the C terminus. The p53 protein is capable of exerting its effects in large part by its ability to act as a transcription factor (El-Deiry *et al*, 1992). Positive regulation of the expression of genes with a p53 consensus sequence is critical for its ability to arrest cells in G_1 and induce apoptosis in some model systems (El-Deiry *et al*, 1992; El-Deiry *et al*, 1993; El-Deiry *et al*, 1994; Zhan *et al*, 1994; Miyashita and Reed, 1995). Transcription independant functions of p53 have also been reported to be involved in maintaining cells in G_0 (Del Sal *et al*, 1995) and inducing apoptosis (Caelles *et al*, 1994; Wagner *et al*, 1994; Haupt *et al*, 1995a; Chen *et al*, 1996; Wang *et al*, 1996; Theiss *et al*, 1997).

Figure 1. Schematic representation of cellular responses to DNA damage mediated through p53 dependent mechanisms. DNA damage induced by a variety of agents results in both the accumulation and increased activity of p53. The p53 protein participates in at least 3 protective mechanisms against DNA damage including DNA repair, cell cycle arrest and apoptosis. Appropriate references are indicated in the text.


DNA damage
p53
DNA repair
Cell cycle arrest
Apoptosis

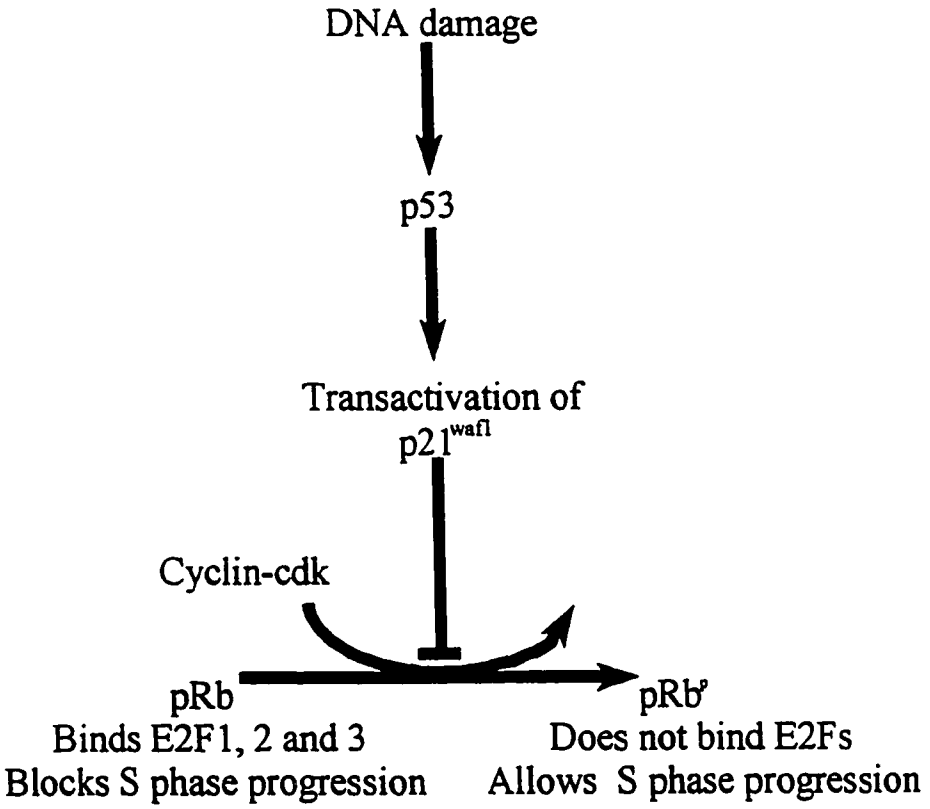
DNA repair and p53

Reports from several laboratories have implicated p53 in repair of UV induced DNA damage. GGR is reportedly reduced in Li-Fraumeni syndrome (LFS) fibroblasts (Smith *et al*, 1995; Ford and Hanawalt, 1995; Mirzayans *et al*, 1996), mutant p53 transgenic mice (Li *et al*, 1996) and p53 null mice (Li *et al*, 1997). *In vitro* repair is reportedly stimulated when extracts from UV irradiated cells are used and this is dependent on p53, indicating that GGR is inducible through a p53 dependent mechanism (Smith *et al*, 1995). TCR has also been reported to be reduced in LFS fibroblasts (Wang *et al*, 1995; Mirzayans *et al*, 1996; McKay *et al*, 1997a and 1997b). We have reported that UVER and HSER of a UV damaged reporter gene is dependent on TCR and p53 suggesting that TCR is also inducible through a p53 dependent mechanism (Francis and Rainbow, 1995; Francis and Rainbow, unpublished results; McKay *et al*, 1997a and 1997b). Since p53 accumulates in response to transcription blocking DNA damage (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996), we have proposed a model in which inducible TCR acts as a negative feedback mechanism to regulate p53 stability (McKay *et al*, 1997b).

Cell cycle arrest and p53

Cell cycle arrest in response to DNA damaging agents including UV has been proposed to result from transcriptional upregulation of p53 responsive genes (El-Deiry *et al*, 1993; Zhan *et al*, 1993). p53 plays a role in arrest in the G_1 and G_2/M stages of the cell cycle in response to DNA damage (Kuerbitz *et al*, 1992; Agarwal *et al*, 1995). The best characterized p53 dependent signaling cascade arrests cells in G_1 (figure 2). The p53 protein

Figure 2. Schematic representation of p53/p21^{waf1}/pRb mediated G_1 arrest. DNA damage induces stabilization and increased activity of p53. Accumulation of p21^{waf1} resulting from transactivation of the p21^{waf1} gene leads to inhibition of cyclin dependent kinase (cdk) activity, preventing phosphorylation of pRb in G_1. The hypophosphorylated form of pRb sequesters E2F transcription factors required for transactivation of S phase specific genes. In the absence of transactivation of E2F responsive genes, cells remain arrested in G_1. pRb^p denotes hyperphosphorylated pRb. References are indicated in the text.


DNA damage
p53
Transactivation of
p21wafl
Cyclin-cdk
pRb
Binds E2F1, 2 and 3
Blocks S phase progression
pRbp
Does not bind E2Fs
Allows S phase progression

can transcriptionally activate $p21^{wafl}$ (El-Deiry *et al*, 1993). This protein is an inhibitor of cyclin E-cdk2, cyclin A-cdk2, and cyclin D1-cdk4 preventing phosphorylation of pRb in G_1 (Harper *et al*, 1993). Hyperphosphorylated pRb is unable to bind and sequester several E2F transcription factors (E2F-1 to -3) (Hiebert *et al*, 1992; Flemington *et al*, 1993; Helin *et al*, 1993). Therefore cyclin/cdk complexes are responsible for release of E2F transcription factors allowing expression of S phase specific genes (DeGregori *et al*, 1997). The p53 protein, through transactivation of $p21^{wafl}$, is capable of preventing S phase progression by preventing cyclin/cdk complexes from phosphorylating pRb (Harper *et al*, 1993). G_1 arrest is thought to preclude replication of damaged DNA. As UV induced lesions in DNA are frequently bypassed during replication (Spivak and Hanawalt, 1992), the mutagenic effect of UV light (Konze-Thomas *et al*, 1982) would be lessened by efficient G_1 arrest thus conferring protection against UV induced skin cancers. In the present work, we demonstrate that $p21^{wafl}$ expression is limited by the repair capacity of the cell following UV treatment (McKay and Rainbow, chapter 8). We suggest that UV induced cell cycle arrest is less efficient following UV compared to IR because transcriptional activation of cell cycle checkpoint genes is inhibited by UV in a fluence dependent manner (McKay and Rainbow, submitted).

Apoptosis

UV induced DNA damage is a potent inducer of apoptosis in normal diploid human fibroblasts (NDF) (Ljungman and Zhang, 1996). Examination of apoptosis in XP and CS fibroblasts indicated that induction of p53 and apoptosis (Yamaisumi and Sugano, 1994; Ljungman and Zhang, 1996) results from persistent DNA damage in active genes. Neither

the signaling cascade leading to p53 accumulation nor downstream effectors leading to apoptosis have been fully elucidated. However, 2 distinct functions of p53 appear to be capable of contributing to apoptosis.

Transcriptional activation of p53 responsive genes appears to be critical to the induction of apoptosis in some model systems (Sabattini *et al*, 1995; Attardi *et al*, 1996). In particular, cell lines expressing the AdE1A protein, which sensitizes cells to DNA damaging agents (Lowe *et al*, 1993), induce apoptosis by a transactivation and p53 dependent mechanism (Sabbatini *et al*, 1995; Attardi *et al*, 1996). A possible target gene involved in p53 dependent apoptosis is *bax* (Zhan *et al*, 1994; Miyashita and Reed, 1995). Expression of *bax* facilitates apoptosis in some model systems (Sakakura *et al*, 1996) but overexpression of *bax* doesn't compensate for p53 deficiency in the induction of apoptosis (Brady et al, 1996).

It is becoming clear that transactivation independent functions of p53 contribute to the induction of apoptosis. Transactivation defective mutant p53 alleles can induce apoptosis in several cell types (Caelles *et al*, 1994; Wagner *et al*, 1994; Haupt *et al*, 1995a; Chen *et al*, 1996; Theiss *et al*, 1997). p53 can also induce apoptosis in the absence of *de novo* RNA or protein synthesis (Caelles *et al*, 1994; Wagner *et al*, 1994). Several mutant p53 alleles including N- (214 amino acids) and C-terminal (72 amino acids) p53 fragments are capable of inducing apoptosis despite the absence of the sequence specific DNA binding domain (Haupt *et al*, 1995a; Wang *et al*, 1996). Clearly, transactivation dependent and transactivation independent functions of p53 regulate apoptosis.

The decision to undergo cell cycle arrest or apoptosis

The decision to undergo apoptosis or cell cycle arrest is not well defined for any cellular stress, however several factors appear to affect the probability to undergo either cell cycle arrest or apoptosis. There is a dose dependent increase in the probability of undergoing apoptosis in response to UV (Ljungman *et al*, 1996; Cotton and Spandau, 1996) and IR (Hu and Hill, 1996). There is cell type and tissue specific variation in the ability to induce p53 and apoptosis (Midgley *et al*, 1995; MacCallum *et al*, 1996). The probability of undergoing apoptosis can vary depending on the DNA damaging agent used despite similar induction of p53 (Ljungman and Zhang, 1996). Furthermore, growth factor modulation can affect the susceptibility of cells to the induction of apoptosis (Canman *et al*, 1995; Lin and Benchimol, 1995). In chapter 8, we demonstrate that expression of $p21^{waf1}$ is reduced in a UV fluence dependent manner. We propose a model in which there is a fluence dependent decrease in the ability of cells to respond to UV by transactivation of UV protective genes, thus favoring p53 dependent, transactivation independent apoptosis (McKay and Rainbow, chapter 8).

Cancer therapy and p53

Chemotherapies and radiation therapies are frequently ineffective due to acquisition of drug or radiation resistance (Perez *et al*, 1993). Several cancer therapies lead to p53 accumulation and the success of therapies may reflect the ability of p53 to induce apoptosis (Lowe *et al*, 1993). In this light, maintenance of p53 function is clinically favourable, however mutations in p53 can lead to disruption of apoptotic function without loss of other p53 functions (Delia *et al*, 1997). One may expect protective functions of p53, such as

transient cell cycle arrest or enhanced DNA repair, to contribute to drug resistance. Several cisplatin resistant cell lines have an increased capacity to repair cisplatin induced lesions (Parker *et al*, 1991; Chao, 1994). Some DNA repair genes appear to be overexpressed in human tumours (Geleziunas *et al*, 1991; Dabholkar *et al*, 1992). Furthermore, repair of cisplatin adducts from transcribed regions of the genome is elevated in cisplatin resistant tumour cell lines (Zhen *et al*, 1992; Johnson *et al*, 1994). Interestingly, oxidative base damage induced by IR may also be repaired by a TCR mechanism requiring the XPG, CSA and CSB proteins (Leadon and Cooper, 1993; Cooper *et al*, 1997) suggesting that enhanced TCR could also contribute to radiation resistance. It would be advantageous to be able to disrupt protective functions of p53 during therapy while stimulating p53 dependent apoptosis.

In the present work, we provide evidence that TCR of UV-damaged DNA is stimulated in a p53 dependent manner in response to DNA damage and heat-shock (McKay and Rainbow, 1996; McKay et al, 1997a; McKay et al, 1997b). These inducible DNA repair responses were found to be commonly disrupted in established tumour cell lines (McKay and Rainbow, 1996; Chapter 7). In chapter 8, we demonstrate the importance of TCR for efficient expression of the $p21^{wafl}$ gene following UV irradiation (McKay and Rainbow, submitted for publication). Overexpression of either $p21^{wafl}$ or pRb can be protective against apoptosis (Gorospe et al, 1997; Haupt *et al*, 1995b). Our results suggest that the efficiency of p53 mediated cell cycle arrest is limited by the DNA repair capacity of the cell. Since most of the tumour cell lines examined lacked inducible DNA repair, these cells would not be expected to efficiently express $p21^{wafl}$ (or other anti-apoptotic genes) following treatment with DNA damaging agents which inhibit transcription. These results suggest that

conventional tumour therapies may be effective in treating tumours with reduced gene specific repair. Conversely, tumour cells which retain the ability to efficiently repair transcription blocking DNA damage may be resistant to such therapies.

Replication defective Ad constructs are commonly used as gene therapy vectors (reviewed in Hitt et al, 1997). It is also reported in this thesis (chapter 8) that transient expression of $p21^{waf1}$ from such a vector (Eastman et al, 1995) is protective against subsequent UV exposure. Similar results were obtained in our lab following cisplatin treatment (Bulmer and Rainbow, unpublished results). Thus, it appears that this approach would not be effective when combined with conventional therapies which induce bulky DNA adducts. Furthermore, recombinant Ad constructs expressing E2F-1 and E2F-4 sensitized cells to UV irradiation, again similar to results were obtained following cisplatin treatment (Bulmer and Rainbow, unpublished results). These results suggest that G_1 arrest is protective against UV and cisplatin treatment.

Hyperthermia is commonly used in conjunction with either radiation or chemotherapy (reviewed in Hahn, 1982). HS triggers the accumulation of p53 (Graeber et al, 1994; Matsumoto et al, 1994; Sugano et al, 1995; Matsumoto et al, 1995; Ohnishi et al, 1995) and induces G_1 arrest through transactivation of $p21^{waf1}$ (Fuse *et al*, 1996; Nitta *et al*, 1997). Thus in addition to resistance to chemotherapy which could conceivably results from HS inducible DNA repair, results presented in chapter 8 suggest that HS induced G1 arrest may be protective against subsequent chemotherapy. It is likely that the efficacy of combined therapies may depend on the sequence and timing of each treatment.

Chapter 2

Materials and Methods

Materials

Cell lines and fibroblast strains

The 293 cell line is a human embryonic kidney cell line transformed with the left end of Ad5. This cell line expresses early region 1 of Ad5 thus permitting propagation and titration of E1 deleted recombinant Ad constructs (reviewed Hitt *et al*, 1997). These cells were graciously provided by Dr. F. L. Graham, McMaster University.

Several primary fibroblast strains as well as the SV40 transformed normal fibroblast cell line, GM637f, were obtained from the National Institute of General Medical Sciences (NIGMS) mutant cell repository (Camden, NJ). These strains are described in table 1. The NDF strain, 423, was provided by Dr. P. Chang, McMaster University. L132 lung epithelial cells were obtained from both Dr J. Arrand, Brunel University, Uxbridge, UK and the American Type Culture Collection (ATCC, Rockville, Ma). SV40 transformed XP-C, XP-A, CS-A and CS-B fibroblasts were provided by Dr R.S. Athwal, Temple University, Philadelphia, PA.

Li-Fraumeni syndrome cells MDAH041 and MDAH087, both the primary fibroblast strains and their spontaneously immortalized sublines, were obtained from Dr M.A. Tainsky, MD Anderson Cancer Centre, Houston, Tx, USA.

Tumour cell lines were obtained from several sources. HeLa (cervical carcinoma), IMR-32 (neuroblastoma), ZR-75-1 (breast carcinoma) and HCT 116 (colon carcinoma) were obtained from the ATCC. SK-N-SH (neuroblastoma) and HT29 (colon carcinoma) were obtained from Dr G. Singh, Hamilton Regional Cancer Centre, Hamilton, Ont. SKOV-3 cells (ovarian carcinoma) were provided by Dr S. Bacchetti, McMaster University. U20S cells

Table 1. Fibroblast strains obtained from the NIGMS.

Cell type	Repository number	Individual
NDF[a]	GM37F	NA
	GM38A	NA
	GM969C	NA
	GM8399	NA
	GM9503	NA
SV40 transformed GM37F	GM637F	NA
XP-A	GM5509B	XP-12BE
XP-B	GM13025	XPCS1BA
XP-B	GM13026	XPCS2BA
XP-C	GM30A	XP3BE
XP-C	GM677	XP2BE
XP-C	GM10881	XP1BE
XP-D	GM3615	XP1BR
XP-G	GM3021	XP2BI
CS-A	GM1856	CS3BE
CS-B	GM739A	CS1AN

[a] abbreviations: NDF (normal diploid fibroblasts), NA (not applicable), SV40 (simian virus 40)

(osteosarcoma) were obtained from Dr P. Whyte, McMaster University. SCC-25 cells (squamous cell carcinoma) were obtained from Dr J. Lazo, University of Pittsburgh School of Medicine, Pittsburgh, PA.

Recombinant adenovirus constructs

Ad5-*lacZ*5 expresses *lacZ* from the deleted E3 region of Ad5. *LacZ* was expressed under control of the E3 promoter and Ad major late promoters. This construct infects and replicates in a wide range of cell types (Mittal *et al*, 1995). This construct was provided by Dr F.L.Graham, McMaster University.

Replication defective viruses expressing a variety of gene products were obtained from several investigators. Two recombinants expressing *lacZ* under control of the human cytomegalovirus (HCMV) immediate early promoter (Ad5HCMVsp1*lacZ*) (Morsy *et al*, 1993) and the murine cytomegalovirus (MCMV) immediate early promoter (Ad5CA35*lacZ*) (Addison *et al*, 1997) were obtained from Dr F.L. Graham, McMaster University.

Ad5SVR112 expresses the early region of SV40 (Massie *et al*, 1986) and AdPyR39 (Gluzman *et al*, 1982) express the large tumour antigen of murine polyomavirus, under control of the Ad5 major late promoter. These constructs were obtained from Dr J. A. Hassell, McMaster University.

Ad-dl70-3 is an E1 and E3 deleted virus which does not express any transgene. AdJL16 and AdJL17 express wildtype and mutant p53, respectively, under control of the HCMV promoter (Bacchetti and Graham, 1993). These constructs were obtained from Dr F.L. Graham.

Ad-E2F1 (Shwarz *et al*, 1995), Ad-E2F4 (DeGregori *et al*, 1997) and Ad-DP1 (DeGregori *et al*, 1997) were obtained from Dr J.R. Nevins, Duke University, Chapel Hill, NC. These constructs express E2F-1, E2F-4 and DP-1 respectively, under control of the CMV promoter.

AdCMV-p21 expresses $p21^{waf1}$ under control of the HCMV promoter (Eastham *et al*, 1995). This construct was made available to us by Dr T.C. Thompson, Baylor College of Medicine, Houston, Tx.

Reagents

ELISA and DNA fragmentation kits were obtained from Oncogene Science, Cambridge, MA. All other enzymes were obtained from GibcoBRL. Chemical reagents were purchased from Sigma (St Louis, Missouri, USA).

Methods

Cell culture

The 293 cells were grown in α-minimal essential medium (α-MEM) supplemented with 10% newborn calf serum (Sigma) and 1% antibiotic/antimycotic (GibcoBRL). All other cell lines and fibroblast strains were grown in α-MEM supplemented with 10% fetal bovine serum (Sigma) and 1% antibiotic/antimycotic (GibcoBRL). Passaging of cells was performed by briefly rinsing cells in phosphate buffered saline (PBS: 140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4 and 1.75 mM KH_2PO_4) then treating with 0.1% trypsin (GibcoBRL) in PBS. Cells were suspended in an appropriate volume of α-MEM and aliquots were placed into

culture flasks.

Virus propagation and titration

Viruses were grown and titred in 293 cells as previously described (Graham and Prevec, 1991). Virus was grown by infecting 70-80% confluent 293 cells in 150 mm dishes with the virus of interest at a multiplicity of infection (moi) of 4 plaque forming units (pfu) per cell. Virus was allowed 60-90 min to adsorb at 37°C with occasional rocking to permit even distribution of virus. Cells were refed with 20 mL of growth medium. Three to five days following infection, cells were harvested by scraping and gently pelleted by centrifugation. Cells were resuspended in 100μL α-MEM/150 mm dish of 293 cells used. Resuspended cells were transferred to microcentrifuge tubes and subjected to three cycles of freezing and thawing. Cell debris was pelleted by centrifugation and the supernatant was transferred to cryovials, and stored at -20°C.

Titration of virus stocks was made on 293 cells. Typically, serial dilutions between 10^6 and 10^9 were performed. Each was used to infect near confluent 293 cells in triplicate in 60 mm dishes in a total volume of 0.5 mL serum free α-MEM. Following 60-90 min infection, cells were overlaid with 10 mL of 0.5 % agarose in minimal essential medium F11 with 1% antibiotic/antimycotic (GibcoBRL), 10 % NCS and 0.1% yeast extract (difco) prewarmed to 44°C.

When plaques were well formed (7 to 10 days), the overlay was removed by inverting the dishes. The monolayers were stained with methylene blue (0.5 % in 70% ethanol). Plaques were counted by eye, with the aid of a light box and where necessary, a dissecting

microscope. Viral titres are expressed as the number of plaque forming units per mL of viral suspension.

Viral infection.

Cells were seeded at $1.9x10^4$, $1x10^5$, $2.5x10^5$ cells per well in 96, 24 or 6 well dishes, respectively. Cells were allowed 20-24 hrs to adhere prior to viral infection. Media was replaced with a minimal volume of serum free α-MEM (40 μL, 200 μL and 500 mL in 96, 24 and 6 well dishes, respectively) with the appropriate viral inocculum. Infections were stopped after 60 to 90 min by replacing the viral suspension with prewarmed α-MEM. Dishes were placed in a 37°C humidified incubator.

UV-irradiation of virus

Virus was suspended in 1.8mL of PBS or serum free α-MEM with a sterile stir bar and placed in a 35 mm dish on ice. The UV source was a General Electric germicidal lamp (model G8T5) emitting predominantly at 254 nm at an incident fluence rate of 2 J/m^2/sec for fluences below 10 KJ/m^2 and 8 J/m^2/sec for fluences above 10 KJ/m^2, as assessed with a UV meter (J-255 shortwave UV meter, Ultraviolet Products, San Gabriel, CA). Irradiation was performed with continous stirring. Aliquots of 200 μL were removed sequentially for each exposure to the virus and diluted appropriately with serum-free α-MEM or PBS. All UV fluences to the virus within a single experiment were cumulative.

Heat shock treatment of cells

The 96 well dishes were sealed with parafilm and submerged in a waterbath at 43±0.25 °C for between 15 and 60 min. Dishes were briefly submerged in a 37°C waterbath prior to viral infection.

UV-irradiation of cells

Medium was replaced with PBS (40 µL; 200 µL, 500 µL and 2 mL for 96 well, 24 well, 6 well and 100mm dishes, respectively) and cells were irradiated at room temperature at a fluence rate of 1 J/m^2/sec as assessed with a UV meter (J-255 shortwave UV meter, Ultraviolet Products, San Gabriel, Ca). Cells were refed with fresh prewarmed medium and placed in a humidified incubator at 37°C.

Enhanced Reactivation of Reporter Gene Activity Assay

Cells were seeded at a density of 1.9x10^4 cells/well in 96 well microtitre plates (Falcon, Lincoln Park, NJ). Between 20 and 24 hrs after seeding, microtitre plates were heat shock treated, UV irradiated or mock treated and then immediately infected (60-90 min at 37°C) with UV-irradiated or unirradiated Ad5HCMVsp1*lacZ* or AdCA35*lacZ* in a total volume of 40 µL in PBS at a multiplicity of infection of between 10 and 25 plaque forming units per cell (pfu/cell). Viral suspensions were aspirated and the cells were refed with prewarmed α-MEM to a total volume of 200 µL.

Infected cells were harvested following 40 to 44 hours for Ad5HCMVsp1lacZ and either 6 or 48 hrs for AdCA35*lacZ*. Infected cell layers were incubated 20 min at 37°C in 75

μL of 250 mM Tris, 1 μM PMSF, 0.5% NP40 (pH 7.8). An equal volume of 200 mM sodium phosphate, 20 mM KCl, 2 mM $MgSO_4$, 100 mM 2-mercaptoethanol (pH 7.5) was added and the mixture was incubated 10 min (Morsy *et al*, 1993). OD_{405} was determined at several times following addition of O-nitrophenol β-D-galactopyranoside (0.1% ONPG pH 7.5) using a 96 well plate reader (EL340, Biotek instruments).

Viral capacity assay

Cells were seeded at $1.9x10^4$ cells/well in 96 well microtitre dishes or $1x10^5$ cells/well in 24 well dishes. Following 24 hrs, medium was replaced with either 40 μL (96 well dishes) or 200 μL (24 well dishes) PBS and cells were irradiated as described above. Experiments included 6 different UV fluences, 1 unirradiated control and a negative control without virus. The PBS was immediately replaced with an equal volume of virus in PBS (40 pfu/cell). Virus was allowed to adsorb 60-90 min at 37°C prior to addition of either 200 μL or 1 mL of α-MEM, depending on well size. Cells were lysed following 48 hrs at 37°C in a humidified incubator. Cells were lysed over a 1 to 2 hr period by addition of either 200 μL or 1 mL of 1mg/mL proteinase K with 1% sodium dodecyl sulfate and 50mM NaCl in 15mM sodium citrate (pH 7.0). DNA was denatured in 0.5 N NaOH and 25 mM EDTA for 30 min at 37°C. Samples were blotted directly to Gene Screen Plus (Dupont, cat. no. NEF-976) using a slot blot apparatus (Schleicher and Schuell, Keene, NH).

Ad2 DNA was radiolabelled with ^{32}P by the random primer method and used to hybridize to viral DNA in cell lysates. Radioactivity was assessed using a phosphorimaging system (model 425B, Molecular Dynamics). Counts determined for the virus free control

were subtracted from counts obtained for all treatments in order to account for non specific binding. Net counts for each treatment were normalized to the net counts for the unirradiated control to determine the surviving fraction of Ad DNA synthesis.

Clonogenic survival assay

Primary fibroblasts were seeded at densities of between 500 and 25 000 per 100 mm dish to generate between 25 and 150 colonies per dish following irradiation. Tumour cells were seeded at densities between 200 and 2000 per well of 6 well dishes in order to generate similar numbers of colonies following irradiation. Cells were allowed to adhere between 4 and 8 hrs prior to UV irradition. Growth medium was replaced with 2 mL of PBS and cells were irradiated as described above. Either 2 weeks (primary fibroblasts) or 7 to 10 days (transformed cells) was required for colonies to form. Medium was replaced with sufficient methylene blue (0.5% in 70% ethanol) to cover the surface of the culture vessel. Methylene blue was removed following 10-15 min and dishes were submerged briefly in tap water to destain. Colonies were counted by eye with the aid of a light box and colony number was confirmed for small colonies with a dissecting microscope. Clonogenic survival is expressed as the ratio of seeding efficiencies for UV treated cells to unirradiated cells.

Survival was also determined for transgene expressing cells. Cells were seeded at $2.5x10^5$ cells per well of 6 well dishes. Twenty four hours following infection, cells were infected with recombinant Ad constructs at a moi of 20 pfu/cell. Following 24 hrs, fibroblasts were seeded to low density as described above and UV irradiated following an additional 4-8 hrs as already described.

Quantitative assessment of $p21^{waf1}$

A $p21^{waf1}$ ELISA assay (cat. no. QIA18, Oncogene Research Products, Cambridge, MA) was used to quantify protein levels. Fibroblasts were seeded at a density of $5x10^4$ cells per well of 24 well dishes. Cells were UV treated or mock treated as described above. Cell lysates were collected at several times following irradiation (between 0 and 48 hrs). Growth medium was replaced with 100 µL of α-MEM and cells were lysed directly into the medium by addition of 20 µL antigen extraction reagent (all reagents were provided by the manufacturer) and incubation on ice for between 30 and 45 min. Lysates were stored at -80°C. Twenty µL of the lysate was used to assess expression levels. Lysates were incubated 2 hrs at room temperature in ELISA plates and then 1 hr with a biotinylated secondary monoclonal antibody. Horseradish peroxidase conjugated streptavidin was added and incubated for 30 min at room temp. Wells were again rinsed three times in wash solution. Tetra-methylbenzidine solution was added and colour was allowed to develop in the dark for 30 min. Stop solution was added and optical densities at both 450 and 540 nm (OD_{450} and OD_{540}) were determined using an ELISA reader (Labsystems multiscan MCC/340). OD_{540} was subtracted from OD_{450} for each sample. The net OD for each treatment was normalized to that for untreated cells collected at the same time.

Expression of $p21^{waf1}$ was also assessed in non adherent UV irradiated fibroblasts. Cells were seeded at a density of $2.5x10^5$ cells per well of a 6 well dish. UV irradiation was performed as already described. Forty eight hours following irradiation, adherent cells and cells suspended in the growth medium were collected separately (as described in the cell detachment assay). Following centrifugation at 1000 x g, cells were resuspended in 100µL

of α-MEM and cell number was determined using a haemocytometer. An equal number of cells (5×10^4) were diluted to a total volume of 100 mL of α-MEM. Cells were lysed directly into the medium and assays were performed as described above.

Cell detachment assay

Cells were seeded at a density of 2.5×10^5 per well of 6 well dishes. Following 20-24 hrs, medium was replaced with 0.5 mL PBS and cells were irradiated or mock irradiated as previously described. Medium was collected 72 hrs following UV irradiation and centrifuged in a table top centifuge at approximately 1000 x g to collect cells from the supernatant. Cells were resuspended in 100μL of PBS. Adherent cells were collected by briefly rinsing in PBS and treating with 1 mL of 0.1 % trypsin in PBS until all cells were detached. Cells were confirmed to be detached by microscopy. To the suspended cells was added approximately 4 mL α-MEM and cells were collected by centrifugation as above. Cells were resuspended in 100 μL of PBS. Cell numbers were determined by direct counting of cells using a haemocytometer. The proportion of cells in the supernatant was expressed as the number of cells counted in the supernatant divided by the total of number of cell counted from both fractions for each treatment.

TUNEL assay

Approximately 10^6 cells were seeded onto 100 mm dishes. Following 24 hrs, medium was replaced with 2 mL of PBS and cells were irradiated as already described. Seventy two hours later, cells in the supernatant were collected separately from adherent cells as already

described. Approximately 2.5 x 10^5 cells were fixed in 100 µL of 3M paraformaldehyde for 30 min in microcentrifuge tubes. These cells were collected by centrifugation and resuspended in 80% ethanol. Suspensions were blotted directly onto slides and allowed to air dry. DNA strand breaks generating 3' hydroxyl groups by endogenous endonucleases were detected in each fraction separately using a terminal deoxynucleotidyl transferase based in situ end labeling method (TUNEL assay) according to the specifications of the manufacturer (cat. No. QIA 33, Oncogene Science, Cambridge, MA). This method is commonly used to quantify the proportion of apoptotic cells in several model systems (reviewed in Allen *et al.*, 1997).

Chapter 3

Use of replication deficient recombinant adenovirus reporter gene constructs to assess repair of UV damaged DNA in mammalian cells.

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1. Introduction

There is accumulating evidence that disruption of DNA repair is associated with carcinogenesis (1-5). Ultraviolet (UV) induced DNA damage as well as bulky adducts induced by several carcinogenic and antineoplastic compounds are repaired by nucleotide excision repair (NER) (3). Several additional links between NER and carcinogenesis have been reported. Deficiencies in NER contribute to elevated incidence of a variety of skin cancers (1,6). Mutations in the p53 tumour suppressor gene, the most commonly altered gene in malignancy (7), have also been shown to result in reduced NER (4,5,8,9). Mismatch repair proteins have recently been implicated in NER (10) and mutations within genes encoding these proteins are associated with hereditary non-polyposis colorectal cancer (2). Decreased NER has also been observed in a variety of tumour and transformed cell lines (11-14). These reports suggest that DNA repair mechanisms are disrupted in tumour cells and further suggest that DNA repair may contribute to resistance to neoplasia.

Repair of DNA damage by NER is not randomly distributed throughout the genome. A transcription coupling mechanism exists which facilitates removal of transcription blocking lesions from transcribed genes (15). Transcription coupled repair (TCR) appears to be protective against UV induced recombination (16) and may therefore reduce genetic instability in cells exposed to genotoxic agents. For this reason, we have been interested in examination of TCR in cells derived from both tumours (14) and unaffected tissue from individuals predisposed to cancer (5). Typically, TCR has been examined using damage specific endonucleases to estimate the number of lesions. The rate of removal of endonuclease sensitive sites from specific restriction fragments is used to quantify repair of

transcribed genes (15). The UV fluence used must generate a specific number of lesions per restriction fragment; therefore, in order to examine multiple UV fluences within a single experiment, repair must be examined in different sized restriction fragments (17). For this reason, single UV fluences are examined in typical experiments (15). In contrast, use of host cell reactivation (HCR) of reporter gene activity allows the rapid assessment of repair of the template strand of an active gene following multiple UV fluences.

HCR of reporter gene activity has been assessed using either recombinant adenovirus (Ad) (5, 14,18,19) or plasmid constructs (8, 20, 21). Transfection of UV damaged plasmid DNA is typically performed in transformed cells, however, many transformed cells have been shown to be reduced in their capacity to repair UV damaged DNA (10-14). Furthermore, the cellular response to DNA damage is stimulated by at least some transfection procedures (22, 23), leading to cell cycle arrest (23) suggesting that transfection may affect the outcome of DNA repair experiments. In contrast, recombinant non-replicating Ad vectors have the ability to infect and express recombinant gene products in most cell types. Furthermore, Ad reporter constructs do not appear to elicit the DNA damage response and fail to inhibit host DNA synthesis following infection (24).

Here we describe a rapid method to assess repair of UV induced DNA damage from the template strand of the bacterial *lacZ* gene introduced into primary human or rodent fibroblasts, transformed fibroblasts, Chinese hamster ovary (CHO) cells or tumour cell lines with a non-replicating recombinant Ad. We have obtained recombinant Ad constructs expressing the *lacZ* gene under control of either the human or murine CMV promoters. The *lacZ* cassettes were inserted into the deleted early region 1 (E1) of Ad (25, 26). Since E1 is

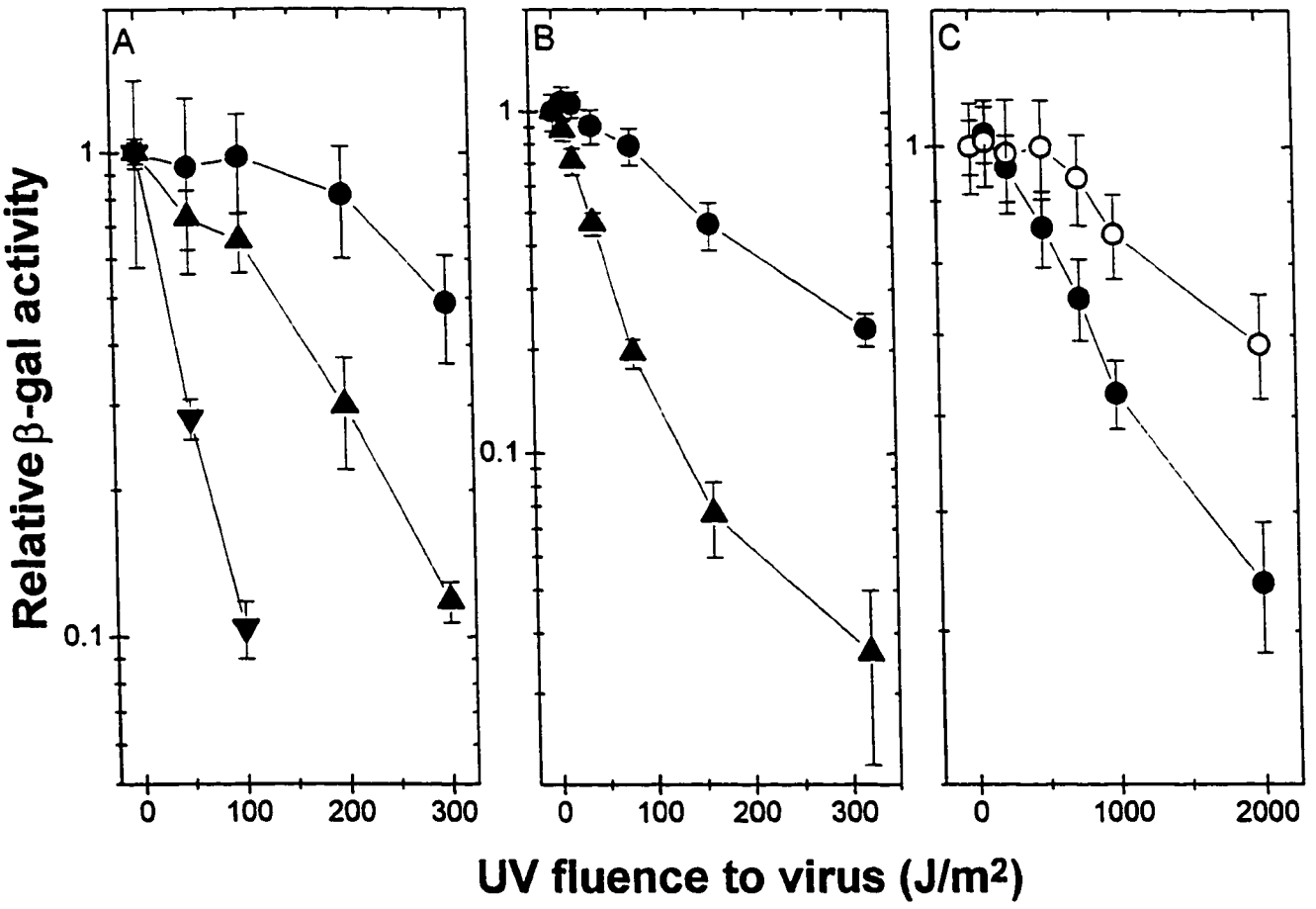
essential for replication, the recombinants are unable to replicate in the absence of E1 transcomplementation. As the construct is unable to replicate in the absence of the E1 gene products, reporter gene expression from a UV damaged virus depends on repair of the transcribed strand of the reporter gene. A similar approach using a recombinant Ad expressing the secreted alkaline phosphatase gene has been reported by Valerie and Singhal (18).

Since the viral genome is approximately 5 orders of magnitude smaller than the human genome, the number of lesions introduced into cells with a UV irradiated virus in our experiments is minimal compared to the number introduced into the host cell genome by UV treatment. NER deficiencies in UV sensitive CHO cells and fibroblasts derived from individuals with the UV sensitive cancer prone syndrome, xeroderma pigmentosum, are detected by this method (figure 1A and B). Repair of the UV-irradiated reporter gene can be further stimulated by pretreatment of repair proficient cells with UV prior to infecting with irradiated virus (figure 1C). We have used this approach to demonstrate that TCR is inducible by cellular stresses such as UV and heat shock by a mechanism which is commonly disrupted in neoplastic cells (5, 14, 19). The procedure is performed entirely in 96 well microtitre plates, can be performed in most cell types, requires a relatively small number of cells and allows separation of constitutive and inducible repair processes.

2. Materials

1. Cell lines and media: 293, tumour cells or CHO cells are grown in α-MEM supplemented with 10% newborn calf serum and 1% antibiotic/antimycotic (Gibco

Figure 1. Host cell reactivation of a UV damaged *lacZ* gene reflects the nucleotide excision repair capacity of both human and rodent cells. (A) Fibroblasts derived from individuals with XP complementation groups A (XP12BE-▼) and C (XP3BE-▲) are reduced in their capacity to repair the UV irradiated AdCA35-1 (see note 1) construct compared to normal diploid fibroblasts (GM3440-●). (B) Similarly, NER deficient CHO cells from complementation group 4 (UV41-▲) are reduced compared to their repair proficient parental cell line (AA8-●). (C) The nucleotide excision repair capacity of normal diploid fibroblasts (●) can be stimulated by pretreatment of the cell monolayer with 15 J/m^2 UV (○) prior to infection with UV-irradiated Ad5HCMVsp1*lacZ*. Each point represents the mean relative β-gal activity (± standard error) from a single experiment performed in at least triplicate.


A
B
C
1
0.1
Relative β-gal activity
0
100
200
300
1000
2000
UV fluence to virus (J/m²)

BRL). Diploid human fibroblasts are grown in α-MEM supplemented with 10% fetal calf serum with similar antibiotic/antimycotic. Some cells of interest may require selective media and these may be substituted appropriately.

2. A recombinant adenovirus expressing the *lacZ* gene under control of the human CMV promoter is commonly used in our lab (25)(note 1). Viruses are propagated and titred on 293 cells (note 2).

3. Agarose MEM F11 overlay: For 200 mL (enough for 20 x 60-mm dishes) autoclave 100 mL of 1% agarose. Separately, combine 74 mL 2x MEM F11, 2 mL 100x antibiotic/antimycotic, 20 mL newborn calf serum and 4 mL 5% yeast extract. Bring MEM F11 solution and agarose to 44°C before mixing and use within about 1 h. Prolonged periods of time at room temperature will allow agarose to congeal.

4. Phosphate buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2PO_4, 0.24 g KH_2PO_4 per L of distilled H_2O (pH 7.8) . Sterilize by autoclaving.

5. Methylene blue: 0.5% methylene blue in 70% ethanol.

6. Phenylmethylsulfonyl fluoride (PMSF): dissolve 0.871 g PMSF in 50 mL isopropanol. Sterilize by filtration, separate into aliquots up to 5 mL and store at -20°C (note 3).

7. Lysing solution: 5 mL Nonidet P40/L of 250 mM Tris (pH 7.8). Sterilize by filtration and store at 4°C.

8. 2-mercaptoethanol (2-ME): 0.744 g KCl, 0.12 g $MgSO_4$, 11.93 g Na_2HPO_4, 2.21 g NaH_2PO_4 and 3.6 mL 2-mercaptoethanol per L distilled H_2O. Sterilize by filtration and store at 4°C.

9. O-nitrophenol β-D-galactopyranoside (ONPG): 11.93 g Na_2HPO_4, 2.21 g NaH_2PO_4

and 4g ONPG are dissolved in 1 L of distilled H_2O. Sterilize by filtration and store at 4°C (note 4).

10. Stop solution (optional): 1 M Na_2CO_3 (106 g/L)

3. Methods

3.1 Propagation and titration of virus

3.1.1 Propagation of virus

1. Methods for efficient construction and propagation of recombinant adenoviruses are described in detail elsewhere (27). Briefly, we propagate recombinant viruses in 293 cells grown as monolayers. 150 mm dishes (note 5) containing 293 cells at or near confluence are infected at a multiplicity of infection (MOI) of 4 plaque forming units (pfu) per cell (note 6). Virus (approximately $1.6x10^8$ pfu/dish) is suspended in serum free α-MEM (4 mL/150 mm dish) and allowed to infect the monolayer for 60-90 min at 37°C with occasional rocking to evenly distribute virus over the cell layer. Cells are refed with 20 mL growth medium and incubated in a humidified 5% CO_2 incubator at 37°C.

2. Cytopathic effects (CPE) may become visible within 3 days. When CPE is observed over the entire plate, scrape cells with a rubber policeman and collect in 50 mL polypropylene tubes. Pellet cells by centrifugation and resuspend in a small volume (0.5 mL/150 mm dish) of 10% glycerol in α-MEM. Three cycles of freezing (-80°C) and thawing (room temperature) are performed to facilitate release of virus. Viral suspension is transferred to microcentrifuge tubes, cell debris is precipitated by

centrifugation and the supernatant is placed in cryovials for storage between -20°C and -80°C. Typical titres are in the range of 10^8 to 10^{10} pfu/mL.

3.1.2 Determination of viral titre

1. 293 cells should be seeded 1 day prior to infection such that the cells approach confluence by the time of infection. Sufficient 293 cells are present in a single 150 mm dish to seed approximately 8 x 60 mm dishes.
2. Dilute virus in serum-free α-MEM sufficiently to expect between 20 and 100 plaques per 60 mm dish. Typically dilutions between 10^6 and 10^{10} are included and each is performed in triplicate. Infect monolayers for 60-90 min in 0.5 mL serum free α-MEM as indicated above. Following infection, cells are overlaid with 10 mL agarose MEM F11 overlay which has been equilibrated to 44°C.
3. Incubate plates at 37°C in a humidified CO_2 incubator. Plaques should be visible within 5-7 days and can be counted at 7-10 days. To count plaques carefully remove agarose overlay by inverting the plates and stain the monolayer with methylene blue (note 7).

3.2. Host cell reactivation of a β-galactosidase activity.

1. Cells of interest are grown in the appropriate growth medium. Cells are seeded at 1.9 x 10^4 cells/well (for 96 well dishes) for primary human and mouse fibroblasts or 3.8 x 10^4 cells/well for transformed human cell lines and CHO cells, approximately 24 hrs prior to viral infection. Under these conditions, cells are confluent at the time of

treatment. If growing cells are specifically required, cells can be seeded at lower density (note 8). Cell layers may be treated with UV or heat shock prior to infection as these cell treatments can stimulate repair of the reporter construct (figure 1C)(5,14, 19)(see section 3.3).

2. An unirradiated aliquot of viral suspension must always be examined in order to normalize expression from UV-irradiated constructs. In addition, an aliquot is irradiated with a lethal UV fluence ($\geq$10 kJ/m^2) (note 9). Expression following this treatment is used to assess background activity . We typically include 6 additional UV fluences between 0 and 2000 J/m^2, however optimal UV fluences are cell type specific. Note differences in UV fluences used in representative experiments in figure 1.

3. The amount of virus required for a given experiment is dependant on the number of wells seeded as well as the MOI selected. We have used MOIs between 1 and 100 pfu/cell (note 10). The virus prep is diluted with serum free α-MEM to a volume of 1.8 mL in a 35 mm petri dish (note 11). Virus is irradiated on ice with continuous stirring (note 12) using a General Electric germicidal lamp (model G8T5) at a fluence rate of 2 J/m^2/sec (8 J/m^2/sec for UV irradiations greater than 10 kJ/m^2) as determined with a J-255 shortwave UV meter (ultraviolet products, San Gabriel, Ca.). Aliquots (200 μL) are removed sequentially from the irradiated viral suspension; therefore, UV fluence is cumulative. Subsequently, each aliquot must be similarly diluted in serum free α-MEM to yield 40 μL/well.

4. For viral infection, growth media is removed and replaced with 40 μL of irradiated or unirradiated viral suspension (note 13). Infection is allowed to proceed for

between 60 and 90 min at 37°C. Virus suspension is aspirated and replaced with 200 µL prewarmed growth media. Infected cells are incubated at 37°C for between 44-48 hours in a humidified 5% CO_2 incubator (note 14).

3.3. Treatment of cells

3.3.1 UV treatment

1. Growth media is replaced with 40 µL PBS.
2. UV irradiation is performed at room temperature at a fluence rate of 1 J/m^2/sec. Columns or rows which are not to be irradiated are shadowed by plate lids. Only single rows or columns are irradiated at one time (note 15).
3. Following irradiation, monolayers are immediately ($\leq$ 30 min) infected with UV irradiated or unirradiated virus (see section 3.2).

3.3.2 Heat shock treatment

1. Dishes are sealed with PVC tape and submerged in a water bath (note 16) at the desired temperature for varying lengths of time. We have observed inducible DNA repair following treatment of 30 min at 43°C.
2. Following heat shock treatment, cells are immediately ($\leq$ 30 min) infected with UV irradiated or unirradiated virus (see section 3.2).

3.4. Beta-Galactosidase Assay

3.4.1 Colour detection

1. Redissolve PMSF by heating stock solution to 37°C. Add PMSF (10 µL PMSF/mL final volume) to lysing solution, 75 µL of lysing buffer is required per well.

2. Aspirate media from cell monolayers and add 75 μL premixed lysing solution. Incubate at 37°C until cells in the monolayer are visibly disrupted (10-15 min) (note 17).

3. Add 75 μL 2-ME and incubate for 5-10 minutes.

4. Add 55 μL ONPG and incubate at 37°C until samples have developed a yellow colour. Measure absorbance on a 96-well plate reader at either 405 or 420 nm at various times following addition of ONPG (note 18).

5. The colour reaction can be attenuated by addition of 1 M Na_2CO_3 (106 g/L), if required.

3.4.2 Treatment of data

1. The mean absorbance (± standard error) is determined for each treatment. Typically, experiments are performed in triplicate or greater.

2. The mean absorbance determined for background activity (note 9) is subtracted from the mean absorbance determined for all other treatments to virus (net β-gal activity). Relative β-gal activity is determined by normalizing net β-gal activity to that of unirradiated virus. Survival curves for reporter gene activity are generated by plotting relative β-gal activity with respect to UV fluence to the virus (figure 1).

4. Notes

1. The choice of construct is critical to the outcome of experiments. Other constructs expressing *lacZ* from alternative promoters have been used in our lab: AdCA35*lacZ* and AdCA11 express *lacZ* under control of the murine CMV (MCMV) (26) and human β-actin promoters, respectively. One advantage of Ad expressing *lacZ* under the MCMV promoter is the ability to examine repair in either human or rodent cells (figure 1A and B) as the HCMV construct expresses β-gal poorly in rodent cells. β-gal expression is greater from the AdCA35*lacZ* construct compared to expression from the Ad5HCMVsp1*lacZ* (26). This has been useful to shorten the duration of the experiment, allowing assessment of β-gal activity following 24 hrs. However, the duration of time following repair is reduced; therefore, survival curves tend to be steeper and inducible responses are not observed to the same extent (unpublished results). A *lacZ* construct driven by the human β-actin promoter has also been used but β-gal expression is insufficient for most cell types (unpublished results).
2. Recombinant Ad constructs with deletions of the essential E1 region are replication defective in the absence of E1 transcomplementation. Virus is propagated and titred in 293 cells (28), available through the American tissue culture collection. These are Ad transformed cells which express E1 genes and are therefore permissive for infection of E1-deleted Ad.
3. PMSF is not stable at 4°C in aqueous solution. For this reason, PMSF should be added to lysing solution immediately prior to use.

4. ONPG solution can be stored at 4°C, however yellow colour gradually develops. Discard yellow solutions. If experiments are not being performed regularly, scale down the size of the ONPG preparations.

5. There is variability between viral preps in terms of β-gal activity/pfu. For this reason, relatively large preps should be prepared which allow use of the same viral suspension for all related experiments. We typically prepare virus stocks using at least 10 x 150 mm dishes.

6. The MOI can affect the final titre obtained. At 4 pfu/cell, one is confident that almost all cells will be infected with replicating virus. If cells are not infected immediately, then CPE does not occur simultaneously over the plate and titres are lower.

7. An alternative to methylene blue staining is crystal violet (2 g crystal violet in 20 mL methanol, 144 mL PBS and 36 mL formaldehyde). Filter through Whatman no. 1 filter paper to remove any particulate matter prior to use.

8. Although lower cell density may be desirable in some experiments, infection with Ad is less efficient at low density.

9. Since some β-gal will be contained in the viral suspension, background activity is assessed by inactivating the reporter gene with a large UV fluence (>10 kJ/m^2). Mean β-gal activity following this treatment is subtracted from all other treatments.

10. β-gal activity is linear with respect to MOI of the reporter construct (figure 2A), however, with high MOIs duration of incubation in ONPG must be reduced (figure 2).

Figure 2. Saturation of absorbance at 405 nm is dependant on both MOI and duration of incubation in ONPG. Normal diploid fibroblasts were infected with various MOIs of Ad5HCMVsp1*lacZ* and allowed 48hrs for expression of β-gal activity. Absorbance at 405 nm was determined at several times following addition of ONPG. (A) β-gal activity was linear with respect to MOI for 200 pfu/cell or less and incubations of 60 (●), 150 (▲) and 360 (▼) minutes in ONPG. (B) Absorbance was linear with respect to duration of incubation in ONPG (up to 4 hrs) for MOIs of 20 (●), 60 (▲) and 200 (▼) for this experiment. Each point represents the mean absorbance at 405 nm (± standard error) for a single experiment performed in triplicate.

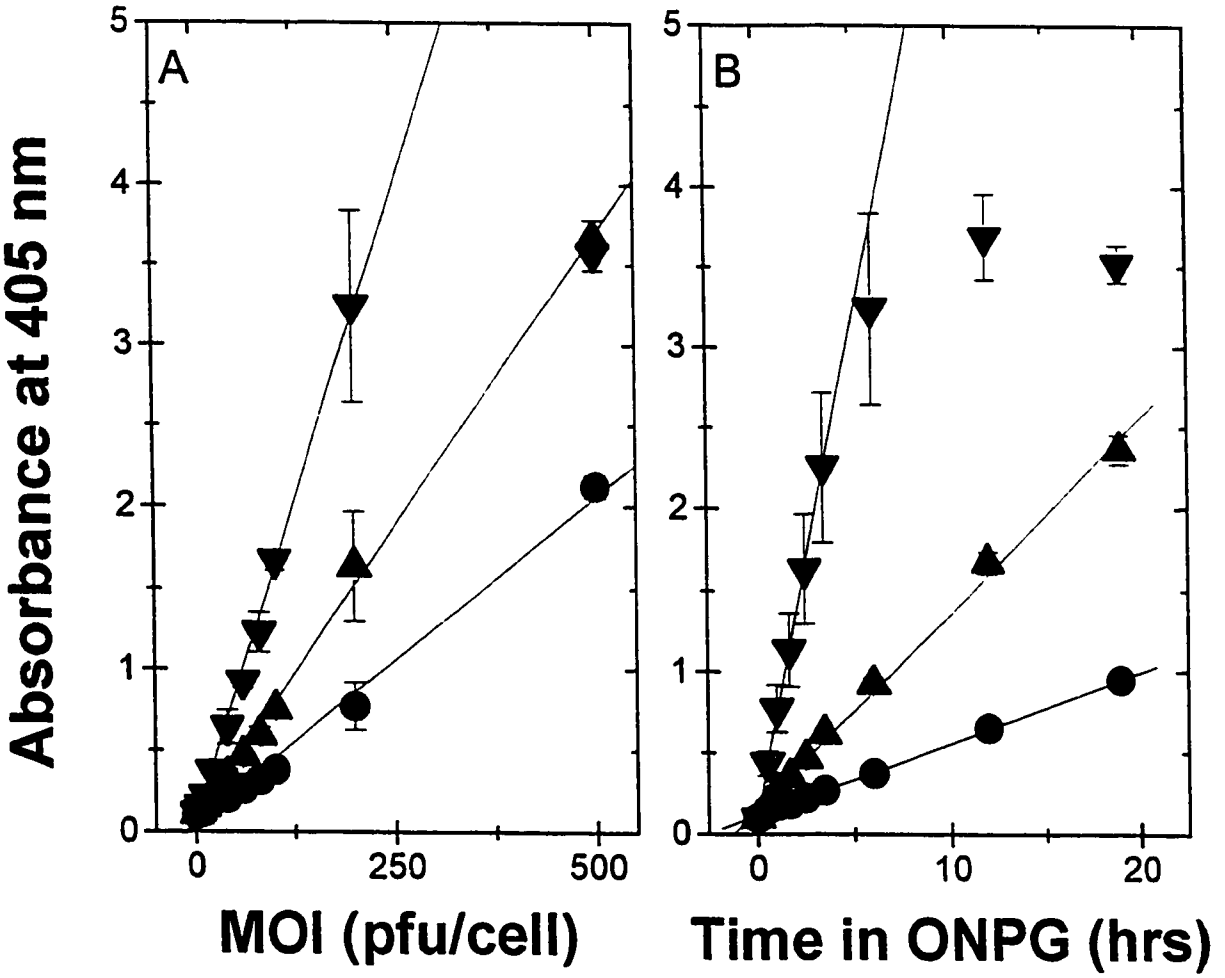
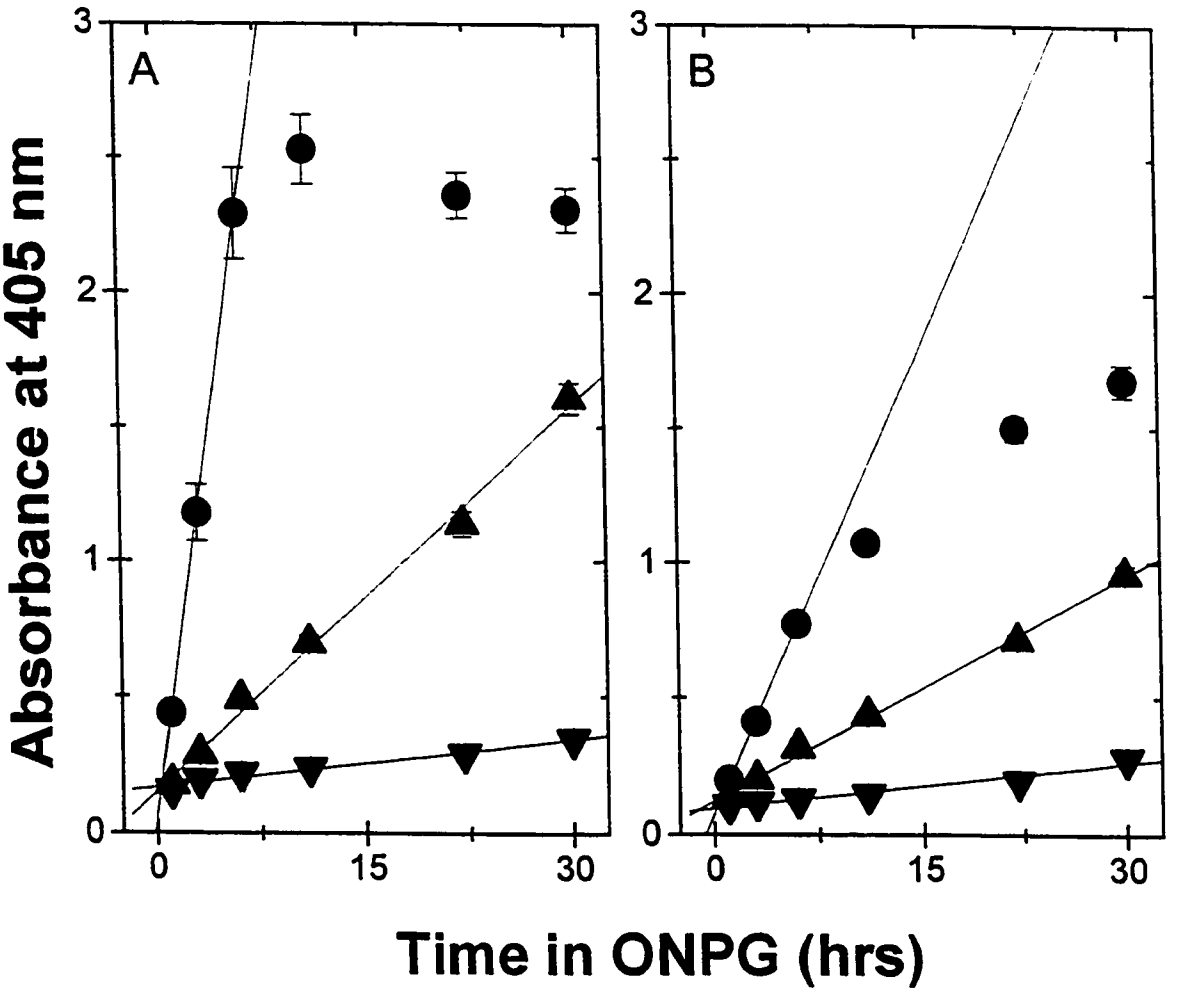

A
B
Absorbance at 405 nm
MOI (pfu/cell)
Time in ONPG (hrs)
0
1
2
3
4
5
0
250
500
0
10
20

Figure 3. The effect of incubation time in ONPG on absorbance at 405 nm. Cell monolayers were infected with either UV irradiated (500 J/m^2-▲ or 15000 J/m^2-▼) or unirradiated (●) Ad5HCMVsp1*lacZ* at an MOI of 20. Cells were allowed 48hrs for repair and expression of β-gal. Absorbance at 405 nm was determined at various times following addition of ONPG in L132 normal diploid lung epithelial cells (A) and HCT116 colon carcinoma cells (B). Absorbance is linear with time for early determinations ($\leq$ 7 hrs for this experiment) but becomes saturated at later times, particularly from samples infected with unirradiated virus. In all experiments, survival curves are generated from data obtained from the linear portion of the saturation curves. Note that at later times, expression from the UV irradiated treatments remain linear and therefore survival curves generated at later times are not an accurate reflection of the repair capacity of these cell lines (note 18). Each point represents the mean absorbance at 405 nm ($\pm$ standard error) for a single experiment performed in triplicate.


A
B
Absorbance at 405 nm
Time in ONPG (hrs)
0
1
2
3
0
15
30

11. A volume of 1.8 mL allows 8 samples of 200 μL to be withdrawn. If greater than 8 samples are required then the volume can be increased. If the volume is increased to greater than 2.5 mL then increase the size of the dish used for irradiation from 35 mm to 60 mm.

12. Water is frozen in 150 mm dishes in order to irradiate virus on a flat ice surface. One dish is sufficient to perform a single experiment. Stir bars are made from paper clips and are sterilized over a flame.

13. Recombinant Ad constructs are commonly used for the expression a variety of gene products in mammalian cells (27). Other recombinant Ad constructs can be introduced into cells with UV-damaged reporter constructs by coinfection in order to assess the effect of exogenous gene expression on DNA repair. A T4 endonuclease V expressing construct has been used in this manner by Valerie and Singhal (18). This technique is amenable to coinfection of recombinant viruses expressing human DNA repair genes (unpublished results) as well as regulators of DNA repair (unpublished results).

14. The duration of the experiment is contingent upon the construct used. Pilot experiments must be performed in order to determine the level of expression from any construct with increasing incubation time (also see note 1).

15. This technique is amenable to use with a number of other DNA damaging agents. Cisplatin has been used in our laboratory (unpublished results).

16. One must ensure that the lower surface of microtitre dishes make contact with the water as an air pocket will insulate the dish. Submerge the dish at between a 30 and

45° angle before placing in a horizontal position. This allows air to escape upon submersion.

17. If monolayers are resistant to lysis, the monolayer can be disrupted by repeated pipetting of solution.

18. Absorbance is linear with respect to time following addition of ONPG (figure 3), however the duration of time over which linearity is observed varies between experiments. We typically measure absorbance several times following addition of ONPG in order to ensure that absorbance values are linear with respect to time for each experiment. If incubation in ONPG is not linear, then survival curves display an increase in survival at low fluence which appears as an increase in the shoulder of the survival curve.

5. Acknowledgements

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Chapter 4

Heat-shock enhanced reactivation of a UV-damaged reporter gene in human cells involves the transcription coupled DNA repair pathway

Bruce C. McKay and Andrew J. Rainbow
Mutation Research, DNA Repair, 363, 125-135.

Preface

Host cell reactivation (HCR) of reporter gene activity is frequently used to assess DNA repair. Previous work from our lab using a recombinant adenovirus expressing *lacZ* under control of the human cytomegalovirus immediate early promoter suggested that HCR of a UV-damaged reporter gene was less efficient in XP-C than in normal fibroblasts (NDF) (Francis and Rainbow, 1995 and unpublished results; McKay and Rainbow, 1996 and chapter 3). XP-C fibroblasts are proficient at TCR of UV induced DNA damage (Venema, 1990; Van Hoffen *et al*, 1995) thus HCR of reporter gene activity was expected to be normal in these cells. Inefficient HCR of reporter gene activity clearly indicates that inhibition of transcription is not sufficient for TCR.

Treatment of recipient cells with UV prior to infection with the UV-damaged reporter construct led to a significant increase in the capacity of NDF and XP-C but not CS-A, CS-B or other XP fibroblast strains to repair the reporter gene. Thus UV-irradiation of XP-C fibroblasts (and NDF) facilitated repair of the reporter gene, strongly suggesting that TCR is inducible in response to UV. UV and heat-shock (HS) stimulate replication of UV damaged DNA viruses but the contribution of DNA repair to this response is unclear. Thus it was of interest to assess the effect of HS on HCR of a UV-damaged reporter gene in NDF, XP-C, CS-A and XP-A fibroblasts. It is reported here that heat-shock could stimulate TCR of the UV-damaged reporter gene in primary cells but not in most transformed cells examined.

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DNA Repair

Heat-shock enhanced reactivation of a UV-damaged reporter gene in human cells involves the transcription coupled DNA repair pathway

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Abstract

A recombinant nonreplicating human adenovirus type 5, Ad5HCMVsp1*lacZ*, expressing the *lacZ* gene under control of the human cytomegalovirus immediate early promoter, was used to assess the effect of heat-shock (HS) on DNA repair of a UV-damaged reporter gene. Host cell reactivation (HCR) of β-galactosidase (β-gal) activity for UV-irradiated Ad5HCMVsp1*lacZ* was used as an indicator of DNA repair in the transcribed strand of an active gene. Repair was examined in heat-shock (HS) pretreated and mock-treated normal fibroblasts, normal lung epithelial cells, xeroderma pigmentosum group A, C, D and G fibroblasts (XP-A, XP-C, XP-D and XP-G), Cockayne's syndrome group A fibroblasts (CS-A), SV40-transformed normal fibroblasts (GM637f) and 5 tumour cell lines (SKOV-3, HeLa, HT29, SCC-25 and U20S). HS enhanced reactivation (HSER) of the reporter gene was detected in normal cells, HT29 tumour cells and XP-C fibroblasts. HSER was reduced or absent in all other XP, CS and tumour cell lines tested. HSER in normal and XP-C cell lines, but not CS-A, XP-A, XP-D or XP-G cells, suggests that HS treatment can enhance the repair of UV-damaged DNA through an enhancement of transcription coupled repair (TCR) or a mechanism which involves the TCR pathway. Since this response was absent in the SV40-transformed fibroblast cell line and 4 of 5 tumour cell lines examined, HSER of β-gal activity for UV-irradiated Ad5HCMVsp1*lacZ* also requires some cellular function(s) affected by transformation.

Keywords: DNA repair; Heat shock; Adenovirus; Enhanced reactivation of virus; UV-irradiation; Nucleotide excision repair

1. Introduction

Host cell reactivation (HCR) of UV-damaged nuclear replicating viruses has been used to assess the DNA repair capacity of a variety of cell types. Xeroderma pigmentosum (XP) (Aaronson and Lytle, 1970; Day, 1974; Abrahams and van der Eb, 1976; Rainbow, 1981), Cockayne's syndrome (CS) (Rainbow and Howes, 1982) and several tumour cells (Rainbow, 1989) have reduced HCR of DNA damaged viruses. Pretreatment of cells with UV, γ-irradiation or heat-shock (HS) enhances reactivation of DNA damaged virus (UVER, γER, HSER) (Bockstahler and Lytle, 1970; Jeeves and Rainbow, 1983a,b; Piperakis and McLennan, 1984; Yager et al., 1985).

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It has been suggested that ER of UV-damaged DNA viruses results from an inducible DNA repair pathway (Rainbow, 1981; Defais et al., 1983). More recent support for inducible DNA repair comes from UVER of UV-damaged reporter genes (Protic et al., 1988; Smith et al., 1995; Francis and Rainbow, 1995).

UV-induced DNA lesions are repaired through two interrelated nucleotide excision repair (NER) pathways. Photolesions are removed more rapidly from the transcribed strand of actively transcribed genes than from the remainder of the genome (Bohr et al., 1985; Venema et al., 1990b; Van Hoffen et al., 1995). Cell lines established from individuals with XP and CS are sensitive to UV irradiation. Both XP and CS fibroblasts exhibit reduced NER. In CS this deficiency is specific to transcription coupled repair (TCR), (Venema et al., 1990b) whereas XP group C (XP-C) cells repair UV-induced lesions in the transcribed strand of active genes but are deficient in global repair (Venema et al., 1990a; Venema et al., 1991; Evans et al., 1993; Barsalou et al., 1994; Van Hoffen et al., 1995). Recent evidence suggests that the ability of XP-C fibroblasts to repair actively transcribed genes involves a UV-inducible pathway (Francis and Rainbow, 1995). Other XP complementation groups are deficient in repair in both pathways (Evans et al., 1993).

It has been suggested that HSER may result from HS enhanced DNA repair (Piperakis and McLennan, 1984; Yager et al., 1985). Recent reports indicate that HS pretreatment of mammalian cells can lead to enhanced resistance to a subsequent exposure to UV light (Maytin et al., 1993, 1994; Kane and Maytin, 1995) and that hsp70 plays a role in cell protection against UV light (Suzuki and Watanabe, 1994; Trautinger et al., 1995, Simon et al., 1995). However, HS treatment immediately prior to UV-irradiation has been reported to inhibit repair of both strands of the adenosine deaminase (ADA) gene in normal human fibroblasts and in fibroblasts deficient in transcription of the ADA gene, but not inhibit repair of the inactive 754 locus (Sakkers et al., 1993, 1995). HS-induced inhibition of repair in the transcribed strand of the ADA gene in XP-C fibroblasts was also observed (Sakkers et al., 1995). It has been suggested that hyperthermia induced intranuclear aggregation probably restricts access of repair complexes to sites of DNA damage in active regions of the genome (Sakkers et al., 1993, 1995). Since HSER suggests that the DNA repair capacity of cells is enhanced by HS treatment, we have taken advantage of a nonreplicating Ad vector carrying the *lacZ* reporter gene, Ad5HCMVsp1*lacZ* (Morsy et al., 1993), to assess gene specific DNA repair in non-heat-damaged UV-irradiated DNA in HS treated normal, repair deficient and tumour cells. We show that normal human fibroblasts, human lung epithelial cells and XP-C fibroblasts express HSER of the reporter gene; however, this response is reduced or absent in CS-A, XP-A, XP-D and XP-G cells. These results suggest that HS can enhance the repair of UV-damaged DNA through an enhancement of TCR or a mechanism which involves the TCR pathway. We show also that HSER of the reporter gene was absent in an SV40-transformed fibroblast cell line and in 4 of 5 tumour cell lines examined, indicating that some cellular function(s) affected by transformation are also involved in the pathway leading to HSER.

2. Materials and methods

2.1. Cells and virus

All human fibroblast strains were obtained from the National Institute of General Medical Sciences repository (Camden, NJ) (see Table 1). HeLa cells were obtained from the American Type Culture Collection (Rockville, MD). U20S cells were obtained from Dr P. Whyte (McMaster University, Hamilton,

Table 1
List of cell lines obtained from the NIGMS repository

NIGMS	Cell line	Description
GM969[c]		normal
GM8399		normal
GM637		SV40 [b]
GM5509	XP12BE	XP-A
GM677	XP2BE	XP-C
GM3615	XP1BR	XP-D
GM3021	XP2BI	XP-G
GM1856	CS3BE	CS-A

[a] NIGMS repository designation.
[b] SV40-transformed normal fibroblasts.

Ont.). HT29 cells were obtained from Dr G. Singh (Hamilton Regional Cancer Clinic, Hamilton, Ont.). SCC-25 were obtained from Dr J. Lazo (University of Pittsburgh School of Medicine, Pittsburgh, PA). SKOV-3 cells were obtained from Dr S. Bacchetti (McMaster University). 293 cells were obtained from Dr F.L. Graham (McMaster University) and the normal lung epithelial L132 cells were obtained from Dr. J. Arrand (Brunel University, Uxbridge, UK). All cultures were maintained in Eagles α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum together with antibiotic-antimycotic (100 μg/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B; Gibco BRL).

Ad5HCMVsp1*lacZ* is a nonreplicating Ad5 derived virus expressing *lacZ* under control of the HCMV immediate early promoter. This construct expresses β-galactosidase (β-gal) in various human cell types without replication of the virus (Morsy et al., 1993). Viral stocks were prepared as described previously (Graham and Prevec, 1991).

2.2. UV-irradiation of virus

UV irradiation of the virus has been described previously (Bennett and Rainbow, 1988). Viral suspensions in 1.8 ml PBS (140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4 and 1.75 mM KH_2PO_4) were irradiated in 35 mm dishes on ice with continuous stirring using a general electric germicidal lamp (model G8T5) emitting predominantly at 254 nm at an incident fluence rate of 2 J/m^2 per s (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 PBS.

2.3. Heat shock enhanced reactivation of the reporter gene

Cells were seeded at a density of 1.9×10^4 cells/well in 96-well microtitre plates (Falcon, Lincoln Park, NJ). Between 20 and 24 h after seeding, microtitre plates were seeled with PVC tape and submerged in a water bath at 43 ± 0.25°C. Immediately following hyperthermic treatment, cells were infected with either irradiated or unirradiated Ad5HCMVsp1*lacZ* in a total volume of 40 μl in PBS at a multiplicity of infection of 10 pfu/cell. Following viral adsorption for 90 min at 37°C, the viral suspensions were aspirated and the cells were refed with prewarmed α-MEM.

Infected cells were harvested at 40 to 44 h after infection. Briefly, infected cell layers were incubated 20 min at 37°C in 250 mM Tris, 1 μM PMSF, 0.5% NP-40 (pH 7.8), followed by 10 min in 100 mM sodium phosphate, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol (pH 7.5) (Morsy et al., 1993). A_{405} was determined at several times following addition of *O*-nitrophenol β-D-galactopyranoside (0.1% ONGP pH 7.5) using a 96-well plate reader (Labsystems multiscan MCC/340).

3. Results

3.1. Heat shock enhanced reactivation in normal cell lines

UV-irradiated and unirradiated Ad5HCMVsp1*lacZ* were used to infect HS treated and untreated normal diploid fibroblasts and normal diploid lung epithelial cells. Representative survival curves for β-galactosidase (β-gal) expression of UV-irradiated Ad5HCMVsp1*lacZ* are presented in Fig. 1. D_0 values were determined from the slope of the straight line exponential equation ($SF = e^{-D/D_0}$)

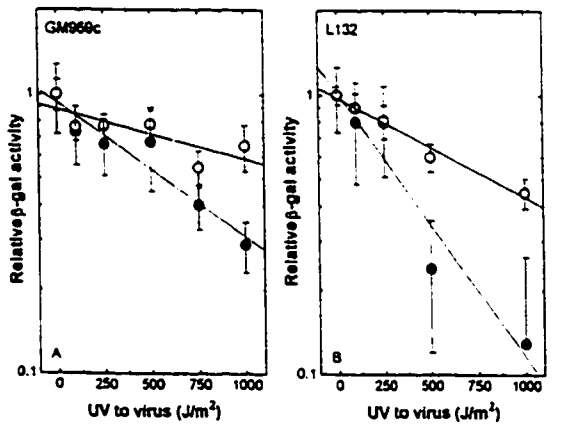

Fig. 1. Effect of HS on HCR of a UV-damaged reporter gene in normal cells. Survival of β-galactosidase activity for untreated (●) and 30 min HS-treated (○) (A) L132 and (B) GM969c cells were examined. Points represent the mean (±SE) of 4 determinations from a single experiment. Mean relative D_0 values obtained for a number of experiments are shown in Table 2.

where SF is the surviving fraction of β-gal activity. To compare enhancement between cell lines, relative D_0 values (D_0 with HS/D_0 without HS) were determined. Relative D_0 values for UV survival of β-gal activity in normal fibroblasts and lung epithelial cells are elevated in cells pretreated with HS (43°C) durations of between 15 and 60 min (Table 2). Relative D_0 values for all normal fibroblast cell lines examined were pooled and compared to normal lung epithelial cells (Fig. 2). There was no significant difference in HSER between normal fibroblasts and lung epithelial cells, relative D_0 values of 1.82 ± 0.36 and 1.57 ± 0.14, respectively, were observed following a HS of 30 min.

3.2. Heat shock enhanced reactivation in repair deficient cell lines

UVER of a reporter construct reflects NER in the transcribed strand of an active gene (Protic et al., 1988; Valerie and Singhal, 1995; Francis and Rainbow, 1995). In order to elucidate the NER pathways involved in HSER, XP and CS fibroblast strains with known repair phenotypes were examined. XP-C cells have normal DNA repair in the transcribed strand of actively transcribed regions of the genome, but are deficient in bulk NER (Venema et al., 1990a; Venema et al., 1991; Van Hoffen et al., 1995), CS-A cells retain the ability to repair bulk DNA, but are deficient in TCR (Venema et al., 1990b) whereas XP-A, XP-D and XP-G are deficient in both pathways (Evans et al., 1993; He et al., 1995). HSER (15 and 30 min) of β-gal expression for UV-irradiated Ad5HCMVsp1*lacZ* was examined in the different fibroblast strains. Representative survival curves for β-gal expression are presented in Fig. 3. It can be seen that significant HSER was detected in the XP-C strain, but not in the other NER deficient strains tested. Similar results were obtained for a number of experiments and the mean HSER was determined for each cell strain (Table 2). HSER of a similar magnitude to that in normal cells was detected in XP-C fibroblasts (relative $D_0 = 2.02 \pm 0.58$). However, HSER in the other NER deficient cell lines following 30 min HS was reduced or absent compared to that in the normal strains. Relative D_0 values for CS-A, XP-A, XP-D and XP-G cell lines were 1.20 ± 0.01, 1.01 ± 0.16, 0.99 ± 0.12 and 1.17 ± 0.06, respectively. Since HSER in XP-C cells approaches that in normal fibroblasts, whereas significant HSER was not detected in CS-A, XP-A, XP-D and XP-G cells, the predominant repair pathway involved in HSER of β-gal activity for UV-irradiated Ad5HCMVsp1*lacZ* is most likely TCR.

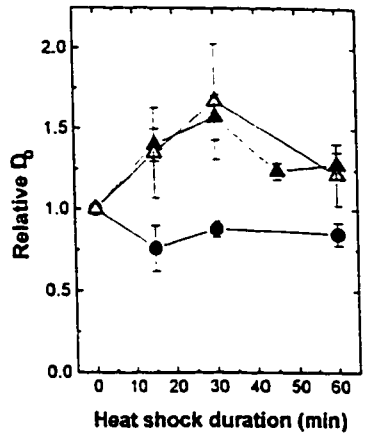

Fig. 2. Mean heat-shock-enhanced reactivation of β-gal expression in normal human and HeLa cells. Points represent the mean relative D_0 value (±SE) for normal fibroblasts (△), L132 cells (▲) and HeLa cells (●) for 2 or more experiments. Data and number of experiments for HS durations of 15, 30 and 60 min are included in Table 2 and Table 3.

Table 2
HSER of β-gal activity in normal and repair deficient cell lines

Cell line	Mean relative D_0 [a]	
	15 min [b]	30 min [b]
GM969c	1.08 ± 0.16 [c] (2 [d])	1.96 ± 0.41 (4)
GM8399	1.62 ± 0.14 (2)	1.53 ± 0.13 (2)
L132	1.40 ± 0.10 (8)	1.57 ± 0.14 (11)
GM1856 (CSA)	ND [e]	1.20 ± 0.01 (2)
GM677 (XPC)	1.13 ± 0.10 (3)	2.02 ± 0.58 (3)
GM5509 (XPA)	0.83 ± 0.15 (2)	1.01 ± 0.16 (3)
GM3615 (XPD)	0.72 ± 0.25 (2)	0.99 ± 0.12 (2)
GM3021 (XPG)	ND	1.17 ± 0.06 (2)

[a] D_0 treated / D_0 untreated.
[b] duration of heat shock treatment in minutes.
[c] Standard error about the mean.
[d] Number of experiments.
[e] Not determined.

3.3. Heat shock enhanced reactivation in transformed cell lines

Since HS is used to enhance the sensitivity of tumours to radiation and antineoplastic agents (Hahn, 1982), we have evaluated HSER in a number of tumour and transformed cell lines. HSER was examined in HT29 (colon carcinoma), HeLa (cervical carcinoma), SKOV-3 (ovarian carcinoma), U20S (osteosarcoma), SCC-25 (squamous cell carcinoma) and GM637f (SV40-transformed fibroblasts) cell lines. HSER was observed in HT29 but was absent in the other 5 tumour and transformed cell lines (Fig. 4, Table 3). The relative D_0 values were 1.44 ± 0.34, 0.88 ± 0.05, 1.00 ± 0.11, 0.82 ± 0.3, 1.03 ± 0.1 and 0.85 ± 0.08, respectively, following a 30 min HS. The relative D_0 for HSER in normal fibroblasts and lung epithelial cells is significantly elevated compared to HeLa cells (Fig. 2). These results indicate that HSER of β-gal activity for UV-irradiated Ad5HCMVsp1*lacZ* depends upon cellular functions affected by transformation in 5 of 6 tumour and transformed cell lines examined.

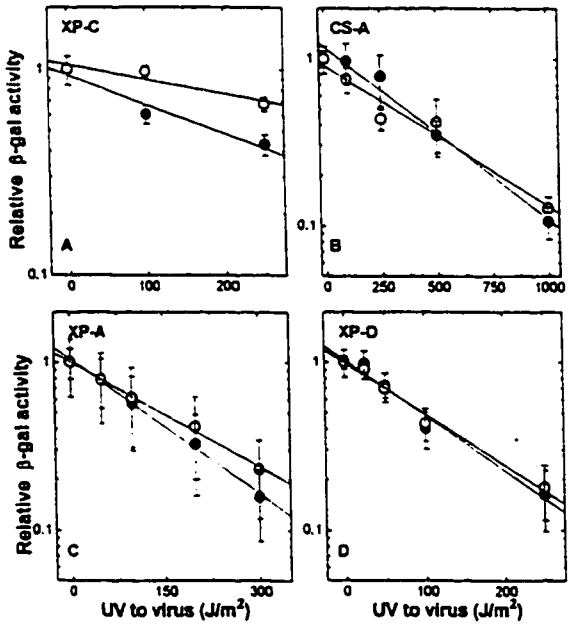

Fig. 3. Effect of HS on HCR of a UV-damaged reporter gene in repair deficient fibroblasts. Survival of β-gal activity for untreated (●) and 30 min HS-treated (○) (A) XP2BE, (B) CS3BE, (C) XP12BE and (D) XP1BR fibroblasts were examined. Points represent the mean (±SE) of 3 determinations from a single experiment. Mean relative D_0 values from a number of experiments are shown in Table 2.

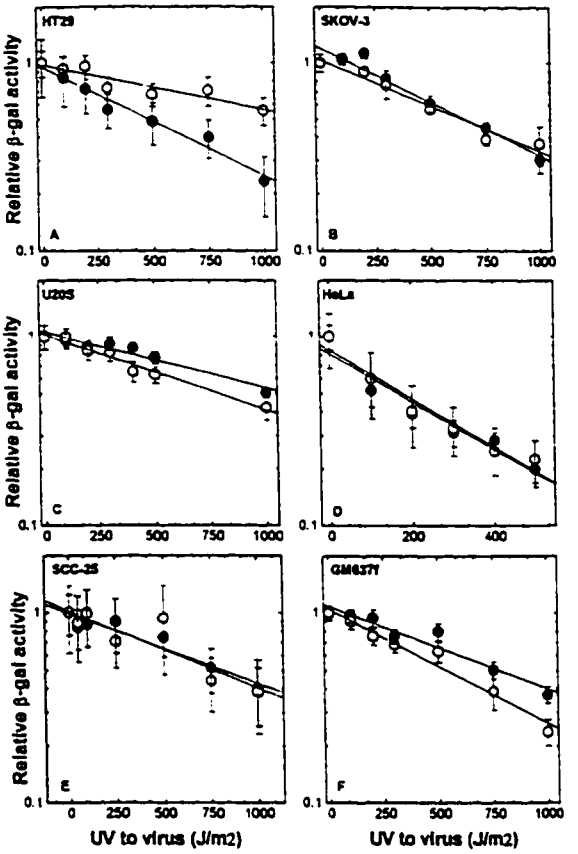

Fig. 4. Effect of HS on HCR of a UV-damaged reporter gene in 4 tumour cell lines and an SV40 transformed cell line. Survival of β-gal activity for untreated (●) and 30 min HS-treated (○) (A) HT29, (B) SKOV-3, (C) U20S, (D) HeLa, (E) SCC-25 and (F) GM637f cells were examined. Points represent the mean (±SE) of 3 or 4 determinations from a single experiment. Mean relative D_0 values from a number of experiments are summarized in Table 3.

4. Discussion

HSER of Ad2 and HSV-1 plaque forming ability has previously been reported in HeLa and Vero cells, respectively (Piperakis and McLennan, 1984; Yager et al., 1985). HS at 45.5°C for between 5 and 20 min

Table 3
HSER of β-gal activity in lung epithelial normal and tumour cells

Cell line	Mean relative D_0		
	15 min [a]	30 min	60 min
L132 [b]	1.40 ± 0.10 [c] (8 [d])	1.57 ± 0.14 (11	1.28 ± 0.08 (3)
SKOV-3	1.11 ± 0.11 (7)	1.00 ± 0.11 (8)	0.88 ± 0.04 (2)
HeLa	0.76 ± 0.14 (4)	0.88 ± 0.05 (6)	0.85 ± 0.07 (2)
U20S	1.15 ± 0.16 (5)	0.82 ± 0.30 (7)	ND [e]
HT29	1.25 ± 0.16 (4)	1.44 ± 0.34 (4)	1.32 ± 0.42 (3)
SCC-25	0.95 ± 0.12 (2)	1.03 ± 0.10 (2)	ND
GM637f	0.88 ± 0.15 (5)	0.85 ± 0.08 (5)	ND

[a] Duration of HS treatment at 43°C in min.
[b] Some of the data for L132 cells is also in Table 2.
[c] Standard error about the mean.
[d] Number of experiments used to determine the mean.
[e] Not determined.

(HeLa cells) and greater than 30 min (Vero cells) enhanced reactivation of the respective viruses. To our knowledge, there are no previous reports on HSER in normal or DNA repair deficient primary fibroblasts. In the present study, we show that HS enhances repair of UV-induced DNA damage in a reporter gene. This is the first evidence that HS may stimulate TCR. Bulk NER and TCR are separate but interrelated pathways. Several repair proteins have been identified by immunoprecipitation in specific complexes. Two such complexes, XPA/XPF/ERCC1/RPA/XPG (Park and Sancar, 1994; He et al., 1995) and XPB/XPD (TFIIH) (Schaeffer et al., 1993, 1994) are required for both pathways. Additional proteins are required for each specific pathway. XP-C binds the human homologue of the yeast DNA repair gene Rad23 (HHR23B) (Masutani et al., 1994) and is required for bulk repair only (Venema et al., 1990a; Venema et al., 1991; Evans et al., 1993; Barsalou et al., 1994; Van Hoffen et al., 1995). CSA and CSB coimmunoprecipitate from cell extracts (Henning et al., 1995) and are required for TCR (Venema et al., 1990b). HSER was examined in XP-A, XP-C, XP-D, XP-G and CS-A fibroblasts in order to assess the role of each of the NER protein complexes in HSER of a reporter gene. HCR of β-gal activity was stimulated by HS in normal human fibroblasts, normal lung epithelial cells and XP-C fibroblasts. The increase in D_0 values for HS treatments approached 2-fold for HS durations of 30 min at 43°C in normal cells and XP-C fibroblasts. HSER of the reporter gene was reduced or absent in other XP and CS fibroblasts. Since XP-C cells are capable of HSER of the reporter gene but CS-A cells have reduced HSER, these results suggest that HS treatment can enhance repair of UV-damaged DNA through an enhancement of TCR or via a mechanism which involves the TCR pathway. HSER of β-gal expression in normal and XP-C cells was of a similar magnitude, suggesting that the inducible mechanism involving the TCR pathway is sufficient to account for the enhancement seen in repair competent cells.

Our laboratory has previously reported that at least two XP-C fibroblast strains were deficient in HCR of the *lacZ* reporter gene but that UV exposure to cells prior to viral infection resulted in enhanced reactivation of the reporter gene to wild-type levels (Francis and Rainbow, 1995). Both HS and UV appear to stimulate the preferential NER pathway. However, we have not ruled out the possibility that bulk repair is also enhanced by HS since repair of a UV-damaged reporter gene only reflects repair of the transcribed strand of an expressed gene. Enhanced TCR of UV-damaged DNA is consistent with the results of Brown and Cerutti (1989) who showed that UVER of SV40 in CV-1 African Monkey cells was dependant on restoration of early gene function. Expression of early genes is required for viral DNA replication; therefore, accelerated repair of early genes is expected to enhance viral reactivation. It is likely that ER of other nuclear replicating viruses is

also dependant on early gene expression. Cellular stresses that induce ER may act similarly through a mechanism of accelerated repair of early genes.

Several similarities exist between cellular responses to HS and DNA damaging agents. DNA damaging agents including ionizing radiation (IR) and UV induce p53 accumulation (Kastan et al., 1991; Maltzman and Czyzyk, 1984). Also, p53 accumulates in both the cytoplasm and nucleus following HS treatment (Graeber et al., 1993; Sugano et al., 1995) in a complex with HSP72 (Matsumoto et al., 1994 and Matsumoto et al., 1995). For some time, heat-shock proteins (HSPs) have been known to associate with mutant p53 (Pinhasi-Kimhi et al., 1986) through a conserved binding domain (Lam and Calderwood, 1992) and more recently, binding of wild-type p53 to HSP72 following HS treatment has been demonstrated (Matsumoto et al., 1995). Both UV- and γ-irradiation induce HSP72 expression (Williams et al., 1989; Muramatsu et al., 1992; Sierra-Rivera et al., 1993) and HSP72 expression correlates with UVER and HSER of HSV-1 in Vero cells (Williams et al., 1989). Furthermore, the expression of HSP72 in human cells and HSP68 in mouse cells provides protection against UV-irradiation (Trautinger et al., 1995; Simon et al., 1995). HS induced stabilization of p53 and expression of HSP72 may involve some common signalling pathways which stimulate DNA repair.

A G_1/S cell cycle checkpoint mediated through a p53 dependant pathway is thought to allow time for DNA repair following UV and IR damage (Lane, 1991). However, a more direct role of p53 in NER has been proposed recently. Disruption of p53 function with HPV16-E6 or expression of mutant p53 decreases NER (Smith et al., 1995). The role of p53 in NER may occur through the p53 dependent induction of GADD45 (Smith et al., 1994). In addition, p53 has been shown to bind XPB, XPD and CSB possibly modulating NER directly (Wang et al., 1995).

Wang et al. (1995) showed that Li-Fraumeni fibroblasts, heterozygous for a p53 mutation at codon 245, have reduced CPD removal from the transcriptionally active DHFR gene compared to normal fibroblasts, suggesting some involvement of p53 in TCR. There is also evidence to suggest that p53 is involved in bulk DNA repair. Smith et al. (1995) demonstrated that in vitro incorporation of radio nucleotides into UV-damaged DNA was decreased when cell extracts from cells transfected with HPV16-E6 are used. Additionally, Ford and Hanawalt (1995) provided evidence that Li-Fraumeni cells expressing only mutant p53 are deficient in bulk repair of CPDs, whereas TCR of CPDs is retained. Inactivation of p53 with HPV-E6 reduced clonogenic survival and HCR of a transfected reporter gene following UV irradiation (Smith et al., 1995). Since HCR of a transfected reporter gene appears to reflect gene specific repair (Stevnsner et al., 1995), the results reported by Smith and coworkers are consistent with a role of p53 in TCR as well as bulk DNA repair. The results presented in this work, which suggest that HSER results from an enhancement of cellular factors involved in TCR, do not exclude the possibility that the bulk DNA repair pathway is inducible by HS since our assay reflects repair of the transcribed strand of the reporter gene. It is possible that both the TCR and bulk NER pathways are inducible, and stimulation of these pathways may depend on similar gene products. Recent results indicate that although both TCR and bulk NER are operative in normal human cells, the relative importance of each pathway depends on the type of lesion as well as the UV dose employed (Van Hoffen et al., 1995).

Since HS is used to enhance the sensitivity of tumours to radiation and antineoplastic agents (Hahn, 1982), we have evaluated HSER in a number of tumour and transformed cell lines. Only HT29 colon carcinoma cells showed HSER, whereas HSER was absent in all other tumour cell lines examined. Mutations in p53 are the most common genetic alterations in cancer cells (Hollstein et al., 1991). Results presented here showing reduced HSER of β-gal activity for UV-irradiated Ad5HCMVsp1 *lacZ* in tumour cells may result from aberrant p53 induction. There is no detectable p53 in SKOV-3 cells (Johnson et al., 1991). HPV-18 E6 expression in HeLa cells affects levels of p53 (Wrede et al., 1991; Hoppe-Seyler and Butz, 1993). U20S cell lines have altered p53 expression due to constitutive over expression of *mdm-2* (Florenes et al., 1994), a common genetic alteration in sarcomas (Oliner et al., 1992). p53 expression was not detected by immunocytochemistry in SCC-25 cells due to a 2 bp deletion and subsequent

frame shift at codon 208 (Caamano et al., 1993). Large T antigen of SV40 binds and functionally inactivates p53 (Quartin et al., 1994 and references therein); therefore, GM637f cells are expected to have reduced p53 function. However, HSER was detected in HT29 cells which have a point mutation at codon 273 ($Arg^{273}His$) (Rodrigues et al., 1990). $Arg^{273}His$ p53 can be detected in the wild-type conformation (Rodrigues et al., 1990), can activate transcription of a reporter construct with p53 recognition sequences (Park et al., 1994) and cooperates inefficiently with activated *ras* to transform primary rat cells (Hinds et al., 1990). Also, $Arg^{273}His$ p53 retains the ability to bind XPB, XPD and CSB, a function suggested to modulate NER (Wang et al., 1995). Taken together these results are consistent with a role for p53 in HSER in which the p53 mutation in HT29 cells does not disrupt the p53 function(s) required for enhanced reactivation of β-gal activity. It may be possible that the absence of a HS induced, p53 dependant NER pathway in some tumor cells compared to normal cells could be exploited to improve protocols for combined hyperthermia and chemotherapy of tumours.

HSER of plaque forming ability for UV-damaged Ad2 in HeLa cells (Piperakis and McLennan, 1984) has been reported. The conditions of treatment differed from those used in the present study. HS treatments of between 5 and 20 min at 45.5°C enhanced viral reactivation (Piperakis and McLennan, 1984). It is possible that HSER of plaque forming ability in HeLa cells either requires more elevated temperatures to illicit a repair response or the enhancement of viral plaque forming ability could result from enhanced global repair not detected by our assay. Alternatively, since replication of Ad requires *E1* gene expression (Jones and Shenk, 1979) whereas the E1 region of Ad5 is deleted in Ad5HCMVsp1*lacZ* (Morsy et al., 1993), the difference in HSER may reflect interactions of the E1 gene products with cellular targets of HPV tumour antigens.

Sakkers et al. (1993, 1995) reported that HS of 30 min at 45°C inhibits CPD removal from both strands of the ADA gene but not the 754 locus in normal fibroblasts. Removal of CPD from the transcribed strand of the ADA gene is delayed in XP-C fibroblasts; however, the repair of lesions following HS approached that of nonheat-treated cells by 24 h (Sakkers et al., 1995). HS induces intranuclear aggregation (Kampinga, 1993) which would be expected to restrict access of DNA repair proteins to sites of DNA damage (Sakkers et al., 1995). TCR and bulk NER appear to be inhibited under these conditions. However, moderate HS has been demonstrated to elicit a protective response against subsequent UV challenge in yeast (Mitchell and Morrison, 1983), murine keratinocytes (Maytin et al., 1993), mouse skin (Kane and Maytin, 1995), murine fibroblasts (Simon et al., 1995) and human keratinocytes (Maytin et al., 1994; Trautinger et al., 1995). In mouse and human cells the protective effect of HS took time to develop, was optimal for a time interval of about 6 h between HS and UV and required ongoing synthesis of mRNA and protein. It is possible that the protection to UV exposure afforded by HS pretreatment results from a HS induced stimulation of the NER pathway as suggested by the present work. Our experiments examined repair in nonheat-damaged DNA over a 40–48 h period following HS at 43°C, previously shown to induce nuclear accumulation of p53 (Sugano et al., 1995). Under these conditions, HS appears to stimulate repair of UV-induced lesions in the transcribed strand of the reporter gene in normal cells but not in most tumour cells examined. These results suggest that increased UV survival following HS pretreatment may result, at least in part, from HS stimulation of the TCR pathway.

IR-induced DNA damage is also repaired preferentially from the transcribed strand of actively transcribed genes (Leadon and Cooper, 1993). CS-A and CS-B fibroblasts were deficient in TCR of IR-induced DNA damage indicating that the CS gene products are required for preferential repair of IR-induced damage (Leadon and Cooper, 1993). Our results indicate that HSER of β-gal expression was also dependant on the presence of a functional CS-A gene product. Preferential repair of UV- and IR-induced damage may be related pathways suggesting that HS may also stimulate repair of IR-induced DNA damage. Also, chemotherapeutic agents such as cisplatin induce bulky adducts which can be repaired by TCR (for review see Bohr, 1994) such that combined modality tumour treatments may be critically affected by a HS inducible DNA repair mechanism in some tumours.

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Chapter 5

Wildtype p53 is required for heat shock and ultraviolet light enhanced repair of a UV-damaged reporter gene

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Preface

In chapter 4 (McKay and Rainbow, 1996), a rough correlation was observed between disruption of heat-shock (HS) inducible transcription-coupled repair and disruption of p53 function. Unpublished results (Hill and Rainbow) indicate that p53 deficient tumour cell lines also lack ultraviolet enhanced reactivation of reporter gene activity. For this reason, it was of interest to assess both UVER and HSER in Li-Fraumeni syndrome fibroblasts with known mutations in the p53 gene. Two primary fibroblasts stains and spontaneously immortalized sublines lacking the wildtype p53 allele were obtained in order to test the role of wildtype p53 in DNA repair.

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ACCELERATED PAPER

Wildtype *p53* is required for heat shock and ultraviolet light enhanced repair of a UV-damaged reporter gene

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We have previously reported the use of a recombinant nonreplicating adenovirus type 5, Ad5HCMVsp1*lacZ*, expressing the *lacZ* gene under control of the human cytomegalovirus (HCMV) immediate early promoter to assess repair of a UV-damaged reporter gene in UV and heat shock (HS) treated cells. Heat shock and UV-enhanced reactivation (HSER and UVER) of β-galactosidase (β-gal) activity for UV-irradiated Ad5HCMVsp1*lacZ* in normal human fibroblasts involved the transcription coupled repair (TCR) pathway. However, this inducible DNA repair response was absent in *p53* deficient tumour cell lines. In order to examine further the requirement for *p53* in HSER and UVER, we have examined host cell reactivation (HCR) of the reporter construct in HS treated, UV treated and mock treated Li-Fraumeni syndrome (LFS) fibroblasts, which are heterozygous for a *p53* mutation, and immortalized LFS cell sublines, which express only mutant *p53*. HCR of β-gal activity for UV-irradiated Ad5HCMVsp1*lacZ* was normal in all LFS cells examined. However, HCR of β-gal activity for UV-irradiated Ad5HCMVsp1*lacZ* was elevated by pretreatment of cells with either UV or HS in normal diploid human fibroblasts, but not in LFS cells. LFS cells appear to be deficient in an inducible pathway which stimulates repair of the reporter gene. These results support a role for *p53* in a HS and UV inducible DNA repair response in human cells which is dependent on TCR.

Introduction

UV-induced lesions were repaired through two interrelated nucleotide excision repair (NER*) pathways: transcription coupled repair (TCR) and bulk NER. TCR removes photolesions from the transcribed strand of active genes more rapidly than the remainder of the genome (1,2). Xeroderma pigmentosum group C (XP-C) cells are TCR competent but lack bulk NER (3,4). We have demonstrated that host cell reactivation (HCR) of a *lacZ* reporter gene expressed from a nonreplicating adenovirus type 5 construct was enhanced by pretreatment of cells with either UV or heat shock (HS) in normal and XP-C fibroblasts (5,6). This response was absent in *p53* deficient tumour cells and SV40-transformed fibroblasts (6,7). Since enhanced reactivation (ER) of reporter genes is dependent on repair of an active gene and UVER and HSER are detected in TCR competent cell lines, we have suggested that TCR may be enhanced by a UV and HS inducible *p53* dependent mechanism (5,6).

Li-Fraumeni syndrome (LFS) is an autosomal dominant disorder characterized by a predisposition to a variety of cancers (8). Germline transmission of mutant *p53* alleles has been implicated in LFS (9). Mutations in *p53* are the most common genetic alterations in human cancers (10). Loss of the wildtype allele contributes to both tumorigenesis in LFS individuals (11) and immortalization of LFS fibroblasts in culture (12). *p53* accumulates in response to DNA damaging agents (13,14) and is thought to protect cells from DNA damage induced malignant transformation through at least three defence mechanisms. Firstly, in response to DNA damaging agents, *p53* mediates cell cycle arrest preventing replication of damaged DNA (14). Secondly, DNA damage can induce apoptosis through a *p53* dependent pathway thus eliminating cells with potentially mutagenic lesions (15). Thirdly, *p53* has been suggested to play both direct and indirect roles in DNA repair (16–18). Deficiencies in any or all of these cellular responses to DNA damage may contribute to genetic instability associated with tumorigenesis.

In addition to its stabilization in response to DNA damaging agents, *p53* accumulates in the nucleus in response to HS and other elicitors of the HS response (19). Heat inducible HSP72 accumulates in association with *p53* following treatment of cells with UV (20,21) and both HS and HSP 72 overexpression appear to stimulate cellular resistance to UV (22–24). Furthermore, HS enhances repair of a UV-damaged reporter gene in normal cells but not SV40 transformed fibroblasts or *p53* deficient tumour cells (6). Common signalling events in response to HS and UV may lead to cellular resistance to UV by stimulating DNA repair.

To examine further the role of *p53* in UV and HS inducible DNA repair, we have examined UVER and HSER of β-gal activity for UV-irradiated Ad5HCMVsp1*lacZ* in two LFS fibroblast cell strains and two spontaneously immortalized LFS cell sublines. HCR of β-gal activity for UV- irradiated Ad5HCMVsp1*lacZ* in LFS cells did not differ significantly from HCR in normal fibroblasts. HCR of β-gal activity in LFS cells was not stimulated by pretreatment of cells with UV or HS whereas normal fibroblasts and lung epithelial cells exhibited UVER and HSER of the reporter gene (6, present study). These results suggest that LFS cells lack an inducible repair response which is dependent on wildtype *p53* function. Furthermore, the deficiency in heterozygous LFS fibroblasts suggests that inactivation of a single *p53* allele may contribute to genetic instability through decreased repair of active genes.

*Abbreviations: HCMV, human cytomegalovirus; HS, heat shock; HSER, heat shock enhanced reactivation; UVER, UV-enhanced reactivation; β-gal, β-galactosidase; TCR, transcription coupled repair; HCR, host cell reactivation; NER, nucleotide excision repair; XP-C, xeroderma pigmentosum group C; ER, enhanced reactivation; LFS, Li-Fraumeri syndrome; α-MEM, α-minimal essential medium.

Materials and methods

Cells and virus

Normal human fibroblast strains GM8399, GM969c and GM9503 were obtained from the National Institute of General Medical Sciences repository (Camden, NJ). Normal human diploid lung epithelial cells (L132) were obtained from Dr J.Arrand, Brunel University, Uxbridge, UK and the normal human fibroblast 423 strain was obtained from Dr P.Chang, McMaster University, Hamilton, Ontario, Canada. LFS fibroblasts, MDAH041 and MDAH087 (hereafter referred to as 041wt/mut and 087wt/mut) are heterozygous for mutations at codons 184 and 248 of *p53* whereas their spontaneously immortalized counterparts (041mut and 087 mut) express only mutant *p53* (12). LFS cells were obtained from Dr M.A.Tainsky, M.D. Anderson Cancer Centre, Houston, TX. Human 293 cells were obtained from Dr F.L.Graham, McMaster University, Hamilton, Ontario, Canada. All cultures were maintained in Eagle's α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum together with penicillin (100 μg/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml) (Gibco BRL).

Ad5HCMVsp1*lacZ* is a nonreplicating Ad5 derived virus expressing *lacZ* under control of the HCMV immediate early promoter. This construct expresses β-gal in most human cell types without replication of the virus (25). Virus was replicated and titred in human 293 cells (26).

Enhanced reactivation of the reporter gene

Enhanced reactivation of the reporter gene was performed (6). Briefly, cells were seeded in 96-well microtitre plates (Falcon, Lincoln Park, NJ) at a density of 1.9×10^4 cells/well, 24 h prior to treatment. For HS treatment, PVC tape was used to seal dishes prior to submersion in a water bath (43 ± 0.25°C). For UV treatment of cells, the medium was aspirated and replaced with 40 μl PBS (140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4 and 1.75 mM KH_2PO_4). Irradiation of cells was performed using a germicidal lamp (general electric model G8T5) emitting predominantly at 254 nm at a fluence rate of 1 J/m²/s (J-255 shortwave UV meter, ultraviolet products, San Gabriel, CA). Immediately following HS or UV treatment, cells were infected (90 min at 37°C) with UV irradiated Ad5HCMVsp1*lacZ* in a total volume of 40 μl. Virus was UV irradiated at an incident fluence rate of 2 J/m²/s.

Infected cells were harvested following 40–44 h. Briefly, infected cell layers were incubated 20 min at 37°C in 250 mM Tris, 1 μM PMSF, 0.5% NP40 (pH 7.8), followed by 10 min in 100 mM sodium phosphate, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol (pH 7.5) (Morsy *et al.*, 1993). OD_{405} was determined at several times following addition of *O*-nitrophenol β-D-galactopyranoside (0.1% ONGP pH 7.5) using a 96-well plate reader (EL340, Biotek Instruments).

Clonogenic survival

Cells were seeded in 6 well dishes (Corning, NY) at a density of between 200 and 1000 cells/well. Cells were either UV-irradiated at an incident fluence rate of 1 J/m²/s or HS treated by submersion of dishes in a 43 ± 0.25°C water bath. Following 6–10 days, cells were stained with methylene blue (5% w:v) and colonies (>25 cells) were counted.

Results

HCR of β-gal activity in LFS cell lines is not enhanced by UV or HS pretreatment

Normal fibroblasts, LFS fibroblasts and immortalized LFS cell sublines were infected with UV-irradiated and unirradiated Ad5HCMVsp1*lacZ*. A UV exposure dependent decrease in survival of β-gal activity was observed. D_0 values were determined from the slope of the straight line exponential equation ($SF = e^{-D/D_0}$) where SF is the surviving fraction of β-gal activity for UV- irradiated Ad5HCMVsp1*lacZ*. As indicated by the D_0 values presented in Table I, HCR of β-gal activity for UV-irradiated Ad5HCMVsp1*lacZ* in normal fibroblasts varied depending on cell strain examined. D_0 values reported for LFS cells fall within the range for normal fibroblasts (Table I) and suggest that repair of the UV-damaged reporter gene is similar in untreated LFS cells and untreated normal human fibroblasts.

HCR of β-gal activity for UV-irradiated Ad5HCMVsp1*lacZ* was also assessed in HS treated, UV treated and untreated normal diploid fibroblasts, LFS fibroblasts and immortalized LFS cells. Typical survival curves for β-gal activity using normal fibroblasts are presented in Figure 1A and D. Increased survival of β-gal activity was observed in HS or UV treated normal diploid fibroblasts, as previously reported (5,6). However, increased HCR of β-gal activity was not observed in similarly treated LFS cells (Figure 1B, C, E and F). The increased HCR of β-gal activity observed in UV and HS treated normal diploid fibroblasts was consistently observed in a number of separate experiments. The relative D_0 values (D_0 treated/D_0 untreated) obtained are presented in Table II. The mean increase in D_0 following either UV or 30 min HS treatment was significantly >1 for normal cells ($P < 0.01$)

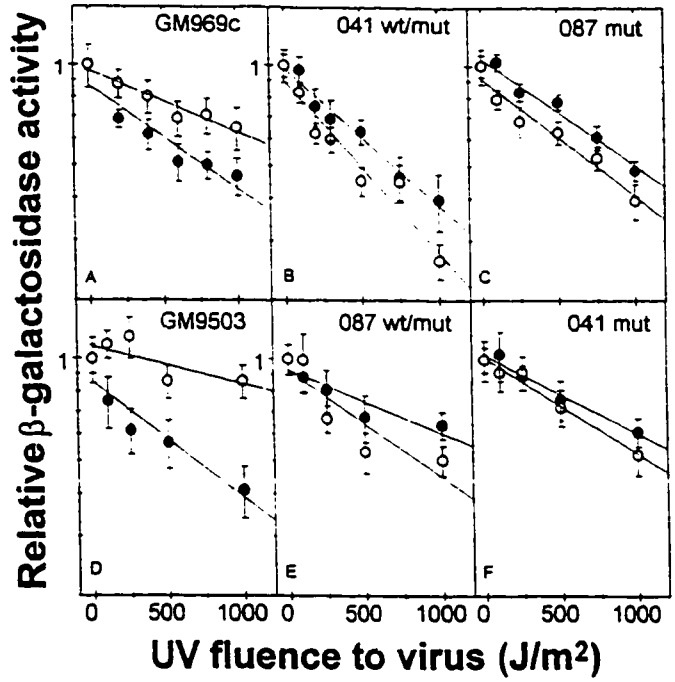

Fig. 1. HSER and UVER of β-gal activity for UV-irradiated Ad5HCMVsp1*lacZ* in normal fibroblasts and LFS cells. HCR of β-gal activity in untreated cells (●) was compared to HCR in cells treated for 30 min at 43°C (○, panels A, B and C) or with 15 J/m² UV (○, panels D,E and F) immediately prior to infection with UV-irradiated or unirradiated Ad5HCMVsp1*lacZ*. Relative β-gal activity is the surviving fraction (SF) of reporter gene activity as measured at OD_{405}. Each point represents the mean ± standard error of at least three determinations from a single experiment. Points were fitted to the straight line exponential equation: $SF = e^{-D/D_0}$. The mean increase in D_0 in response to cell treatment, for a number of experiments on each cell line, is reported in Table II.

Table I. D_0 values for HCR of β-gal activity in normal fibroblasts and LFS cell lines

Cell lines	D_0[a] (J/m²)
Normal fibroblasts[b]	1277 ± 174 (14)
GM8399	791 ± 310 (2)
GM969c	1049 ± 217 (6)
GM9503	1333 ± 206 (4)
423	1861 ± 419 (2)
041wt/mut	1107 ± 153 (5)
087wt/mut	1554 ± 122 (13)
041mut	1420 ± 141 (8)
087mut	1021 ± 262 (5)

[a]Mean D_0 (± SE) determined from several experiments. The number of experiments is given in parentheses.
[b]Mean of all normal diploid fibroblasts (GM969c, GM8399, GM9503 and 423).

Table II. Relative D_0 values for HCR of β-gal activity in HS and UV treated cell lines

Cell lines	HS treated		UV treated	
	15 min[a]	30 min	10 J/m^2	15 J/m^2
Normal[c]	1.33 ± 0.15	1.84 ± 0.26[d]	1.56 ± 0.14[d]	1.76 ± 0.27[d]
041mut	1.17 ± 0.01	0.75 ± 0.09	0.88 ± 0.15	0.95 ± 0.11
087mut	1.11 ± 0.08	0.97 ± 0.13	1.08 ± 0.07	0.98 ± 0.12
041wt/mut	1.17 ± 0.21	0.94 ± 0.15	0.98 ± 0.08	1.00 ± 0.05
087wt/mut	0.90 ± 0.05	0.88 ± 0.03	1.30 ± 0.17	0.91 ± 0.09

[a]Duration of HS treatment at 43°C.
[b]Normal fibroblast cell lines examined were: GM969c and GM8399 for HSER and GM969c, GM9503 and 423 for UVER.
[c]Relative D_0 (D_0 with pretreatment/D_0 untreated) ± standard error for a minimum of three experiments.
[d]Significantly >1 ($P<0.01$) by one-tailed *t* test.

Table III. HS and UV survival of colony forming ability in normal diploid lung epithelial and immortalized LFS fibroblast cell lines

Cell lines	HS 15 min[a]	30 min	UV 10 J/m^2[b]	20 J/m^2
L132	0.86 ± 0.06[c]	0.80 ± 0.05	0.42 ± 0.07	0.06 ± 0.02
041mut	1.00 ± 0.02	0.76 ± 0.06	0.57 ± 0.04	0.09 ± 0.03
087mut	0.92 ± 0.03	0.62 ± 0.13	0.78 ± 0.07	0.19 ± 0.05

[a]Duration of HS treatment at 43°C.
[b]UV (254 nm) fluence in J/m^2.
[c]HS or UV survival ± standard error for a minimum of three experiments.

but not LFS cells. These results suggest that an inducible DNA repair response acting on an active gene is absent in *p53* deficient LFS cells.

Clonogenic survival

To ensure that the absence of UVER and HSER over the range of HS and UV treatments examined was not the result of hypersensitivity to these treatments, we have evaluated the clonogenic survival of 041mut and 087mut cells following UV and HS treatments similar to those giving rise to HSER and UVER of β-gal activity in normal cells (6 and present study). As reported by Ford and Hanawalt (18), UV survival of LFS cells lines was elevated compared to normal diploid L132 cells through this dose range (Table III). Colony survival following HS treatment decreased with duration of exposure in all cell lines to a similar extent (Table III). MDAH087mut and MDAH041mut cells do not appear more sensitive to HS than L132 cells through the HS treatments that elicit HSER β-gal activity in normal cells (6). The absence of HSER and UVER in LFS cell lines does not appear to reflect an increased sensitivity to HS or UV treatments.

Discussion

HCR of the UV-irradiated reporter gene varied among several normal fibroblast strains (Table I). Similarly, HCR of a UV-irradiated chloramphenicol acetyl transferase reporter gene was found to vary among cultured peripheral lymphocytes from different donors (27,28). These results indicate that there is heterogeneity in the ability of cells from different individuals to repair UV induced DNA damage. We were unable to detect a difference between HCR of β-gal activity in untreated normal fibroblasts and untreated LFS cells, heterozygous or hemizygous for mutant *p53*. Mean relative D_0 values for LFS cells fell within the range observed for normal fibroblasts indicating a normal level of repair for the reporter gene construct in untreated LFS cells under the conditions of our assay.

HCR of the UV-damaged reporter gene in normal diploid fibroblasts cells was stimulated by prior HS (30 min at 43°C) or UV (10 and 15 J/m^2) treatment (5,6). This response to cellular stresses was reduced or absent in all LFS cells examined. The absence of UVER and HSER in the LFS fibroblasts heterozygous for a *p53* mutation indicates that these *p53* mutations are dominant with respect to inducible repair of the reporter gene. Since we reported that HSER and UVER are dependent on TCR (5,6), the results presented here suggest that reduced TCR in *p53* deficient cells (17,29,30) stems from a deficiency in an inducible component of TCR. To our knowledge, this is the first demonstration that *p53* or *p53* dependent signalling is required for inducible repair of UV lesions from an actively transcribed gene.

The inability of Ford and Hanawalt (18) to detect a deficiency in TCR in LFS cells is in apparent contrast to results from several laboratories (17,29, this study). The discrepancy observed for TCR in LFS cells may reflect differences in cell treatment since Van Hoffen and coworkers (31) have demonstrated that the relative contribution of TCR and bulk repair is variable depending on cell treatment. Alternatively, the requirement for elimination of replicated DNA in the endonuclease sensitive site assay used by Ford and Hanawalt (18) may create a bias in the examination of repair in G_1/S checkpoint deficient hemizygous LFS cell lines. Elimination of 5-bromodeoxyuridine labelled DNA may result in examination of DNA obtained from a subset of cells which does not reflect the repair phenotype of the entire cell population. Ford and Hanawalt reported a reduction in bulk DNA repair in LFS cell lines, heterozygous and hemizygous for mutant *p53* (18), which is consistent with work from other labs (16,29). Thus it appears that both TCR and bulk NER are affected by *p53*.

P53 is stabilized in response to stalled RNA polymerase II following UV or α-amanitin treatment (32) and accumulates in the nucleus following HS or UV (19). *P53* binds three subunits of the transcription and repair factor TFIIH as well as the CSB gene product required for TCR (17,33). The cyclin dependent kinase-activating kinase, MO15, phosphorylates the C terminal domain of RNA pol II (34–36) and may phosphorylate other cellular proteins such as *p53* at the site of a stalled polymerase (37). A similar kinase activity is stimulated by HS (38) which is consistent with a possible involvement of MO15 in the cellular response to HS. Cellular responses to UV and HS may share common signalling pathways. In this way, HS could confer cellular resistance against subsequent UV exposure (22–24) by stimulation of an inducible *p53* dependent DNA repair response.

Lifetime exposure to UV (39) and decreased NER (27,28), as assessed by HCR of a UV-irradiated reporter gene in peripheral lymphocytes, are risk factors in the development of nonmelanoma skin cancers. Mutations in *p53* are common in both basal cell carcinoma (40) and squamous cell carcinomas (41,42). Since *p53* mutations have been detected in sun exposed skin adjacent to basal cell carcinomas (43) and transgenic mice expressing mutant *p53* are predisposed to squamous cell carcinomas (44), deficiencies in *p53* may facilitate skin cancer development. Interestingly, the *p53* mutations in LFS cell lines exhibiting decreased repair of active genes, Arg^{248}Trp (present study) and the Gly^{245}Asp (17,29), are hotspot mutations in

UV induced nonmelanoma skin cancers (40,41,45). Skin cells carrying *p53* mutations would be expected to be reduced in their ability to remove UV damage upon subsequent sunlight exposure. Genetic alterations required for tumour promotion may be enhanced by the repair deficient phenotype of cells heterozygous for *p53* mutations. This offers a mechanism for the tumour promoting effects of lifetime UV exposure.

DNA damage induced by oxidative stress, ionizing radiation and chemical carcinogens is removed preferentially from active genes (46–49). Therefore, the repair of oxidatively damaged DNA bases in active chromatin is thought to be dependent, in part at least, on the gene products required for TCR of UV lesions (46–48). As oxidative damage to bases occurs as a consequence of normal metabolism (50), a deficiency in the induction of preferential repair of these lesions might contribute to carcinogenesis associated with LFS. Also, TCR of UV- and cisplatin-induced lesions is elevated in several UV (51) and cisplatin resistant cell lines (52); therefore, it would be of clinical importance to determine the contribution of inducible repair of active genes to both radioresistance and drug resistance.

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Chapter 6

Capacity of UV-Irradiated Human Fibroblasts to Support Adenovirus DNA Synthesis Correlates with Transcription-Coupled Repair and is Reduced in SV40-Transformed Cells and Cells Expressing Mutant p53

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Preface

In chapters 4 and 5, evidence was presented which supports a role for p53 in transcription coupled repair of a UV-irradiated reporter gene. Unpublished results (Winrow and Rainbow) suggested that the capacity of UV-irradiated cells to support Ad DNA synthesis reflected the capacity of human or rodent cells to support adenovirus DNA synthesis. This observation was confirmed in this report using a modified assay. The correlation between TCR and the capacity of UV-irradiated cells to support viral DNA synthesis (Winrow and Rainbow, unpublished observations; this chapter) was exploited to assess the role of p53 in TCR. Whereas enhanced reactivation is dependent on repair of UV-induced lesions in a reporter construct, capacity is dependent on repair of genomic DNA and thus provides a rapid method to assess the physiological relevance of enhanced reactivation.

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ABSTRACT

We have examined the capacity of UV-irradiated human diploid fibroblasts to support adenovirus (Ad) DNA synthesis in order to assess repair of UV-damaged DNA. The capacity of UV-irradiated xeroderma pigmentosum group C (XP-C) fibroblasts to support Ad DNA synthesis was similar to that of UV-irradiated normal diploid fibroblasts, following UV exposures of greater than 9 J/m². In contrast, XP-A, Cockayne syndrome groups A and B (CS-A and CS-B) fibroblasts were reduced in their capacity to support Ad DNA synthesis compared to normal diploid fibroblasts following a similar UV treatment. These results demonstrate that the capacity of UV-irradiated fibroblasts to support Ad DNA synthesis correlates with their ability to remove UV-induced DNA damage from active genes by transcription-coupled repair (TCR). We also demonstrate that simian virus 40 (SV40)-transformed human fibroblasts, Li–Fraumeni syndrome (LFS) fibroblasts, heterozygous for mutations in one allele of the p53 gene and immortalized LFS cell lines expressing only mutant p53 are reduced in their capacity to support Ad DNA replication following similar UV treatments. These results suggest that the capacity of UV-irradiated cells to support viral DNA synthesis involves TCR of UV-damaged DNA and is disrupted by SV40 transformation and expression of mutant p53. We propose a model in which p53-dependent TCR regulates p53 stability in response to UV.

INTRODUCTION

The primary DNA lesions induced by far UV radiation are repaired by the nucleotide excision repair (NER)† pathway that can be divided into the subpathways of transcription-coupled repair (TCR) and bulk NER (1). Transcription-coupled repair removes photoproducts rapidly from the transcribed strand of active genes by a process coupled to RNA polymerase II (RNAPII)-dependent transcription (2–4) whereas bulk NER removes UV photoproducts less rapidly from the entire genome. Fibroblasts from individuals with the UV-sensitive disorders xeroderma pigmentosum (XP) and Cockayne syndrome (CS) have been well characterized with respect to their ability to repair UV-induced DNA damage. The CS cells are capable of repairing UV damage by bulk NER but are deficient in TCR (5,6). In contrast, fibroblasts derived from individuals of XP complementation group C (XP-C) retain the ability to repair UV-induced DNA damage by TCR but are deficient in bulk NER (7,8). Other NER-deficient XP cell strains are reduced in their ability to repair UV-damaged DNA by both subpathways (9–13).

Recent evidence indicates that the p53 tumor suppressor may participate in TCR (14–18). We have reported that TCR of a UV-damaged reporter gene is inducible by either heat shock (HS) (17) or UV (18,19) treatment of cells, by a mechanism that is dependent on wild-type p53 (18). Because similar gene products are required for repair of UV, ionizing radiation, oxidative stress and chemical carcinogen-induced DNA damage from actively transcribed genes (20–22) and because p53 mutations are common in neoplasia (23), decreased repair resulting from disruption of p53 function could contribute to genetic instability associated with carcinogenesis. Consistent with this hypothesis, we have previously reported that several human tumor cell lines (17) and Li–Fraumeni syndrome (LFS) fibroblasts expressing mutant p53 alleles (18) are reduced in their capacity to support inducible TCR of a UV-damaged reporter gene.

The capacity of UV-irradiated cells to support replication of nuclear replicating viruses has been used to assess DNA repair (24–28). The capacity of several UV-irradiated CS and XP fibroblast strains to support viral replication is reduced compared to normal fibroblasts (24,26,28). Ultraviolet-induced DNA damage is thought to reduce expression of cellular genes required for viral infection, thus limiting viral replication. We report here that the ability of UV-irradiated cells to support adenovirus (Ad) DNA synthesis correlates with TCR of UV-damaged DNA in human diploid fibroblasts. Furthermore, the capacity of UV-irradiated fibroblasts to support Ad DNA synthesis is reduced in LFS and simian

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†*Abbreviations:* Ad, adenovirus; cdk, cyclin-dependent kinase; CS, Cockayne syndrome; CS-A, CS complementation group A; DHFR, dihydrofolate reductase; HS, heat shock; HSV, herpes simplex virus; LFS, Li–Fraumeni syndrome; NER, nucleotide excision repair; PBS, phosphate-buffered saline; pfu, plaque-forming unit; RNAPII, RNA polymerase II; SV40, simian virus 40; SV40LT, SV40 large tumor antigen; TCR, transcription-coupled repair; TFIIH, transcription factor IIH; XP, xeroderma pigmentosum; XP-A, xeroderma pigmentosum complementation group A.

virus 40 (SV40)-transformed fibroblasts. These results suggest that the capacity of UV-irradiated cells to support viral DNA synthesis is dependent on both TCR and p53 and is disrupted by SV40 transformation.

MATERIALS AND METHODS

Cells and virus. Normal and SV40-transformed normal human, CS and XP fibroblast strains were obtained from the National Institute of General Medical Sciences repository (Camden, NJ). All other SV40-transformed fibroblasts were provided by Dr. R. S. Athwal, Temple University, Philadelphia, PA. The LFS fibroblasts, MDAH041 and MDAH087 (hereafter referred to as 041wt/mut and 087wt/mut), are heterozygous for mutations at codons 184 and 248 (respectively) of p53, whereas their spontaneously immortalized counterparts (041mut and 087mut) express only mutant p53 (29). The LFS cell lines were obtained from Dr. M. A. Tainsky, M.D. Anderson Cancer Center, Houston, TX. Two hundred ninety-three cells were obtained from Dr. F. L. Graham, McMaster University. All cultures were maintained in Eagles α-minimal essential medium supplemented with 10% fetal calf serum together with penicillin (100 mg/mL), streptomycin (100 mg/mL) and amphotericin B (250 ng/mL) (Gibco BRL).

Ad5–lacZ5 is a recombinant Ad5-derived virus with the *lacZ* gene inserted in place of early region 3. This construct replicates in a wide range of human cell lines (30). Virus was replicated and titered in 293 cells as previously described (31).

Capacity of UV-irradiated cells to support viral DNA synthesis. Adenovirus DNA synthesis in UV-irradiated cells was quantified as previously reported (32). Briefly, cells were seeded at a density of either 1.9×10^4 cells per well in 96 well microtiter dishes or 1×10^5 cells per well in 24 well dishes (Falcon, Franklin Lakes, NJ). Cells were allowed to adhere 24 h prior to UV treatment. Medium was replaced with either 40 μL (96 well dishes) or 200 μL (24 well dishes) phosphate-buffered saline (PBS: 140 m*M* NaCl, 2.5 m*M* KCl, 10 m*M* Na_2HPO_4 and 1.75 m*M* KH_2PO_4) and cells were irradiated at 1 J/m²/s (J-225 shortwave UV meter, Ultraviolet Products, San Gabriel, CA) at 254 nm (General Electric germicidal lamp G8T5). The PBS was immediately replaced with the same volume of *Ad5–lacZ5* in PBS at a multiplicity of infection of 40 plaque-forming units (pfu)/cell. Virus was allowed to adsorb 90 min at 37°C prior to addition of either 200 μL (96 well dishes) or 1 mL (24 well dishes) of growth medium. Following 48 h, cells were lysed (1–2 h in 1 mg/mL proteinase K, 1% sodium dodecyl sulfate, 50 m*M* NaCl, 15 m*M* sodium citrate, pH 7.0), denatured (30 min at 37°C in 0.5 *N* NaOH, 25 m*M* EDTA), slot blotted to Gene Screen Plus (Dupont cat. no. NEF-976), hybridized with ^{32}P-labeled Ad2 DNA and quantified with a phosphorimaging system (model 425B, Molecular Dynamics) as previously reported (32).

Cells that were UV-irradiated in 96 well dishes consistently had a greater capacity to replicate Ad DNA compared to cells treated similarly in 24 well dishes, suggesting that the UV fluence was affected by the surface area of the cell monolayers. A number of experiments with GM637f cells, using both 96 and 24 well dishes concurrently, were performed to obtain a correction factor to allow comparison of results generated in 96 well dishes to other published data. Capacity curves were fitted to the linear quadratic function (ln [relative viral DNA] = $-[ax + bx^2 + c]$) and solved for the UV fluence required to reduce viral DNA synthesis to 37%. The mean ratio of D_{37} values (mean [D_{37} in 96 well dish/D_{37} in 24 well dish] = 1.60 ± 0.02) was used to correct UV fluence in all experiments.

RESULTS

The capacity of UV-irradiated cells to support Ad DNA synthesis correlates with TCR

The UV-irradiated normal, CS-A, CS-B, XP-A and XP-C fibroblasts were assessed for their ability to support Ad DNA synthesis. The mean responses of normal (four strains), XP-C (three strains), XP-A (GM5509), CS-A (GM1856) and CS-B (GM739) fibroblast strains are presented in Fig. 1A.

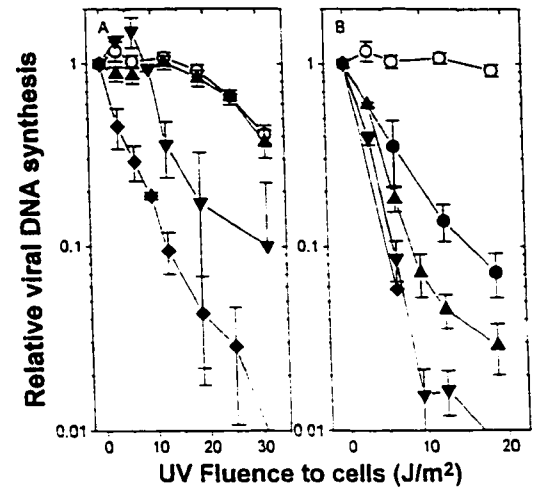

Figure 1. The capacity of UV-irradiated fibroblasts to support Ad DNA synthesis. (A) Representative capacity curves for UV-irradiated normal (○—four strains), XP-C (▲—three strains), CS-A (▼—GM1856) and XP-A (♦—GM5509b) fibroblasts are presented. The capacity of UV-irradiated CS-A and XP-A strains is reduced compared to normal diploid fibroblasts. (B) Furthermore, all SV40-transformed fibroblasts examined, normal (●), XP-C (▲) CS-A (▼) and CS-B (♦) cell lines, are reduced in their capacity to support Ad DNA synthesis compared to normal diploid fibroblasts (○). Each point represents the mean ± standard error of several independent experiments performed in triplicate. Mean D_{37} values for individual fibroblast strains and the number of experiments for each cell type are indicated in Table 1.

All curves were fitted to the linear quadratic function (ln [relative viral DNA] = $-[ax + bx^2 + c]$). This equation was used to determine the UV fluence required to reduce Ad DNA synthesis to 37% (D_{37}) for each experiment. Mean D_{37} values for XP-C fibroblasts were similar to values obtained for normal diploid fibroblasts (Table 1), whereas mean D_{37} values for CS-A, CS-B and XP-A cell strains were significantly reduced compared to those obtained for normal fibroblast strains (Table 1). Thus the capacity of UV-irradiated fibroblasts to support Ad DNA synthesis as assessed by D_{37} correlates with TCR.

However, it can be seen that UV-irradiated XP-C fibroblasts show a reduced capacity for Ad DNA synthesis compared to CS fibroblasts at relatively low UV fluences of 3 and 6 J/m² (Fig. 1A, Table 2), whereas CS cell strains are reduced compared to normal and XP-C strains in their capacity to support Ad DNA synthesis following UV exposure at fluences of 12 J/m² and greater (Fig. 1A, Table 2). The XP-A fibroblasts were reduced compared to normal fibroblasts for all UV exposures examined (Fig. 1A). These results indicate that disruption of either TCR and/or bulk NER lead to fluence-specific differences in the capacity of UV-irradiated fibroblasts to support Ad DNA replication.

SV40 transformation reduces the capacity of UV-irradiated cells to support Ad DNA synthesis

We have assessed the effect of SV40 transformation on the capacity of UV-irradiated normal, CS-A, CS-B and XP-C cell lines to support Ad DNA synthesis. The capacity of all SV40-transformed cell lines to support Ad DNA synthesis following UV exposure was reduced compared to that of

Table 1. Capacity of UV-irradiated normal, XP and CS fibroblast strains to support Ad DNA synthesis

Clinical phenotype	Cell line	Mean D_{37} ± SEM	No. of experiments
Normal	GM9503	30.20 ± 3.75	5
	GM969c	43.75 ± 4.38	4
	GM37f	37.94 ± 1.56	3
	GM38A	37.90 ± 5.10	4
	Mean of normals	36.03 ± 2.37	16
XP-C	GM677 (XP2BE)	32.93 ± 1.78	4
	GM30a (XP3BE)	33.85 ± 3.92	2
	GM10881 (XP1BE)	28.16 ± 6.61	2
	Mean of XP-C	32.95 ± 2.27	8
CS-A	GM1856B (CS3BE)	13.23 ± 6.61*	3
CS-B	GM739A (CS1AN)	17.16 ± 4.46*	2
XP-A	GM5509b (XP12BE)	5.70 ± 0.78*	6
SV40 transformed	GM637f (normal)	6.38 ± 0.71*	7
	XP-C	4.29 ± 1.02*	5
	CSA (CS3BE)	2.73 ± 0.54*	5
	CSB (CS1AN)	2.01 ± 0.20*	2
LFS	041wt/mut	20.19 ± 5.88*	4
	041mut	11.90 ± 1.18*	6
	087wt/mut	23.81 ± 3.30*	6
	087mut	13.41 ± 4.25*	4

*D_{37} values are significantly reduced compared to mean D_{37} values for XP-C fibroblasts ($P < 0.025$).

their nontransformed counterparts (Fig. 1B, Table 1). The SV40 transformation disrupts the capacity of UV-irradiated fibroblasts to support Ad DNA synthesis.

UV-irradiated LFS cells are reduced in their capacity to support Ad DNA synthesis

The capacity to replicate Ad DNA was also assessed in UV-irradiated LFS cells heterozygous and hemizygous for p53 mutations. The UV-irradiated 041wt/mut, 087wt/mut, 041mut and 087mut cells were decreased in their capacity to replicate Ad DNA compared to similarly treated normal and XP-C fibroblasts (Fig. 2). Furthermore, immortalized LFS cell lines expressing only mutant p53, were reduced in their capacity to a greater extent than heterozygous LFS fibroblasts, indicating that either the absence of the wild-type allele or subsequent alterations associated with immortalization may contribute to a reduction in the ability of UV-irradiated fibroblasts to support Ad DNA synthesis. Mean D_{37} values for all LFS fibroblasts and cell lines were significantly reduced compared to mean D_{37} values for normal fibroblasts ($p < 0.025$, one-tailed t-test) (Table 1).

DISCUSSION

Using a series of NER-deficient human fibroblast strains, we have demonstrated that the capacity of UV-irradiated fibroblasts to support Ad DNA synthesis correlates with TCR for UV exposures of greater than 9 J/m². XP-C fibroblasts, which retain the ability to repair UV-induced DNA damage from active genes, were capable of replicating Ad DNA to a similar extent as normal diploid fibroblasts following UV exposures greater than 9 J/m². Because Ad DNA replication

Table 2. Capacity of XP-C, CS-A and CS-B fibroblasts to support Ad DNA synthesis following UV fluences of 6 and 12 J/m²

Cell type	Capacity (6 J/m²)	Capacity (12 J/m²)
XP-C (3 strains)*	0.87 ± 0.09†	1.02 ± 0.09
CS-A (CS3BE)	1.51 ± 0.29	0.36 ± 0.12‡
CS-B (CS1AN)	1.19 ± 0.12	0.76 ± 0.03‡

*Strains: XP1BE, XP2BE, XP3BE.
†Significantly less than the capacity of similarly treated CS-A or CS-B fibroblasts, one-tailed t-test ($P < 0.01$).
‡Significantly reduced compared to the mean D_{37} for XP-C fibroblasts at the indicated UV fluence, one-tailed t-test ($P < 0.01$).

requires host gene products (33), UV-induced lesions in critical cellular genes may reduce the rate of Ad DNA synthesis in UV-irradiated fibroblasts. It has previously been reported that UV-irradiated XP-C fibroblasts were capable of supporting herpes simplex virus (HSV) plaque formation to a similar extent as normal fibroblasts, whereas XP-A, XP-B and XP-D fibroblasts were not (24). Together, these results suggest that persistent DNA damage in active cellular genes inhibits replication of both Ad and HSV.

Previous work from our laboratory suggests that TCR of a UV-damaged reporter gene is inducible by UV and HS treatment to the cell (17–19). It is therefore possible that the magnitude of the UV fluence given to the cell may affect the relative contribution of bulk repair and TCR of cellular DNA. Because there is a clear correlation between TCR in cellular DNA and the capacity of UV-irradiated cells to support viral DNA synthesis, this assay may be a valuable approach to assess the relative rates of TCR in cellular DNA at a variety of UV fluences to the cell.

The detailed results obtained for the capacity curves of CS-A, CS-B, XP-C and normal fibroblast strains at various

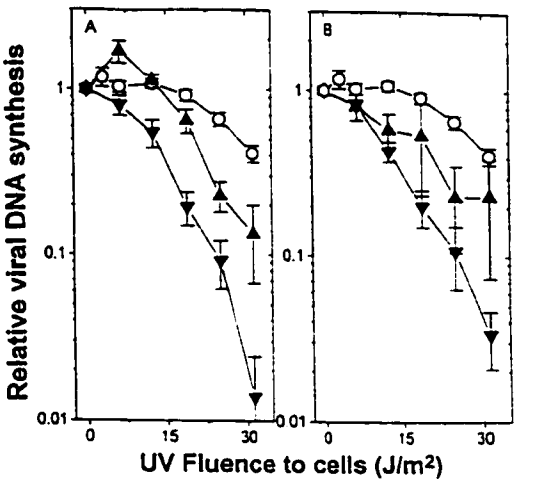

Figure 2. The capacity of UV-irradiated LFS fibroblasts and immortalized sublines to support Ad DNA synthesis. The UV-irradiated LFS fibroblasts, 087 (A) and 041 (B), were assessed for their capacity to support Ad DNA synthesis. Both LFS heterozygous fibroblasts (▲) and hemizygous immortalized sublines (▼) were reduced compared to normal diploid fibroblasts at UV fluences greater than 9 J/m² (○). Each point represents the mean ± standard error for several independent experiments performed in triplicate and the number of experiments for each cell type is indicated in Table 1.

UV fluences are consistent with a fluence-dependent variation in the relative contribution of TCR and bulk NER in cellular DNA, as previously reported (6). At low UV exposures, we did not observe a deficiency in the capacity of UV-irradiated CS cells to support Ad DNA synthesis, consistent with the hypothesis that bulk NER is sufficient to repair transcribed genes at low UV fluence. In contrast, at UV fluences greater than 9 J/m^2, CS strains were reduced in their capacity to support Ad DNA synthesis compared to both normal and XP-C fibroblast strains. Because the capacity of UV-irradiated normal and XP-C fibroblasts are similar at higher UV fluences, it appears that cellular capacity only reflects TCR at UV fluences that are sufficient to induce TCR (18,19). The apparent sensitivity of XP-C fibroblasts at low UV fluence (3 and 6 J/m^2) and subsequent recovery at higher UV fluence is consistent with previous reports from our laboratory that TCR of a UV-damaged reporter gene is inducible by UV fluences to the cell of between 10 and 25 J/m^2 (18,19). Similar UV exposures are typically used to assess TCR of cellular genes (3,5,8,11,34,35), indicating that TCR is operative under these conditions. Low-dose hypersensitivity and subsequent recovery at higher doses has been reported for both human and rodent cell lines following ionizing radiation at doses below 1 Gy (36,37) and recovery from low-dose hypersensitivity has been suggested to represent inducible DNA repair (38,39).

Adenovirus encodes two tumor antigens: E1A, which leads to increased p53 accumulation, and E1B, which binds to p53 and inhibits p53-dependent apoptosis (40). Two lines of evidence suggest that the correlation between TCR of UV-damaged DNA and the capacity of UV-irradiated cells to support Ad DNA synthesis is not affected by E1A and/or E1B expression of the infecting Ad. First, rapid preferential repair of the template strand of the dihydrofolate reductase (DHFR) gene was observed in Ad-transformed human cells that express the Ad E1 region (41), indicating that neither E1A nor E1B expression disrupts TCR. Second, the capacity of XP-C fibroblasts to support HSV plaque formation was similar to that of normal fibroblasts for UV irradiation immediately prior to infection, whereas the capacity of similarly treated UV-irradiated XP-A, XP-B and XP-D fibroblasts was substantially reduced compared to that in normal fibroblasts (24). In contrast to SV40, Ad, human papilloma virus and hepatitis B virus, no HSV-encoded protein has been demonstrated to disrupt p53 function (42). Because the results presented in this work are similar to the results reported for the capacity of UV-irradiated XP cell strains to support HSV plaque formation (24) and because TCR has been detected in a cell line stably expressing E1A and E1B (41), it is unlikely that E1A and/or E1B expression significantly affects the capacity of UV-irradiated fibroblasts to support Ad DNA synthesis.

The precise role of p53 in NER is as yet unclear; however, there is evidence to support involvement of p53 in both TCR (15–18) and bulk NER (14,16,35,43). The p53 protein accumulates in response to both DNA damage in actively transcribed genes and inhibition of transcription by α-amanitin, suggesting that stalled RNAPII may stimulate the accumulation of p53 in response to UV-damaged DNA (44,45). Stabilization of p53 may involve a phosphorylation signal mediated by the cyclin-dependent kinase (cdk)-activating kinase at the site of stalled RNAPII (15,46–50). Increased activity and/or accumulation of p53 would then be expected to facilitate DNA repair (14,18), induce G1 arrest (51) and/or induce apoptosis (45). Because the capacity of UV-irradiated LFS fibroblasts and immortalized LFS sublines to support Ad DNA synthesis is reduced compared to that of UV-irradiated normal and XP-C fibroblasts, the results of the present study support a role for wild-type p53 in the repair of active cellular genes (15–18). The p53 protein was originally identified by its association with the SV40 large T antigen (SV40LT) (52). In this study we showed that UV-irradiated SV40-transformed cells showed a reduced capacity for Ad DNA synthesis compared to that of their nontransformed fibroblast counterparts. These results are consistent with a disruption of DNA repair in SV40-transformed human cells as reported previously (17,53,54).

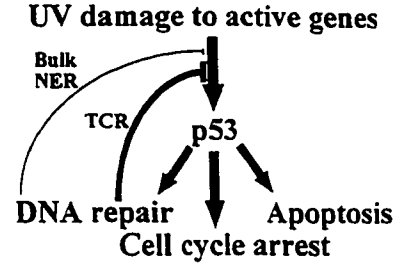

Figure 3. Inducible TCR regulates p53 stability following UV exposure. Persistent UV-induced DNA damage in active genes leads to stabilization of p53 (44,45) and induction of apoptosis (45). Stimulation of TCR (18,19) would contribute to the rapid removal of lesions from active genes, thus reducing upstream signalling through p53. Disruption of inducible TCR would result in a lower threshhold for DNA damage-induced cell cycle arrest and apoptosis (45) in cells retaining the ability to arrest or undergo apoptosis in response to DNA damage. In contrast, inducible bulk NER (14) removes DNA damage less rapidly from active genes and would not be expected to contribute as greatly to regulation of p53 stability. The relative contribution of TCR and bulk repair is indicated by the intensity of the corresponding arrows. Arrowheads indicate positive interactions, whereas perpendicular bars represent negative interactions.

Because persistent UV-induced DNA damage in active genes leads to p53 accumulation (44,45) and apoptosis (45), p53-dependent upregulation of TCR may be considered as a negative feedback mechanism that regulates p53 stability following UV exposure. This scheme is diagrammed in Fig. 3. Increased activity and/or stability of p53 resulting from transcription-blocking DNA damage could result in apoptosis (45), cell cycle arrest (51) or inducible repair of UV damage from both transcribed genes (18) and nontranscribed DNA (14). Transcription-coupled repair would result in a rapid reduction in the amount of transcription-inhibiting DNA damage, whereas stimulation of bulk repair would remove transcription-blocking lesions at a much slower rate. This model would predict that removal of transcription-blocking lesions in XP-C fibroblasts would result in normal induction of p53 in response to UV (44,45). Conversely, CS cells would be expected to have persistent transcription-blocking DNA damage, leading to prolonged p53 stabilization and apoptosis (45). This outcome is consistent with the absence of sunlight-induced skin cancers in CS individuals.

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Chapter 7

Ultraviolet- and Heat-Shock-Enhanced Reactivation of an Ultraviolet-damaged reporter gene is frequently attenuated in human cancer cell lines.

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Preface

In chapters 4, 5 and 6, evidence was presented which is highly suggestive that transcription coupled repair of UV-induced DNA damage is inducible through a p53 dependent mechanism. In addition, heat shock enhanced reactivation (HSER) was reduced or absent in all but 1 transformed cell line examined (chapter 4). It was of interest to determine if HSER and UV enhanced reactivation could be detected in tumour cell lines expressing wildtype p53. A series of histologically diverse, wildtype p53 expressing tumour cell lines were obtained and assessed for their capacity to support HSER and UVER of reporter gene activity.

Abstract

We have previously reported that transcription coupled repair of ultraviolet (UV)-damaged DNA is inducible in response to either UV or moderate heat-shock through a p53 dependent mechanism (*Carcinogenesis*, 18, 245-249). Here we report that both UV- and HS-inducible DNA repair is absent in a series of established tumour cell lines. HCT116, U20S, ZR-75-1, SK-N-SH, IMR-32 and HeLa cells all express wildtype p53 yet lack inducible TCR suggesting that genetic alterations other than p53 mutations lead to disruption of UV- and HS-inducible DNA repair and further suggests that disruption of this inducible DNA repair response is more frequent than p53 mutations.

Introduction

The central role of the p53 tumour suppressor gene product in protection against malignant transformation is highlighted by both the elevated incidence of p53 mutations in sporadic cancers (Hollstein, 1991) and the elevated incidence of malignancy in individuals carrying germline p53 mutations (Malkin, 1994). In addition to the well established role for p53 in mediating cell cycle arrest (Kastan et al, 1991) and apoptosis (Lowe et al, 1993) following genotoxic stress, several authors have proposed both direct and indirect roles for p53 in the repair of UV-induced DNA damage (Smith et al, 1995; Wang et al, 1995; Ford and Hanawalt, 1995; Mirzayans et al, 1996; McKay et al, 1997a; McKay et al, 1997b).

UV-induced lesions are removed by nucleotide excision repair (NER) (reviewed in Friedberg et al, 1995) which can be further subdivided into transcription coupled repair (TCR) of DNA damage from the template strand of active genes (Bohr et al, 1985; Mellon

et al, 1987) and global genome repair (GGR) which removes lesions less rapidly from the entire genome. TCR is thought to involve recruitment of DNA repair enzymes to the site of DNA damage in response to RNA polymerase II (RNAPII) stalled at a lesion (reviewed in Hanawalt, 1994). We have previously demonstrated that either HS or UV treatment of cells leads to stimulation of repair of a UV-damaged reporter gene introduced into normal diploid human cells using a recombinant adenovirus (Francis and Rainbow, 1995; McKay and Rainbow, 1996; McKay et al, 1997a). We demonstrated the requirement for the CS-A, XP-A, XP-D and p53 gene products but not the XP-C gene product (Francis and Rainbow, 1995; McKay and Rainbow, 1996; McKay et al, 1997a; Francis and Rainbow, unpublished results). These data suggest that the recruitment of DNA repair proteins to sites of transcription blocking UV lesions is not passive but is inducible through a p53 dependent mechanism (Francis and Rainbow, 1995; McKay and Rainbow, 1996; McKay et al, 1997a; McKay et al, 1997b; Francis and Rainbow, submitted).

TCR deficient fibroblasts accumulate p53 following lower UV fluences than TCR proficient fibroblasts indicating that persistent UV-induced DNA damage in active genes contributes to p53 accumulation and apoptosis (Yamaizumi and Sugano, 1995; Ljungman and Zhang, 1996). An attractive model is emerging in which inducible p53-mediated TCR negatively regulates p53 stability in response to UV exposure thus providing protection against UV induced apoptosis (Ljungman and Zhang, 1996; McKay et al, 1997b). Cisplatin induced DNA damage is also repaired by TCR and some cisplatin resistant cell lines efficiently remove cisplatin adducts from active genes suggesting that enhanced repair may contribute to drug resistance (Zhen et al, 1992). As p53 mutations are not universal in tumour cells, it

was of interest to assess inducible repair in a variety of tumour cell lines including wildtype p53 expressing cells. We report here that UVER and HSER are reduced in most tumour cell lines examined, including wildtype p53 expressing cells. These results suggest that additional genetic alterations in tumour cell lines, in addition to p53 mutations, result in disruption of DNA repair.

Materials and Methods

Cells and viruses

Normal human fibroblast strains were obtained from the National Institute of General Medical Sciences repository (Camden, NJ). HCT116, U20S, ZR-75-1, IMR-32 and SK-N-SH cells all express wild type p53 (Waldman et al, 1995; Kuerbitz et al, 1992; Gudas et al, 1995; Davidoff et al, 1992). U20S and 293 cells were obtained from Drs P. Whyte and F.L. Graham, McMaster University, respectively. All other tumour cells and L132 lung epithelial cells were obtained from the American Tissue Culture Collection cell repository (Rockville, Md).Cultures were maintained in Eagles α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum together with penicillin (100 μg/mL), streptomycin (100 μg/mL) and amphotericin B (250 ng/mL) (Gibco BRL). Virus was replicated and titred in 293 cells as previously described (Graham and Prevec, 1991).

Enhanced reactivation of β-gal activity

Enhanced reactivation of β-gal activity was assessed as previously described (McKay and Rainbow, 1996). Briefly, $1.9x10^4$ cells/well were seeded in 96 well microtitre plates

(Falcon, Lincoln Park, NJ), 24 hrs prior to HS (43 ± 0.25 °C) or UV (with a germicidal lamp, General Electric model G8T5, at 254 nm, 1 J/m^2/sec, McKay et al, 1997b) treatment. Immediately following HS or UV treatment, cells were infected (90 min at 37°C) with unirradiated or UV-irradiated (fluence rate of 2 J/m^2/sec) Ad5HCMVsp1*lacZ* at a multiplicity of infection between 10 and 25 pfu/cell. Ad5HCMVsp1*lacZ* is a nonreplicating Ad5 derived virus expressing *lacZ* under control of the human cytomegalovirus immediate early promoter (Morsy et al, 1993). Infected cells were harvested following 40 to 44 hours as previously described. The OD_{405} (EL340 plate reader, Biotek Instruments) was determined at several times following addition of O-nitrophenol β-D-galactopyranoside (0.1% w:v).

Clonogenic survival

Cells were seeded at between 500 and 10000 cells per 100 mm dish for primary fibroblasts or per well of 6 well dishes for immortalized cells. Cells were allowed to adhere between 4 and 8 hrs prior to UV irradiation in either 2 mL (100 mm dishes) or 0.5 mL (6 well dishes) of PBS. Colonies were stained (0.5% methylene blue in 70% ethanol) either 2 weeks (primary fibroblasts) or 7 to 10 days (immortalized cells) following UV treatment.

Results

Tumour derived cell lines lack HSER and UVER of a UV-damaged reporter gene.

Normal diploid fibroblasts, normal lung epithelial cells and several tumour cell lines were infected with either UV-irradiated or unirradiated replication defective Ad (Ad5HCMVsp1*lacZ*) immediately following UV, HS or mock treatment of cells. As

previously reported (Francis and Rainbow, 1995; McKay et al, 1996; McKay et al, 1997a), pretreatment of normal diploid cells with either UV or HS resulted in an increase in HCR of β-gal activity. In contrast, no increase in HCR of β-gal activity was observed in the 6 tumour cell lines examined following UV (figure 1 B and C and data not shown). Seven of eight tumour cell lines were also deficient in HSER of the UV damaged reporter construct (figure 1E and F and data not shown). Survival curves were fit to the straight line exponential equation ($\ln SF = -D/D_0$) where SF is the surviving fraction of reporter gene activity. The relative increase in D_0 (D_0 treated/D_0 untreated) was determined for each experiment and the mean increase in D_0 for each cell line is presented in table 1. As previously reported (McKay and Rainbow, 1996; McKay et al, 1997a), a significant increase in D_0 was observed following UV treatments of 12 J/m^2 (table 1) and HS treatments of 30 min at 43°C (table 1) for normal diploid cells. Whereas no tumour cell lines were found to express UVER following UV fluences between 6 and 18 J/m^2 (table 1 and data not shown), HCR of β-gal activity in HT29 cells was stimulated by HS treatment of cells prior to viral infection (table 1, McKay and Rainbow, 1996). Although UVER and HSER are related functions (McKay et al, 1997a), these results suggest that they are separable in HT29 cells.

Wildtype p53 status correlates with UV sensitivity.

Clonogenic survival of NDF and tumour cell lines was determined for UV treatments up to 30 J/m^2. As seen in figure 2A, substantial variation was observed in the sensitivity of tumour cell lines to UV irradiation as assessed by clonogenic survival. The sensitivity of cell lines correlates with wildtype p53 in the established tumour cell lines examined. To further

Figure 1. Ultraviolet and heat shock enhanced reactivation of a UV damaged reporter gene is reduced in wildtype p53 expressing tumour cell lines. L132 (A), ZR-75-1 (B), GM969c (C) and SK-N-SH (D) cells were UV irradiated (12 J/m^2, circles in A and B), heat treated (30 min at 43°C, circles in C and D) or mock irradiated (squares) immediately prior to infection with a UV-irradiated or unirradiated Ad5HCMVsp1*lacZ*. β-gal activity was assessed at 48 hrs following infection and expressed as the proportion of reporter gene activity relative to that obtained from the unirradiated reporter construct. Each point represents the mean (± standard error) for a minimum of 3 determination from a single experiment. Pooled results from several independent experiment are presented in table 1.

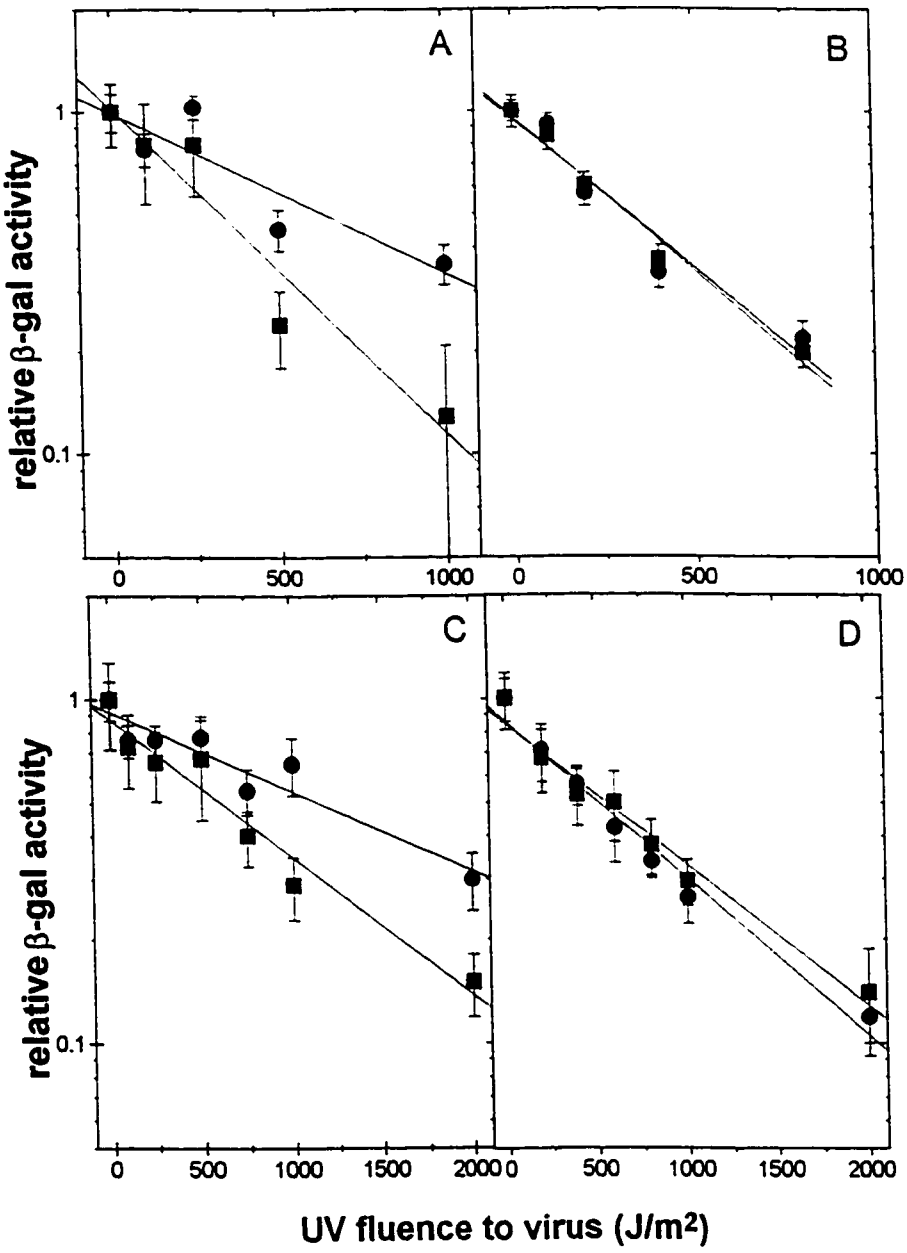

A
B
C
D
relative β-gal activity
relative β-gal activity
1
0.1
1
0.1
0
500
1000
0
500
1000
0
500
1000
1500
2000
0
500
1000
1500
2000
UV fluence to virus (J/m2)

Table 1. Comparison of enhanced reactivation and clonogenic survival.

cell line/strain	UVER (rel D_o)[A]	HSER (rel D_o)[A]	colony survival (D_{37})
NDF (+/+)[B]	2.3 ± 0.6 (9)[C]	1.7 ± 0.2 (8)	8.3 ± 0.9 (12)
L132 (+/+)	1.7 ± 0.3 (5)	1.4 ± 0.1 (16)	9.8 ± 1.1 (7)
HeLa (+/+)	ND[D]	0.9 ± 0.1 (6)	10.1 ± 1.3 (3)
IMR-32 (+/+)	0.8 ± 0.1 (4)	0.6 ± 0.2 (3)	6.7 ± 1.0 (2)
SK-N-SH (+/+)	1.0 ± 0.3 (2)	0.7 ± 0.2 (2)	4.9 ± 0.3 (4)
U20S (+/+)	1.0 ± 0.1 (4)	0.8 ± 0.3 (7)	6.9 ± 0.6 (5)
ZR-75-1 (+/+)	1.0 ± 0.0 (2)	0.8 ± 0.2 (3)	ND
HCT116 (+/+)	0.9 ± 0.1 (4)	0.9 ± 0.1 (6)	13.2 ± 1.3 (5)
HT29(-/n)	0.9±0.2 (2)	1.6 ± 0.3 (5)	16.0 ± 1.6 (4)
SKOV-3 (n/n)	ND	1.0 ± 0.1 (8)	13.7 ± 0.9 (2)

[A] Relative D_o (D_o treated/D_o untreated) for UV treatments of 12 J/m^2 and HS treatments of 30' at 43°C.

[B] p53 genotype. +, - and n indicate wildtype, mutant and null alleles, respectively.

[C] The number of experiments performed.

[D] ND indicates not determined.

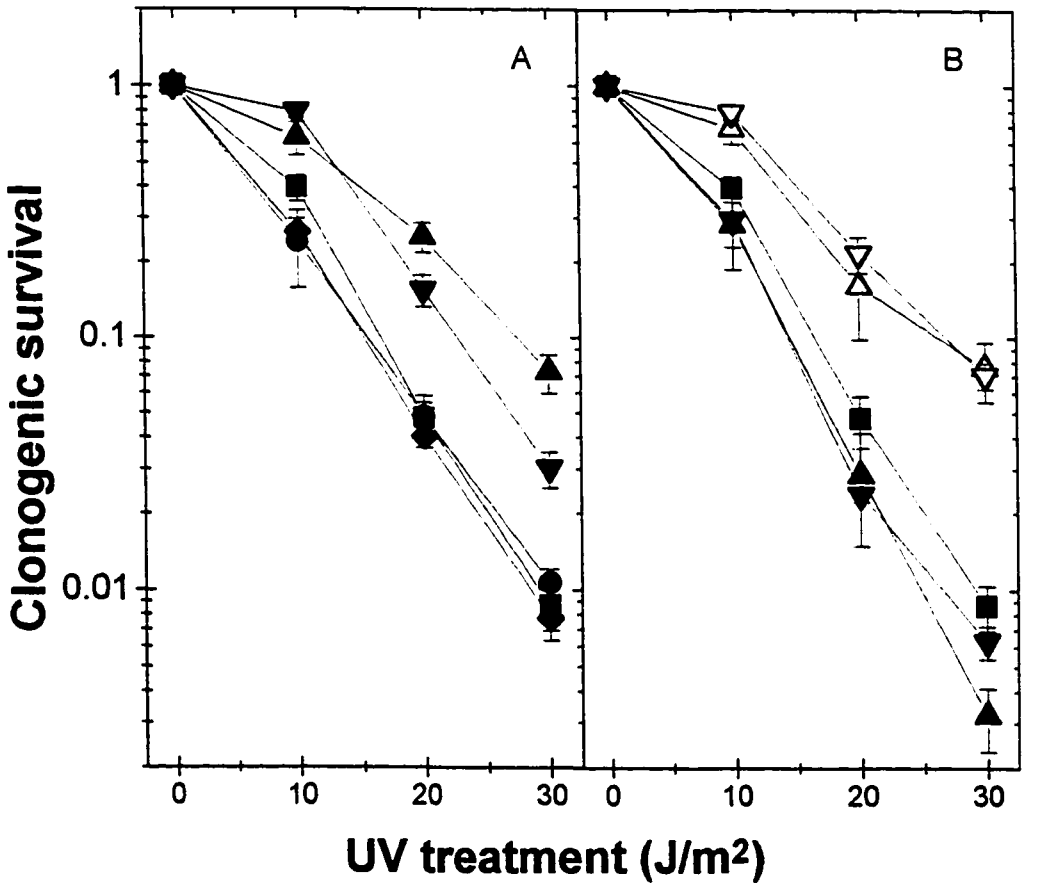
test this relationship, clonogenic survival was assessed in Li-Fraumeni syndrome (LFS) fibroblasts, heterozygous for mutant p53 and hemizygous immortalized sublines expressing only mutant p53 (figure 2B). Heterozygous cell lines displayed similar UV sensitivity to NDF whereas hemizygous sublines were significantly more resistant to UV exposure. All curves were fit to the linear quadratic function $[\ln(SF)=-(ax+bx^2+c)]$ and solved for the UV dose required to reduce survival to 37% (D_{37}). Pooled data is presented in table 1. Wildtype p53 expressing tumour cell lines (except HCT116), NDF and heterozygous LFS cells were similarly sensitive to UV compared to hemizygous LFS and mutant p53 expressing tumour cells. Clearly the presence of wildtype p53 confers sensitivity to UV irradiation.

Discussion

We have previously reported that enhanced reactivation of reporter gene activity in response to either HS or UV treatment and the capacity of UV irradiated cells to support Ad DNA synthesis are dependent on both TCR and p53 (Francis and Rainbow, 1995; McKay and Rainbow, 1996; McKay et al, 1997a; McKay et al, 1997b). Other authors have also suggested that TCR (Wang et al, 1995; Mirzayans et al, 1996) and GGR (Ford and Hanawalt, 1995; Mirzayans et al, 1996; Li et al, 1997) require wildtype p53. We demonstrate here that disruption of HSER and UVER is common in established tumour cell lines. Absence of these inducible DNA repair responses was observed in wildtype p53 expressing cell lines indicating that genetic alterations other than mutational inactivation of p53 inhibit HSER and UVER of reporter gene activity.

Only a limited number of tumour cell lines have been assessed for their capacity to

Figure 2. Wildtype p53 correlates with sensitivity to UV. (A) The UV sensitivity of SKOV-3 (inverted triangles), HT29 (triangles), IMR-32 (circles), U20S (diamonds) and normal diploid fibroblasts (GM38A-squares) were assessed by a standard clonogenic survival assay. (B) MDAH041 (triangles) and MDAH087 (inverted triangles) heterozygous for mutations in p53 (closed symbols) and immortalized sublines (open symbols) were assessed by clonogenic survival. The normal diploid fibroblast line from A is included for comparison (squares). Cell lines lacking wildtype p53 (HT29, SKOV-3 and hemizygous LFS cells) are significantly more resistant UV treatment than cells expressing at least 1 wildtype p53 allele (GM38A, IMR-32, SK-N-SH, U20S and heterozygous LFS fibroblasts). Each point represents the mean of several independent experiments performed in triplicate.


A
B
1
0.1
0.01
Clonogenic survival
0
10
20
30
UV treatment (J/m²)

support TCR of UV-induced DNA damage. SW480, HEC2-4 and HCT116 3-6 (HCT116 with chromosome 3) are capable of TCR whereas HCT116, LoVo, HEC1A and HEC59 did not exhibit strand selective repair of UV induced DNA damage (Mellon et al, 1996; Leadon and Avrutskaya, 1997). TCR has also been reported to be reduced in several HeLa derived sublines (Evans et al, 1996). TCR of UV-induced DNA damage appears to be commonly disrupted in tumour cell lines. This is consistent with disruption of UVER and HSER of a UV-damaged reporter gene in tumour cells (McKay and Rainbow, 1996; this study).

Clonogenic survival of these tumour cell lines did not correlate with their ability to support inducible DNA repair. Survival more accurately reflected the status of p53 in these cells. Wildtype p53 expressing tumour cell lines (except HCT116) and heterozygous LFS fibroblasts were as UV sensitive as NDF. Several mechanisms to disrupt p53 function, in addition to p53 mutations, have been proposed. Some of these such as HPV-E6 expression in HeLa cells (Wrede et al, 1991), mdm-2 overexpression in U20S cells (Florenes et al, 1994) and nuclear exclusion in IMR-32 and SK-N-SH cells (Moll et al, 1996) may disrupt p53 function and thus affect UVER and HSER in these cell types. However, the correlation between UV sensitivity and the presence of wildtype p53 suggests function(s) of p53 is (are) retained in these cells, likely permanent cell cycle arrest and/or apoptosis.

Despite the requirement for wildtype p53 for UVER and HSER (McKay et al, 1997a), it appears that the presence of wildtype p53 is not in itself sufficient for either UVER or HSER. As TCR of oxidative base damage requires similar gene products to TCR of UV-induced DNA damage (Leadon and Cooper, 1993; Cooper et al, 1997; Leadon and Avrutskaya, 1997), disruption of TCR may contribute to the malignant phenotype of tumour

cells.

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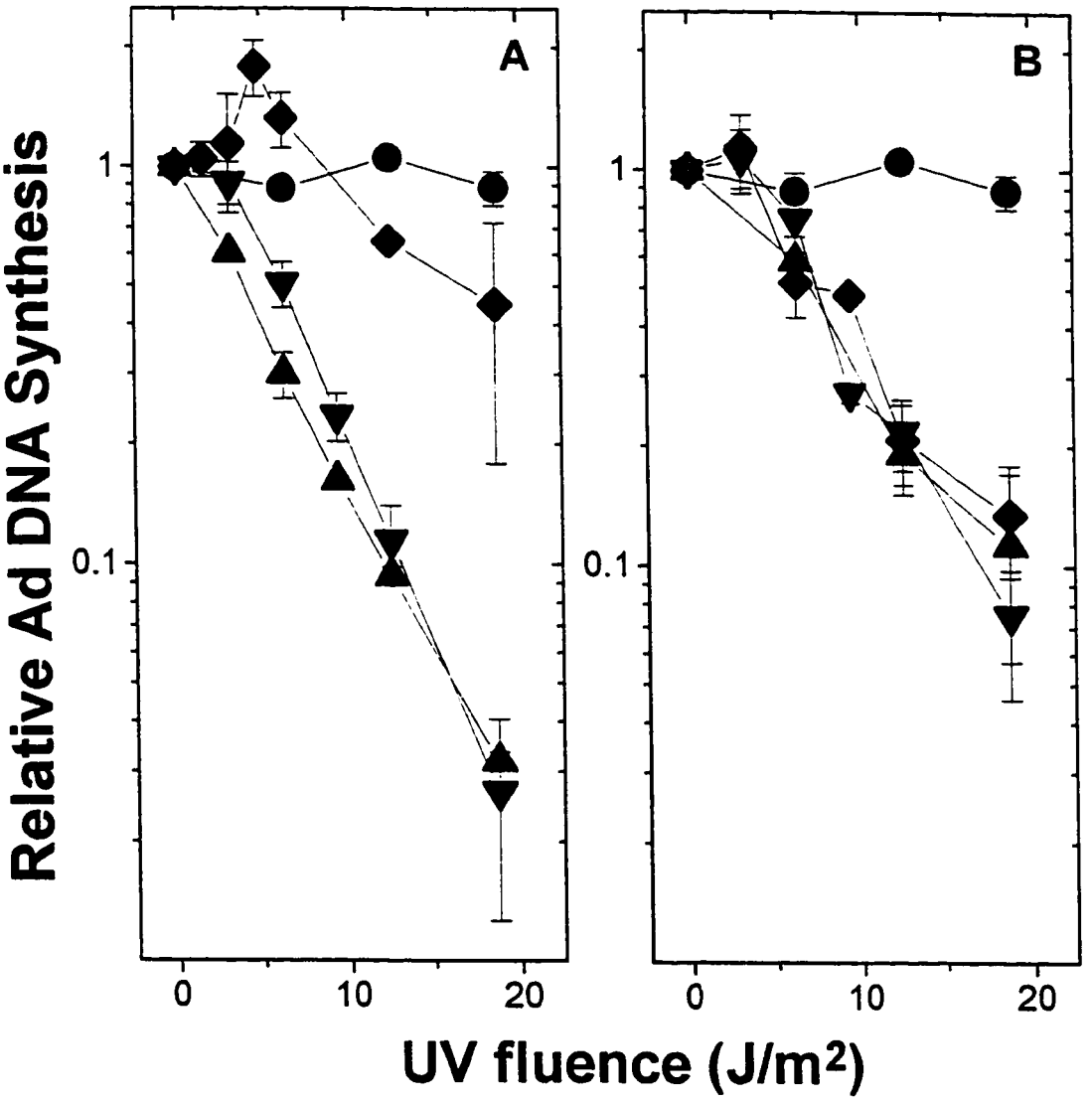
Addendum

The capacity of UV irradiated tumour cells to support Ad DNA synthesis was also assessed in a wide variety of tumour cell lines. It was demonstrated that the capacity to support Ad DNA synthesis is dependent on TCR and p53 in primary diploid fibroblasts. Here, it is reported that the capacity of tumour cell lines to support Ad DNA synthesis is greatly impaired compared to normal fibroblasts (figure 3). These results are consistent with the absence of inducible repair of the UV damaged reporter gene following UV or HS treatment in these cells (McKay and Rainbow, 1996; chapter 6). However, normal lung epithelial cells (L132 cells) are reduced in their capacity to support Ad DNA synthesis compared to normal diploid fibroblasts (D_{37} of 5 J/m2 compared to 36 J/m^2) and accumulate in the culture medium within 48 hrs yet these cells support HSER (McKay and Rainbow, 1996) and UVER (figure 1C). It is clear that factors other than DNA repair phenotype contribute to the capacity of cells to support Ad DNA synthesis.

The Ad E1A protein is required for viral DNA replication (reviewed in Horwitz, 1990) and is thus expressed during the course of capacity experiments, but not in HSER, UVER or clonogenic survival experiments. AdE1A sensitizes cells to p53 and DNA damage induced apoptosis (Lowe et al, 1993; Sabbattini et al, 1995; Attardi et al, 1996). Fibroblasts typically exhibit delayed apoptosis whereas many other cell types including tumour cells undergo apoptosis more rapidly (reviewed in Olive and Durand, 1997). The relative sensitivity of tumour cells and L132 cells may reflect a different timecourse for UV induced apoptosis in the presence of E1A expression. It is uncertain what contribution TCR plays in the capacity of these cell types to support Ad DNA synthesis following UV exposure.

Figure 3. Capacity to support Ad DNA synthesis is reduced in tumour cells relative to normal diploid fibroblasts. Cell monolayers were UV-irradiated and immediately infected with Ad5*lacZ*-5. Viral DNA was quantified following 48 hrs and normalized to the amount of viral DNA in unirradiated controls. (A) HT29 (diamonds), HCT116 (inverted triangles) and U20S cells (triangles) were reduced in their capacity to support Ad DNA synthesis compared to normal diploid fibroblasts (circles-mean of GM9503, GM38A, GM37f and GM969c). (B) SK-N-SH (diamonds), SKOV-3 (inverted triangles) and HeLa cells (triangles) were all reduced compared to normal diploid fibroblasts (circles-same curve as A). Each point represents the mean (± standard error) of at least 3 experiments performed in triplicate.


A
B
Relative Ad DNA Synthesis
1
0.1
0
10
20
UV fluence (J/m²)

Chapter 8

Persistent DNA damage induced by ultraviolet light inhibits $p21^{waf1}$ expression: implications for DNA repair, UV sensitivity and the induction of apoptosis.

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Preface

The p53 protein is regulated at the level of protein stability (reviewed in Ko and Prives, 1996). Cells accumulate p53 in large part by increasing the half-life of the protein in response to cellular stresses. A cryptic sequence specific DNA binding function is unmasked by DNA damage or a monoclonal antibody that binds the C-terminal domain of p53 (Hupp et al, 1993). Thus p53 activity is regulated post transcriptionally by at least two distinct mechanisms. This allows increased p53 activity in the presence of various cellular stresses, some of which inhibit RNA synthesis (Yamaizumi and Sugano, 1994; Chernova et al, 1995).

Ultraviolet light is a potent inhibitor of transcription and inhibition of transcription by UV correlates with p53 accumulation in primary fibroblasts (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996). Furthermore, inhibition of RNA synthesis by α-amanitin treatment (Yamaizumi and Sugano, 1994) or depletion of nucleotide pools (Chernova et al, 1995) leads to p53 accumulation. The best characterized role of p53 is as a transcription factor regulating expression of a set of responsive genes (reviewed in Ko and Prives, 1996). However, it appears counterintuitive for p53 to act solely as a transactivator of responsive genes under conditions where RNA synthesis is inefficient. Recently, transactivation independent functions of p53 have been suggested to contribute to apoptosis (Caelles et al, 1994; Wagner et al, 1994; Haupt et al, 1995a; Chen et al, 1996; Wang et al, 1996; Theiss et al, 1997).

In chapters 4, 5 and 6, evidence is presented which indicates that p53 facilitates

transcription coupled repair (TCR) of UV induced DNA damage. A model is presented in chapter 6 which suggests that inducible TCR would be expected to act as a negative feedback mechanism to regulate p53 stability. As UV induced DNA damage poses a block to transcription, it was of interest to use NDF, XP-C, XP-A and CS-A fibroblasts to assess the role of TCR in permitting expression of p53 regulated genes following UV exposure. Inhibition of $p21^{waf1}$ expression in TCR deficient cells would suggest that in addition to a role in regulation of p53 stability, TCR would regulate the ability of p53 to transactivate target genes.

Abstract

The p53 tumour suppressor gene product can act as a transcription factor to increase expression of several genes following DNA damage. Ultraviolet light (UV) induced DNA damage poses a direct block to transcription yet UV reportedly induces both accumulation of p53 and increased expression of p53 responsive genes. In order to examine expression of p53 responsive gene products in the presence of persistent DNA damage, we have performed detailed $p21^{wafl}$ expression studies in normal diploid fibroblasts (NDF) and nucleotide excision repair defective xeroderma pigmentosum group A and C (XP-A and XP-C) and Cockayne syndrome group A (CS-A) fibroblasts using a quantitative ELISA assay. We observed a fluence dependent reduction in $p21^{wafl}$ expression in all fibroblast strains. Recovery of $p21^{wafl}$ expression was delayed or absent in transcription coupled repair (TCR) deficient fibroblasts (XP-A and CS-A) compared to TCR proficient fibroblasts (normal and XP-C). Both a delay and an absence of $p21^{wafl}$ induction following UV correlated with sensitivity to UV induced apoptosis. To examine the possible consequences of attenuated $p21^{wafl}$ expression, we assessed the UV sensitivity of NDF transiently expressing gene products which either disrupt or stimulate the p53/$p21^{wafl}$/pRb pathway. Infection of NDF with recombinant adenoviruses expressing E2F-1, E2F-4 or the large tumour antigens of either SV40 or polyomavirus led to a significant sensitization of NDF to UV. In contrast, expression of $p21^{wafl}$ led to an increase in UV resistance in NDF. We propose a model in which fluence dependent reduction in transcription of p53 responsive genes facilitates apoptosis by transactivation independent mechanisms in response to UV irradiation.

Introduction

Nucleotide excision repair (NER) removes ultraviolet light (UV) induced DNA damage as well as bulky lesions induced by a variety of genotoxic agents (reviewed in Freidberg *et al.*, 1995). Cells derived from individuals with xeroderma pigmentosum (XP) and Cockayne's Syndrome (CS) are sensitive to UV exposure and are characterized by DNA repair defects. Genetic heterogeneity exists within each of these disorders and cell fusion studies have led to the classification of the XP and CS strains into several complementation groups (XP-A to XP-G, XP-V, CS-A and CS-B) (Freidberg *et al.*, 1995). UV induced cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone dimers (6-4PP) in the template strand of active genes block transcription (Mayne and Lehmann, 1982; Protic-Sabljic and Kraemer, 1985; Protic-Sabljic and Kraemer, 1986; Donahue *et al.*, 1994). A transcription coupled repair (TCR) subpathway of NER rapidly removes lesions from the template strand of active genes to allow transcription (Bohr *et al.*, 1985; Mellon *et al.*, 1987). The NER defect in CS cells results in a specific reduction in TCR whereas the NER defect in XP-C fibroblasts results in reduced repair of the remainder of the genome (global genome repair or GGR). Other NER deficient fibroblast strains (XP-A through XP-G except XP-C) are reduced in their capacity to repair UV induced DNA damage by both TCR and GGR (reviewed in Freidberg *et al.*, 1995). TCR deficient fibroblast strains (including CS-A, CS-B, XP-A) are unable to restore normal transcription following UV irradiation (Mayne and Lehmann, 1982; Yamaizumi and Sugano, 1994) whereas XP-C fibroblasts are as efficient as NDF in this regard (Yamaizumi and Sugano, 1994). It has been suggested that TCR allows expression of genes essential for UV survival (Bohr *et al.*, 1985; Mellon *et al.*, 1987).

The p53 tumour suppressor gene product is the most commonly altered gene in malignancy (Hollstein *et al.*, 1991). The antineoplastic effect of p53 is conferred through stimulation of at least 3 different mechanisms: DNA repair (Smith *et al.*, 1995; Wang *et al.*, 1995; Ford and Hanawalt, 1995; Mirzayans *et al.*, 1996; McKay *et al.*, 1997a and b), cell cycle arrest (Kastan *et al.*, 1991) and apoptosis (Lowe *et al.*, 1993). Accumulation and increased activity of p53 can occur in response to both strand breaks (Nelson and Kastan, 1994) and inhibition of transcription (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996). CS and XP-A fibroblasts accumulate p53 in response to low UV fluences compared to either NDF or XP-C fibroblasts (Yamaizumi and Sugan, 1994; Ljungman and Zhang, 1996) suggesting that induction of p53 by UV results from persistent DNA damage in active genes. Accumulation of p53 at low UV fluences in XP-A and CS fibroblasts correlates with the induction of apoptosis in these cells (Ljungman and Zhang, 1996). Together, these results suggest that inhibition of transcription promotes apoptosis in human fibroblasts.

A key regulatory feature of p53 is its ability to act as a transcription factor, regulating transcription of p53 responsive genes. Transcriptional regulation is thought to play a central role in p53 mediated cell cycle arrest (reviewed in Ko and Prives, 1996). Transactivation of the cyclin dependent kinase (cdk) inhibitor, $p21^{wafl}$ (El-Deiry *et al.*, 1993; El-Deiry *et al.*, 1994) plays a role in p53 dependent G_1 arrest. Overexpression of $p21^{wafl}$ leads to cell cycle arrest (Harper *et al.*, 1995) whereas disruption of the $p21^{wafl}$ gene attenuates G_1 arrest following DNA damage (Brugarolas *et al.*, 1995; Deng *et al.*, 1995). The $p21^{wafl}$ gene product inhibits phosphorylation of pRb by cyclin/cdk complexes leading to a predominance of the hypophosphorylated form of pRb (reviewed in Beijersbergen and Bernards, 1996).

Hypophosphorylated pRb sequesters members of the E2F family of transcription factors reducing expression of genes required for S-phase progression (reviewed in Beijersbergen and Bernards, 1996).

Although the role of transcriptional activation of p53 responsive genes is well established for G_1 arrest, the role of p53 dependent transcriptional regulation in the induction of apoptosis is less clear (Caelles *et al.*, 1994; Wagner *et al.*, 1994; Haupt *et al.*, 1995a; Sabbatini *et al.*, 1995; Attardi *et al.*, 1996; Wang *et al.*, 1996; Chen *et al.*, 1996; Theis *et al.*, 1997). Upregulation of p53 regulated genes such as *bax* can facilitate apoptosis (Miyashita and Reed, 1995), however, apoptosis can be induced by p53 expression in the presence of inhibitors of transcription and translation (Caelles *et al.*, 1994; Wagner *et al.*, 1994). Expression of p53 alleles which cannot transactivate p53 responsive genes can still induce apoptosis in a number of genetic backgrounds (Caelles *et al.*, 1994; Haupt *et al.*, 1995a; Chen *et al.*, 1996; Wang *et al.*, 1996). It is apparent that one or more activities of p53, other than transcriptional activation, can contribute to p53 dependent apoptosis.

Persistent transcription blocking DNA damage induces accumulation of p53 and apoptosis in a fluence dependent manner (Ljungman and Zhang, 1996). It seems paradoxical that transactivation of p53 responsive genes is dependent on the presence of transcription blocking DNA damage. For this reason, we have examined the timecourse for induction of $p21^{waf1}$ in detail following several UV fluences. We observed a fluence dependent decrease in $p21^{waf1}$ expression immediately following UV exposure in NDF, XP-A, XP-C and CS-A fibroblasts. The duration of the reduction and the extent of induction of $p21^{waf1}$ expression at later times reflects the capacity of the cells to repair UV induced DNA damage by TCR.

Since $p21^{waf1}$ is required for efficient p53 mediated G_1 arrest in response to DNA damage, we have used recombinant Ad constructs expressing $p21^{waf1}$, SV40LT, PyLT, E2F-1 and E2F-4 to either facilitate or decrease the capacity of cells to arrest in G_1 in response to UV treatments. Expression of SV40LT, PyLT, E2F-1 or E2F-4 conferred sensitivity to UV treatment whereas $p21^{waf1}$ expression increased resistance to UV. Changes in UV sensitivity reflected differences in the sensitivity to the induction of apoptosis suggesting that decreased efficiency of G_1 arrest at high UV fluence may be a determining factor in the commitment of cells to undergo apoptosis in response to UV irradiation.

Results

High UV fluences inhibit $p21^{waf1}$ expression.

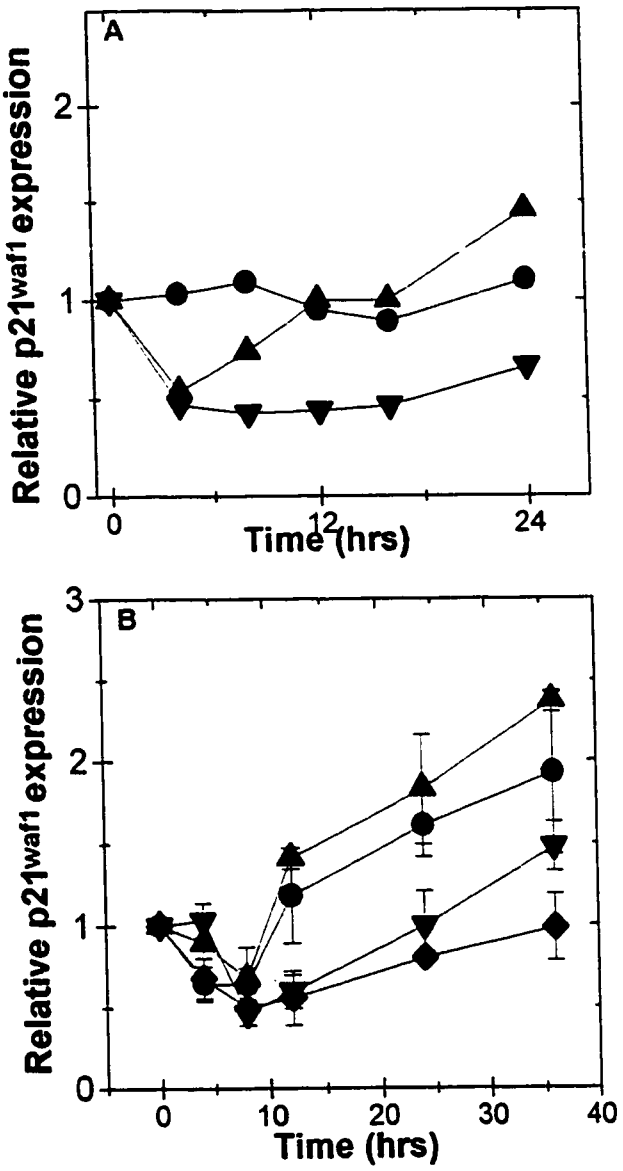
Recovery of RNA synthesis following UV treatment is inversely correlated with UV induced apoptosis in diploid fibroblasts (Ljungman and Zhang, 1996). For this reason, it seems counter intuitive that transactivation functions of p53 will mediate apoptosis in these cells. Several recent reports suggest that $p21^{waf1}$ expression can be protective against p53 dependent apoptosis (Polyak *et al.*, 1996; Gomez-Monzano *et al.*, 1996; Gorospe *et al.*, 1997). As the ability to express p53 regulated gene products such as $p21^{waf1}$ (Sheikh *et al.*, 1997) may be reduced in TCR deficient fibroblasts, we have determined in detail the timecourse for induction of $p21^{waf1}$ in NDF, XP-C, CS-A and XP-A fibroblasts following a variety of UV fluences using a quantitative ELISA assay.

Although induction of $p21^{waf1}$ was observed in NDF following UV irradiation of 30 J/m^2 following 12 to 36 hrs (Figure 1A and B), $p21^{waf1}$ expression was reduced compared to

unirradiated controls at 4 and 8 hrs following UV fluences of 30 and 50 J/m^2 but not 10 J/m^2 (Figure 1A). The magnitude of the reduction in p21^{wafl} expression over the first 4-8 hrs following UV treatment was similar in all fibroblasts strains regardless of repair phenotype for UV fluences of 30 J/m^2 or greater (Figure 1B). The initial CPD and 6-4PP frequencies reported for diploid fibroblasts in the ADA, c-myc, DHFR, δ-globin, MDR1, MDR2 and p53 genes (Venema *et al.*, 1990a; Venema *et al.*, 1990b; Evans *et al.*, 1993; Van Hoffen *et al.*, 1995; Tolbert and Kantor, 1996) are proportional to UV fluence. The mean initial dimer frequencies reported were approximately 7.2x10^{-3} CPD/kb/J/m^2 and 1.4x10^{-3} 6-4PP/kb/J/m^2. Since the p21^{wafl} transcription unit is approximately 8600 nucleotides (GenBank accession no. Z58996), one would expect greater than 2 lesions per copy of the p21^{wafl} transcription unit following a fluence of 30 J/m^2. Using the Poisson distribution, one would expect less than 22 % and 5.5 % of cells to have a single copy of the p21^{wafl} gene free of UV induced dimers immediately following 30 and 50 J/m^2, respectively. The observed decrease in p21^{wafl} expression at 4 and 8 hrs following UV treatment is independent of the DNA repair phenotype of the cell and likely results from the short halflife of p21^{wafl} (Poon *et al.*, 1996) in combination with inhibition of transcription at high UV fluences.

Expression of p21^{wafl} recovered following 30 J/m^2 within 12 hrs in NDF and XP-C fibroblasts (Figure 1). Recovery from UV inhibition of p21^{wafl} expression was more rapid in XP-C compared to CS-A fibroblasts demonstrating that efficient recovery of p21^{wafl} expression requires TCR. Induction of p21^{wafl} was observed in UV irradiated NDF, XP-C and CS-A fibroblasts following 30 J/m^2 (Figure 1A and B), however the time required for induction of p21^{wafl} following this fluence was delayed for CS-A fibroblasts compared to XP-

Figure 1. Expression of $p21^{wafl}$ is decreased following elevated UV fluences in human fibroblasts. A quantitative ELISA assay was used to quantify $p21^{wafl}$ expression at several times following UV irradiation, $p21^{wafl}$ levels in UV irradiated samples were normalized to unirradiated controls collected at the same time. A. NDF were irradiated with 10 (circles), 30 (up triangles) or 50 J/m^2 (down triangles) and each point represents a single determination from the same experiment, similar results were obtained in a total of 6 experiments. B. NDF (circles), XP-C (up triangles), CS-A (down triangles) and XP-A (diamonds) were treated with 30 J/m^2. Each point represents the mean ($\pm$ standard error) of several independant experiments.


A
Relative p21waf1 expression
2
1
0
0
12
24
Time (hrs)
B
Relative p21waf1 expression
3
2
1
0
0
10
20
30
40
Time (hrs)

C cells and NDF (Figure 1B) indicating that TCR facilitates induction of $p21^{waf1}$ following UV. The level of expression of $p21^{waf1}$ in UV treated CS-A fibroblasts was significantly reduced compared to NDF and XP-C fibroblasts between 12 and 24 hrs following UV treatment and did not recover completely by 36 hrs (Figure 1B). Induction of $p21^{waf1}$ was not observed in NDF following 50 J/m^2 within 36 hrs (Figure 1A), as previously reported (Reinke and Lozano, 1997), nor did the level of $p21^{waf1}$ expression recover to the level detected in unirradiated controls (Figure 1A). These results clearly demonstrate that both recovery of $p21^{waf1}$expression and induction of $p21^{waf1}$ following UV exposure reflect the rate and extent of repair of UV induced DNA damage in active genes.

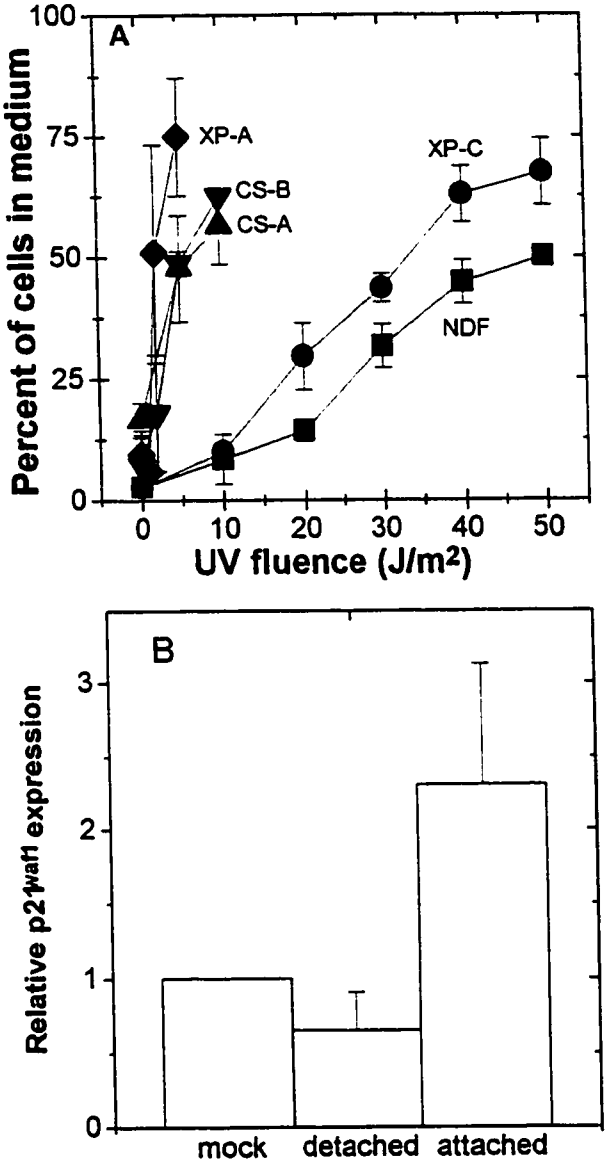
In order to examine $p21^{waf1}$ expression in fibroblasts undergoing apoptosis, we took advantage of a recent report indicating that the proportion of cells collected from the growth medium can be used as a quantitative estimate of apoptosis in fibroblasts (Weissinger *et al.*, 1997). We have evaluated this approach in 3 ways. Firstly, detached cells were non viable following a fluence of 30 or 50 J/m^2 (<10 % viable) whereas adherent cells remained viable (93 and 94 % viable, respectively) as assessed by trypan blue exclusion. Secondly, greater than 80% of detached NDF were apoptotic as assessed by TUNEL staining whereas less than 10 % of cells which remained adherent stained TUNEL positive following these UV fluences. In addition, we have quantified cell detachment in NDF, XP-A, XP-C, CS-A and CS-B fibroblasts (Figure 2). The proportion of detached fibroblasts following UV irradiation yielded strikingly similar results to those reported by Ljungman and Zhang (1996) using both DNA fragmentation and flow cytometry. These results indicate that fibroblasts which accumulate in the culture medium following UV irradiation are undergoing apoptosis.

We have assessed expression of $p21^{wafl}$ in both detached and adherent NDF following a UV fluence of 30 J/m^2. Near confluent fibroblasts were UV treated and detached and adherent cells were collected separately, 48 hrs following exposure to 30 J/m^2. Cell number was determined for each treatment and an equivalent number of cells was used to assess $p21^{wafl}$ expression. Expression of $p21^{wafl}$ was significantly reduced in detached cells compared to adherent cells ($p<0.05$, Figure 2). Expression of $p21^{wafl}$ appears to be decreased in apoptotic cells and increased in adherent cells (Figure 2). In summary, sensitivity to UV induced apoptosis inversely correlates with the ability of fibroblast strains to remove UV induced DNA damage by TCR (Ljungman and Zhang, 1996; Figure 2). Fibroblast strains with reduced capacity to repair by TCR do not express $p21^{wafl}$ as efficiently as TCR competant strains following UV treatment. Delayed recovery of $p21^{wafl}$ expression in NDF correlates with UV fluence dependent increase in apoptosis. Apoptotic NDF do not express elevated levels of $p21^{wafl}$ whereas surviving cells do. These results are consistant with UV induced apoptosis occuring in a population of cells with decreased $p21^{wafl}$ expression resulting from persistant transcription blocking DNA damage.

Alteration of the p53/$p21^{wafl}$/pRb pathway affects UV sensitivtity of normal diploid fibroblasts.

The p53, $p21^{wafl}$ and pRb proteins make up a cell cycle regulatory cascade which negatively regulates transactivation by the E2F family of transcription factors (reviewed in Beijersbergen and Bernards, 1996). Since high UV fluences decrease expression of $p21^{wafl}$ immediately following UV treatment and $p21^{wafl}$ is a key regulator of p53 dependent cell cycle

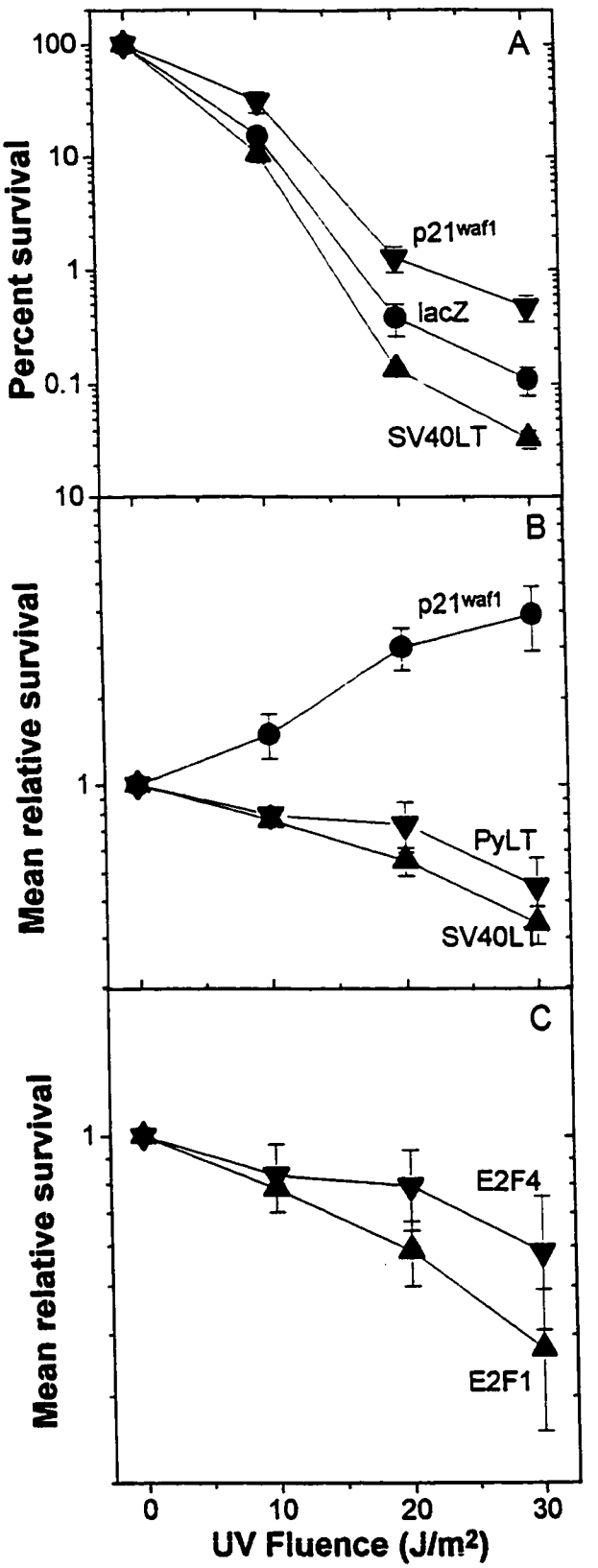
Figure 2. Non-adherent UV irradiated fibroblasts are apoptotic and reduced in the level of $p21^{wafl}$ expression. A. NDF (squares), XP-C (circles), CS-A (triangles), CS-B (down triangles) and XP-A (diamonds) fibroblasts were UV irradiated at the indicated UV fluences. The number of detached and adherent cells were determined as described in the materials and methods. % of cells in the medium was calculated as the number of cells in the medium compared to the total number of cells counted in both fractions. Each point represents the mean (± standard error) of 5, 4, 2, 2 and 3 determinations for NDF, XP-C, CS-A, CS-B and XP-A fibroblasts, respectively. B. Attached and detached NDF fibroblasts were collected separately 48 hrs following UV irradiation. Cell number was determined for each and an equivalent number of cells was used to assess $p21^{wafl}$ levels. Data is expressed relative to unirradiated controls collected at the same time and represents the mean (± standard error of 2 independent experiments) for both detached cells and adherent cells.


A
Percent of cells in medium
100
75
50
25
0
XP-A
CS-B
CS-A
XP-C
NDF
0
10
20
30
40
50
UV fluence (J/m2)
B
Relative p21waf1 expression
3
2
1
0
mock
detached
attached

arrest (El-Deiry *et al.*, 1993), we have assessed the effect of expression of several transgenes which are known to either inhibit or stimulate the p53 dependent G_1 checkpoint. The interaction of both SV40LT and PyLT with pRb disrupts p53 dependent G_1 arrest (Quartin *et al.*, 1994; Doherty and Freund,1997). SV40LT has also been shown to disrupt $p21^{waf1}$ induced cell cycle arrest (Harper *et al.*, 1993). E2F-1 expression overcomes cell cycle arrest in response to both ionizing radiation (DeGregori *et al.*, 1995a) and expression of the cyclin dependant kinase inhibitors $p21^{waf1}$, $p27^{kip1}$ or $p16^{ink4}$ (DeGregori *et al.*, 1995b). E2F-4 is capable of inducing S phase in G_0 arrested cells (DeGregori *et al.*, 1997) but has not yet been assessed for its ability to overcome DNA damage or p53 induced G_1 arrest. In contrast, overexpression of $p21^{waf1}$ is sufficient to induce G_1 arrest (Harper *et al.*, 1993). Clonogenic survival was assessed in NDF infected with recombinant Ad constructs at a moi of 20 pfu/cell expressing each of these gene products 24 hrs prior to UV challenge. Whereas $p21^{waf1}$ expression conferred an increase in resistance to UV irradiation (Figure 3A), infection of cells with recombinant Ad constructs expressing SV40LT (Figure 3A and B), PyLT (Figure 3B), E2F-1 (Figure 3C) and E2F-4 (Figure 3C) conferred an increase in sensitivity to UV-irradiation. These results suggest that the p53/$p21^{waf1}$/pRb pathway regulates sensitivity of NDF to UV irradiation.

As UV inhibited expression of $p21^{waf1}$ in UV irradiated cells, we wanted to assess the effect of UV irradiation on $p21^{waf1}$ expression in AdCMVp21 infected cells. Increased expression of $p21^{waf1}$ was not observed in AdCMVp21 infected NDF (Figure 4A). Following UV irradiation of Ad5HCMVsp1*lacZ* infected cells, a reduction in $p21^{waf1}$ expression following 30 J/m^2 was observed to a similar extent to uninfected cells and expression similarly

Figure 3. Modulation of the p53/p21^{waf1}/pRb pathway using recombinant Ad contructs affects clonogenic survival of NDF following UV treatment. NDF were infected with recombinant viruses expressing p21^{waf1}, SV40LT, PyLT, E2F-1 or E2F-4, 24 hrs prior to UV challenge. A. Clonogenic survival was increased by infection with the p21^{waf1} expressing virus (down triangles) but was reduced by infection with the SV40LT expressing virus (up triangles) compared to the control virus (circles). Each point represents the mean ($\pm$ standard error) for a single experiment performed in triplicate. B and C. Relative survival was determined by normalizing clonogenic survival to the survival of cells infected with the *lacZ* control virus in each experiment. The mean relative survival of NDF infected with the PyLT (down triangles in B), SV40LT (up triangles in B), E2F-1 (up triangles in B) and E2F-4 (down triangles in C) contructs decreased with increasing UV fluence whereas infection with the AdCMVp21 construct (circles in B) led to a dose dependent increase in the mean relative survival. Each point represents the mean ($\pm$ standard error) determined from at least 3 independent experiments performed in triplicate.


A
100
10
1
0.1
10
Percent survival
p21waf1
lacZ
SV40LT
B
1
Mean relative survival
p21waf1
PyLT
SV40LT
C
1
Mean relative survival
E2F4
E2F1
0
10
20
30
UV Fluence (J/m2)

recovered within 12 hrs (Figure 4B). In contrast, the reduction in $p21^{wafl}$ expression observed in UV irradiated AdCMVp21 infected cells was not significant ($p>0.10$, 1 tailed t test) (Figure 4B). Expression of $p21^{wafl}$ in Ad5HCMVsp1*lacZ* infected cells was significantly reduced ($p<0.05$, 1 tailed t test) compared to AdCMVp21 infected cells 4 hrs but not 12 hrs following UV exposure (Figure 4B). These results suggest that UV resistance is conferred by exogenous $p21^{wafl}$ expression without a large increase in $p21^{wafl}$ levels. Expression of $p21^{wafl}$ from the AdCMVp21 construct appears to compensate for inhibition of endogenous $p21^{wafl}$ gene expression by UV.

The effect of SV40LT and E2F-1 expression on UV induced cell detachment was also assessed by determining the fraction of detached cells 72 hrs following UV treatment. Expression of E2F-1 and SV40LT led to an increase in the proportion of UV irradiated NDF in the culture medium following either 10 or 30 J/m^2 (Figure 5A and B). As apoptoic fibroblasts accumulate in the culture medium, these results suggest that disruption of G_1 arrest by SV40LT expression (Quartin *et al.*, 1994) and E2F-1 overexpression (DeGregori *et al.*, 1995a; DeGregori *et al.*, 1995b) sensitizes cells to UV induced apoptosis.

Discussion

UV inhibits $p21^{waf1}$ expression following high UV fluence

Using a quantitative ELISA assay, we have demonstrated that $p21^{wafl}$ expression is reduced and induction of $p21^{wafl}$ is delayed in human fibroblasts following high UV fluences (30 and 50 J/m^2). These UV fluences are expected to generate a mean of 2.2 and 3.6 UV lesions per $p21^{wafl}$gene, respectively, based on lesion frequencies previously reported for

Figure 4. The effect of AdCMVp21 infection on $p21^{waf1}$ expression. NDF were infected with AdCMVp21 or Ad5HCMVsp1*lacZ* at an moi of 20 pfu/cell. A. Expression of $p21^{waf1}$ was determined at several times following infection. Each point represents the mean (± standard error) determined from 4 independent experiments. The absence of error bars indicates that the error is within the symbol. B. NDF infected with the AdCMVp21 and Ad5HCMVsp1lacZ contruct were UV irradiated with 30 J/m^2, 24 hrs following infection. At 4 and 12 hrs following infection, $p21^{waf1}$ expression was determined and normalized to mockirradiated controls. Each column represents the mean (± standard error) determined from either 2 (12 hrs) or 4 (4hrs) independent experiments.

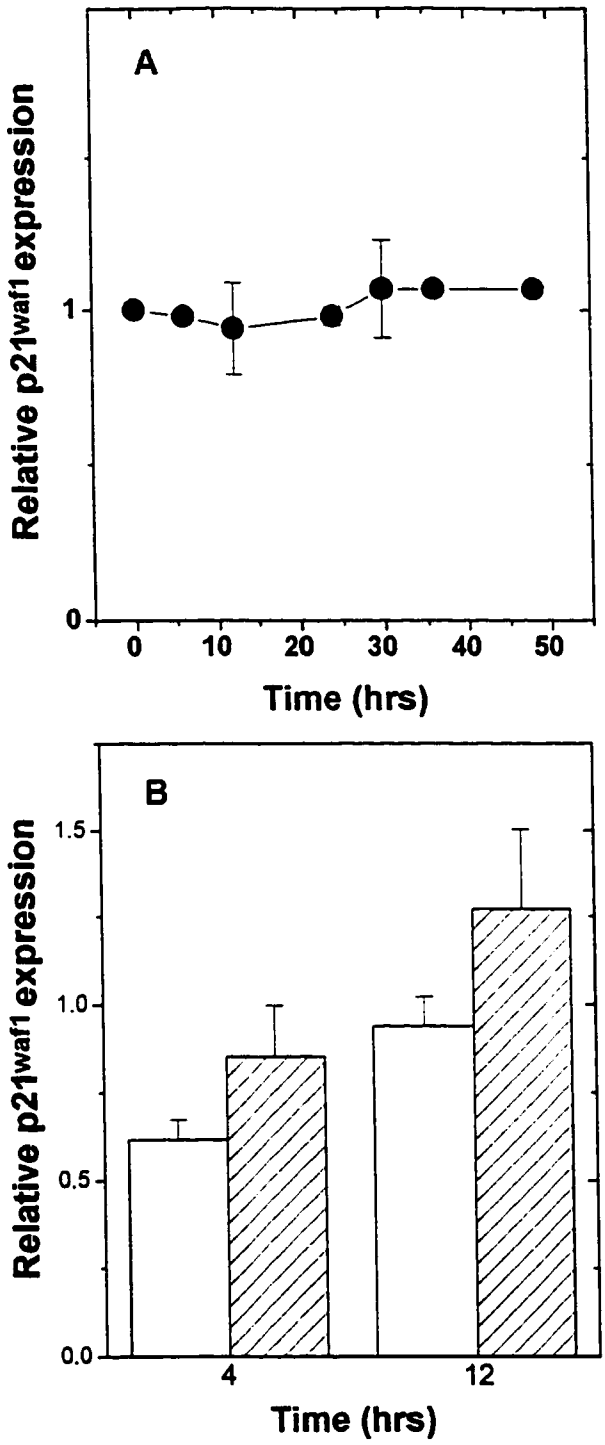
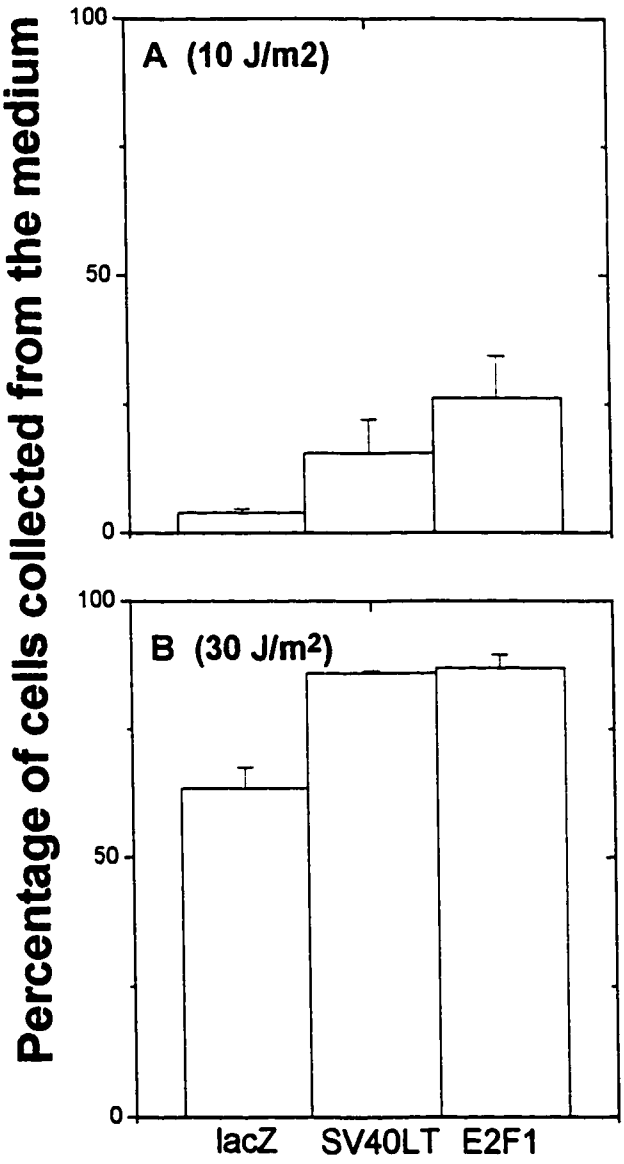

A
Relative p21waf1 expression
1
0
0
10
20
30
40
50
Time (hrs)
B
Relative p21waf1 expression
1.5
1.0
0.5
0.0
4
12
Time (hrs)

Figure 5. Infection of NDF with Ad5SVR112 and Ad-E2F1 leads to increased cell detachment following UV irradiation. Infection of NDF with either the SV40LT or E2F-1 expressing construct leads to increased cell detachment compared to cells infected with the *lacZ* construct following either 10 J/m^2 (A) or 30 J/m^2 (B). Each column represents the mean ($\pm$ standard error) for at least 3 independent determinations.


Percentage of cells collected from the medium
100
50
0
A (10 J/m2)
100
50
0
B (30 J/m2)
lacZ
SV40LT
E2F1

several cellular genes (Venema *et al.*, 1990a; Venema *et al.*, 1990b; Evans *et al.*, 1993; Van Hoffen *et al.*, 1995; Tolbert and Kantor, 1996). Using the Poisson distribution, one predicts approximately 22% of cells to have a single undamaged $p21^{wafl}$ allele following a UV treatment of 30 J/m^2. Thus, UV induced DNA damage would be expected to block transcription of $p21^{wafl}$ genes immediately following UV treatment because UV induced dimers pose a block to transcript elongation (Mayne and Lehmann, 1982; Protic-Sabljic and Kraemer, 1985; Protic-Sabljic and Kraemer, 1986; Donahue *et al.*, 1994). A 50% reduction in $p21^{wafl}$ expression was observed in all fibroblast strains between 4 and 8 hrs following UV fluences of 30 or 50 J/m^2. Inhibition of transcription (Donahue *et al.*, 1994) coupled with a short half life for $p21^{wafl}$ (Poon *et al.*, 1996) likely results in the rapid decrease in $p21^{wafl}$ levels at these UV fluences.

We observed a fluence dependent delay in recovery of $p21^{wafl}$ expression which correlated with the ability of cells to repair UV induced DNA damage by the TCR pathway. NDF and XP-C fibroblasts recovered from inhibition of $p21^{wafl}$ expression within 12 hrs following 30 J/m^2 whereas CS-A and XP-A cells are greatly delayed in recovery of $p21^{wafl}$ expression. Removal of CPD by TCR following either 15 or 30 J/m^2 is less efficient than estimates following 10 J/m^2, indicating that TCR of CPD is less efficient at higher UV fluence (Van Hoffen *et al.*, 1995; Tu *et al.*, 1997). Decreased efficiency of TCR with increasing fluence could contribute to fluence dependant reduction in $p21^{wafl}$ expression. The duration of the delay in the induction of $p21^{wafl}$ appears to increase with UV fluence as reported for $p21^{wafl}$, mdm-2, cyclin G and *bax* (Perry *et al.*, 1993; Poon *et al.*, 1996; Lu *et al.*, 1996; Ljungman and Zhang, 1996; Reinke and Lozano, 1997). We suggest that UV induced DNA

damage causes delayed induction of p53 responsive genes by blocking transcript elongation.

Murine fibroblasts stably or transiently expressing *lacZ* under control of a p53 responsive promoter have been used to assess both transcriptional activity of p53 and either p53 accumulation by double immunofluorescent staining (Lu *et al.*, 1996) or apoptosis as assessed by morphological criteria (Chernov and Stark, 1997). In stable transfectants, cells with high levels of p53 following 50 J/m^2 did not express the highest levels of β-gal. Since the transgene including the promoter was at least 6.5 kb in length and there was only a single copy of the transgene per cell, the majority of cells would have at least 1 CPD per transgene following 50 J/m^2. The inverse correlation observed between p53 levels and transcriptional activation of the p53 responsive reporter gene following UV treatment probably results from persistent transcription blocking DNA damage because cells with persistent transcription blocking DNA damage accumulate p53 to a greater extent (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996). Using a transient transfection protocol, apoptotic cells efficiently expressed β-gal following a UV fluence of 24 J/m^2 (Chernov and Stark, 1997). The presence of β-gal activity in apoptotic cells (Chernov and Stark, 1997) is a clear indication that p53 in apoptotic cells is capable of transcriptionally activating target genes. We demonstrated that detached cells which stain positive by the TUNEL assay are reduced in p21^{waf1} expression whereas adherent cells have prolonged p21^{waf1} induction. Together, these results indicate that high UV fluences inhibit expression of p21^{waf1} due to persistent transcription blocking DNA damage, not an inability to transactivate p53 responsive genes.

G_1 arrest is protective against p53 dependent apoptosis.

Expression of $p21^{waf1}$ leads to inhibition of cdk activity ultimately preventing S phase progression by preventing expression of E2F responsive genes (Harper *et al.*, 1993; reviewed in Beijersbergen and Bernards, 1996). G_1 arrest in response to DNA damage or p53 overexpression is attenuated in cells with disruptions of either the $p21^{waf1}$ or pRb genes (Deng *et al.*, 1995; Brugarolas *et al.*, 1995; Polyak *et al.*, 1996; Morgenbesser *et al.*, 1994). We demonstrated that disruption of pRb function with either SV40LT or PyLT sensitizes NDF to UV irradiation. These results suggest that disruption of the G_1 checkpoint confers sensitivity to UV in NDF.

G_1 arrest mediated by expression of the cdk inhibitors, $p21^{waf1}$, $p27^{kip1}$ or $p16^{ink4a}$ can also be overcome by E2F-1 overexpression (DeGregori *et al.*, 1995b). S phase can be induced from G_0 arrest by either E2F-1 or E2F-4 overexpression (Shwarz *et al.*, 1995; DeGregori *et al.*, 1997) indicating that E2F-1 and E2F-4 are potent stimulators of S phase induction. E2F-1 overexpression can also induce apoptosis (Wu and Levine, 1994), however E2F-4 overexpression cannot (DeGregori *et al.*, 1997). We observed a decrease in clonogenic survival following UV treatment of NDF expressing either E2F-1 or E2F-4. UV sensitivity does not simply result from synergism between UV stimulated p53 and E2F-1 expression because both E2F-1 and E2F-4 sensitized cells to UV treatment. This is the first report of any E2F transcription factor inducing sensitivity to a DNA damaging agent. In addition, these results suggest that pRb related proteins, p107 and p130 play a role in UV resistance because E2F-4 binds p107 and p130 but not pRb (reviewed in Beijersbergen and Bernards, 1996). Taken together, these results suggest that overcoming functions of the pRb

family of proteins leads to UV sensitivity and further suggests that G_1 arrest is protective against UV irradiation.

Several reports indicate that overexpression of either $p21^{wafl}$ (Gomez-Manzano *et al.*, 1996; Gorospe *et al.*, 1997) or pRb (Haupt *et al.*, 1995b; Haas-Kogan *et al.*, 1995) is protective against apoptosis induced in response to p53 overexpression or DNA damage. These reports support a role for $p21^{wafl}$ and pRb in protection against p53 dependent apoptosis. As $p21^{wafl}$ expression and pRb hypophosphorylation are both stimulated by p53 dependent signalling, p53 mediated G_1 arrest appears to be protective against p53 dependent apoptosis as recently suggested (Polyak *et al.*, 1996). As we observed a reduction in $p21^{wafl}$ expression following UV in a fluence dependant manner, one would expect a fluence dependent reduction in the antiapoptotic functions of both $p21^{wafl}$ and pRb.

We examined UV resistance in $p21^{wafl}$ expressing NDF. Infection of NDF with the AdCMVp21 construct conferred resistance to UV as assessed by clonogenic survival. A similar increase in UV survival has been reported in colon carcinoma cells expressing $p21^{wafl}$ from a tetracycline regulated promoter (Sheikh *et al.*, 1997). A decrease in clonogenic survival has been reported in $p21^{wafl}$ deficient cells following treatment with UV (McDonald *et al.*, 1996), adriamycin (Waldman *et al.*, 1996), ionizing radiation (Waldman *et al.*, 1996), cisplatin (McDonald *et al.*, 1996; Fan *et al.*, 1997) and nitrogen mustard (Fan *et al.*, 1997) suggesting that expression of $p21^{wafl}$ is protective against a variety of DNA damaging agents. Whereas $p21^{wafl}$ overexpression in a colon carcinoma cell line conferred resistance to UV treatment (Sheikh *et al.*, 1997), we did not observe an increase in $p21^{wafl}$ expression within 48hrs following infection of NDF with the AdCMVp21 construct. The size of the $p21^{wafl}$

transgene is less than 1/10 that of the endogenous primary $p21^{wafl}$ transcript. Consistent with the small target size of the transgene, we did not observe a significant reduction in $p21^{wafl}$ expression following 30 J/m^2. These results indicate that expression of the $p21^{wafl}$ transgene confers UV resistance without significant induction of $p21^{wafl}$ but rather by eliminating the fluence dependent reduction in $p21^{wafl}$ expression. Thus, inefficient transcription of antiapoptotic genes at high UV fluence may contribute to UV induced apoptosis.

The role of $p21^{wafl}$ in repair of UV induced DNA damage

Altered sensitivity to DNA damaging agents (including UV) resulting from $p21^{wafl}$ disruption (McDonald *et al.*, 1996; Waldman *et al.*, 1996; Fan *et al.*, 1997) correlates with reduced repair of carcinogen treated reporter genes. Also, forced expression of $p21^{wafl}$ led to increased reactivation of UV damaged reporter constructs (Sheikh *et al.*, 1997). Several groups have suggested the involvement of p53 in repair of UV induced DNA damage (Smith *et al.*, 1995; Wang *et al.*, 1995; Ford and Hanawalt, 1995; Mirzayans *et al.*, 1996; McKay *et al.*, 1997a; McKay *et al.*, 1997b). Both TCR (McKay *et al.*, 1997a) and GGR (Smith *et al.*, 1995) appear to be inducible processes through p53 dependent mechanisms. A requirement of transactivation of p53 regulated genes has been suggested for inducible DNA repair (Smith *et al.*, 1995), however, following a UV fluence of 30 J/m^2, the majority of CPD and 6-4PP are repaired from both active and inactive regions of the genome within 8 hrs (Van Hoffen *et al.*, 1995). Since $p21^{wafl}$ expression is reduced following this UV fluence in all fibroblast strains examined for greater than 8 hrs, this repair must occur in the absence of $p21^{wafl}$

induction and likely occurs in the absence of transactivation of other p53 regulated genes. Decreased expression of $p21^{wafl}$ following UV irradiation is not consistent with its requirement for repair of UV induced DNA damage in diploid human fibroblasts. It is possible that $p21^{wafl}$ expression is not rate limiting for TCR or GGR in NDF.

Reduced expression of antiapoptotic genes at high UV fluence favours apoptosis by a transactivation independent mechanism.

NDF are competant to undergo apoptosis (Ljungman *et al.*, 1996) but there are conflicting reports of the ability of NDF to arrest in G_1 following moderate UV treatment (van Laar *et al.*, 1994; de Laat *et al.*, 1996; Poon *et al.*, 1996). UV induced G_1 arrest occurs in NDF treated with low UV fluences (Kaufman and Wilson, 1994). XP-A fibroblasts do not have a distinct G_1 arrest (Imray *et al.*, 1983), despite increased sensitivity to p53 induction (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996) suggesting that unrepaired DNA damage attenuates G_1 arrest. In contrast, ionizing radiation induces a prolonged G_1 arrest in NDF coincident with induction of $p21^{wafl}$ (DiLeonardo *et al.*, 1994). Despite induction of p53, these cells are resistant to apoptosis (DiLeonardi *et al.*, 1994; Ljungman *et al.*, 1996).

The number of DNA lesions induced in response to UV is far greater than that produced by an equitoxic dose of IR (Fornace, 1982). Thus there is a greater probability of sustaining DNA damage within a transcription unit following UV treatment compared to IR treatment. UV induced DNA damage inhibits transcription (Mayne and Lehman, 1982) whereas an equitoxic dose of IR does not (Whitmore *et al.*, 1961). Inhibition of transcription of specific genes such as $p21^{wafl}$ may play a role in commitment to undergo apoptosis following UV.

Transcriptional regulation of genes such as *bax* can induce apoptosis in some model systems (Miyashita and Reed, 1995) and transactivation appears to be required for p53 dependent apoptosis in Ad E1A expressing cell lines (Sabbatini *et al.*, 1995; Attardi *et al.*, 1996). As observed for $p21^{waf1}$, it is unlikely that expression of *bax* will be efficient in response to UV fluences which induce apoptosis in human fibroblasts. Several observations suggest that p53 is capable of mediating apoptosis in the absence of transactivation of target genes. Overexpression of p53 can induce apoptosis in the presence of both transcriptional and translational inhibitors (Caelles *et al.*, 1994; Wagner *et al.*, 1994). Apoptosis can be induced by forced expression of mutant p53 which cannot induce transcription of p53 regulated genes (Caelles *et al.*, 1994; Haupt *et al.*, 1995a; Wang *et al.*, 1996; Chen *et al.*, 1996; Theiss *et al.*, 1997), including either C-terminal or N-terminal truncation mutants (Haupt *et al.*, 1995a; Wang *et al.*, 1996). Clearly, p53 can induce apoptosis without transactivation of its target genes.

We suggest that there is a fluence dependent decrease in expression of p53 responsive genes resulting from transcription blocking DNA damage (Figure 6). As p53 can mediate apoptosis in the absence of transcription and translation (Caelles *et al.*, 1994; Wagner *et al.*, 1994), p53 dependent apoptosis could occur in cells with transcription blocking DNA damage. A fluence dependent decrease in the ability to express antiapoptotic genes such as $p21^{waf1}$ would be expected to favour p53 dependent apoptosis by one or more transactivation independent mechanisms.

Materials and Methods

Cells, viruses and UV treatments

Normal, XP-A (XP12BE), XP-C (XP2BE), CS-A (CS3BE) and CS-B (CS1AN) fibroblasts were obtained from the National Institute of General Medical Sciences repository (Camden, NJ). Repository reference numbers are GM38A, GM5509, GM677, GM1856 and GM739, respectively. 293 cells were obtained from Dr F.L. Graham (McMaster University). Cells were maintained in α-minimal essential medium supplemented with fetal calf serum together with penicillin (100mg/mL), streptomycin (100 mg/mL) and amphotericin B (250 ng/mL) (GibcoBRL).

Replication defective recombinant adenoviruses Ad5HCMVsp1*lacZ*, AdCA35*lacZ*, AdSVR112, AdSVR39, Ad-E2F-1, Ad-E2F-4 and AdCMVp21 (see table 1) were obtained from Dr F.L. Graham (McMaster University), Dr J.A. Hassel (McMaster University), Dr J.R. Nevins (Duke University) and Dr T.C. Thompson (Baylor College of Medicine). All viruses were propagated and titred in 293 cells as previously reported (Graham and Prevec, 1991). Viral titres are expressed as plaque forming units (pfu) per mL. Viral infections were performed in 40 μL, 200 μL or 500 μL of serum free α-MEM for 96 well, 24 well and 6 well dishes, respectively. Viral suspensions were added to cell monolayers and incubated for 60 min at 37°C to allow adsorption. Infected monolayers were returned to 37 °C following addition of prewarmed growth medium.

For UV treatment of cells, medium was replaced with 40 μL, 200 μL, 500 μL or 2000 μL phosphate buffered saline (PBS: 140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4 and 1.75 mM KH_2PO_4) for 96, 24, 6 well dishes or 100 mm dishes, respectively. Irradiation of cells

Figure 6. Model for UV fluence dependent increase in the probability to undergo apoptosis following UV exposure. UV irradiation induces transcription blocking DNA damage which stimulates accumulation of p53. Transactivation dependent functions (TDF) and transactivation independent functions (TIF) of p53 contribute to p53 activity. TDF can induce G_1 arrest, stimulate DNA repair and induce apoptosis. TIF of p53 can induce apoptosis and facilitate TCR. High UV fluences inhibit expression of p53 responsive genes such that one would expect a fluence dependent decrease in the efficiency of TDF of p53. As TIF of p53 can occur in the presence of transcription blocking DNA damage, high UV fluences in NDF, and moderate UV fluences in NER deficient fibroblasts would favour apoptosis by transactivation independent mechanisms.

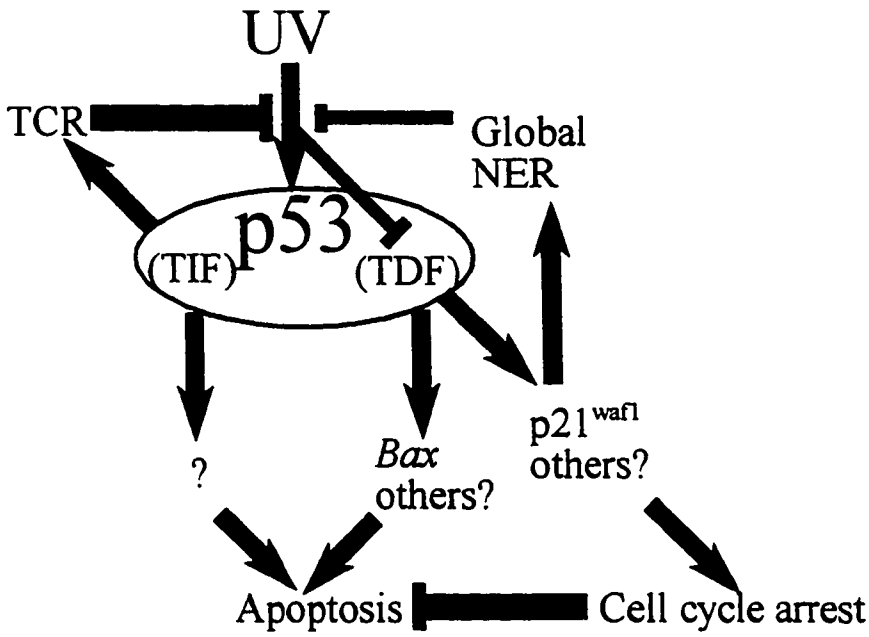

UV
TCR
Global
NER
(TIF)
p53
(TDF)
?
Bax
others?
p21waf1
others?
Apoptosis
Cell cycle arrest

Table 1. Recombinant Ad contructs.

Virus	gene product	promoter	reference
Ad5HCMVsp1*lacZ*	*lacZ*	HCMV	Morsy *et al*, 1993
Ad5SVR112	SV40LT	Ad major late	Gluzman *et al*, 1982
Ad5PyR39	PyLT	Ad major late	Massie *et al*, 1986
Ad-E2F1	E2F1	HCMV	Schwarz *et al*, 1995
Ad-E2F4	E2F4	HCMV	DeGregori *et al*, 1997
AdCMVp21	$p21^{waf1}$	HCMV	Eastham *et al*, 1995

was performed at room temperature at a fluence rate of 1 $J/m^2/sec$ from a germicidal lamp emitting predominantly at 254nm (General Electric G8T5). Fluence rate was assessed with a UV meter (model J255, Ultraviolet products, San Gabriel, Ca). UV irradiation of virus was performed with the same UV source, on ice with constant stirring, at a fluence rate of 2 $J/m^2/sec$. Cells were immediately refed with growth medium.

Apoptosis

Fibroblasts were seeded at $2.5x10^5$ cells per well of 6 well dishes. Twenty four hours later, cells were UV irradiated. Experiments with recombinant viruses were performed separately, cells were allowed 24 hrs to seed prior to viral infection and were incubated an additional 24 hrs to allow transgene expression prior to UV irradiation. Following UV treatment, prewarmed growth medium was immediately replaced and cells were placed in a humidified incubator for 72 hrs. Detached cells were collected from the growth medium by centrifugation whereas attached cells were rinsed in PBS, trypsinized and collected by centrifugation. Cells were resuspended in 100 μL PBS and cell number was determined by direct counting of both detached and adherent cells. The proportion of detached cells is expressed as the number of detached cells relative to the total number of cells.

Viability of cells was assessed by trypan blue exclusion for both attached and detached cells 72 hrs following UV irradiation. TUNEL staining was performed separately on detached and adherent cells. Cells were collected as above and fixed for 30 min. in 3 % paraformaldehyde. TUNEL staining was performed according to the specification of the manfacturer (cat. no. QIA 33, Oncogene Science, Cambridge, Ma). The proportion of

TUNEL positive cells was determined by counting a minimum of 200 cells for each treatment. The proportion of apoptotic cells is expressed as the number of TUNEL positive cells compared to the total number of cells counted.

Expression of $p21^{waf1}$

Cells were allowed to seed in 24 well dishes at $5x10^4$ cells per well for 24 hrs prior to either viral infection or UV irradiation. Cells infected with recombinant Ad constructs were allowed an additional 24 hrs for transgene expression prior to UV treatment. Cell lysis and $p21^{waf1}$ detection by ELISA was performed according to the specifications of the manufacturers (cat. no. QIA 18, Oncogene Science, Cambridge, Ma). Expression of $p21^{waf1}$ for UV irradiated samples was normalized to mockirradiated controls collected simultaneously. Expression of $p21^{waf1}$ from detached and attached fibroblasts, as collected above, was performed using 1×10^5 cells determined by direct counting.

Clonogenic survival

Cells were seeded in 6 well dishes at $5x10^5$ cells per well. Infection or mock infection of cell monolayers was performed 24 hrs following seeding. Cells were allowed an additional 24 hrs to allow expression of the transgene. Cells were then seeded to low density (between 500 and 10000 per 100 mm dish). Approximately 6 hrs following seeding to low density, medium was replaced with PBS and cells were irradiated at UV fluences between 0 and 30 J/m^2. Medium was immediately replaced and colonies were allowed to form for approximately two weeks. Colonies were stained with methylene blue (0.5 % methylene in

70% ethanol) and colonies greater than 20 cells were counted. The surviving fraction was expressed as the relative seeding efficiency of UV irradiated versus mock irradiated cultures.

Acknowledgements

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Addendum

Persistent UV induced DNA damage in actively transcribed genes leads to accumulation of p53 (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996) and the induction of apoptosis (Ljungman and Zhang, 1996). NER deficient XP-A fibroblasts accumulate p53 following UV treatments of less than 5 J/m^2 whereas TCR deficient CS-A and CS-B require at least 5 J/m^2 and TCR proficient XP-C and NDF require UV fluences greater than 10 J/m^2 (Ljungman and Zhang, 1996). Following 4 J/m^2, XP-A fibroblasts are capable of increased mdm-2 expression compared to unirradiated controls suggesting that accumulated p53 in NER deficient cells is transcriptionally active (Ljungman and Zhang, 1996). Consistent with these results, induction of p21^{wafl} was not observed in NDF or XP-C fibroblasts following UV treatments of 5 or 10 J/m^2 whereas p21^{wafl} induction was observed following both 5 and 10 J/m^2 in XP-A and following 10 J/m^2 in CS-A fibroblasts (figure 7). Induction of p21^{wafl} was greatest following 5 J/m^2 in XP-A fibroblasts, 10 J/m^2 in CS-A fibroblasts and 30 J/m^2 in NDF and XP-C fibroblasts (figure 7). Thus cells which are hypersensitivity to the induction of p53 following UV treatment also induce p21^{wafl} at low UV fluence.

Despite accumulation of p53 in these cells following high UV fluences, unrepaired lesions in the p21^{wafl} gene inhibit transactivation of p21^{wafl} in XP-A and CS-A fibroblasts at lower UV fluences than XP-C and NDF (figure 7 and figure 1B). Thus p21^{wafl} expression and expression of other p53 responsive genes are positively and negatively affected by the presence of transcription blocking DNA damage. The ability of p53 to transactivate target genes will reflect in large part the size of the gene. Since p21^{wafl} is small in comparison to

mdm-2, one would expect that UV induced inhibition of $p21^{wafl}$ expression would require a higher UV fluence than the fluence required to block mdm-2 expression. This concept is sufficient to account for recent reports suggesting that $p21^{wafl}$ and mdm-2 are differentially regulated in response to UV (Reinke and Lozano, 1997; Wu and Levine, 1997). In light of the protective role reported for $p21^{wafl}$ against p53 mediated apoptosis (Gomez-Monzano et al, 1996; Gorospe et al, 1997), these results suggest that attenuation of $p21^{wafl}$ expression by unrepaired UV lesions, with the concomitant accumulation of p53, favours apoptosis through (a) transactivation independent function(s) of p53.

Recombinant Ad vectors are frequently used to deliver transgenes for a variety of gene therapy strategies (reviewed in Hitt et al, 1997). As described in this chapter, expression of $p21^{wafl}$ led to a significant increase in UV survival of NDF (figure 3). This vector has been proposed as a possible gene therapy vector to prevent replication of tumour cells (Eastman et al, 1995) however these results and similar results from Bulmer and Rainbow for cisplatin sensitivity (unpublished results), suggest that combining this gene therapy approach with conventional therapies may be detrimental. Ad constructs expressing p53 have been suggested as a means of sensitizing tumour cells to conventional cancer therapies. Transient p53 expression from a recombinant Ad vector (Bacchetti and Graham, 1995) led to a significant increase in UV sensitivity in NDF at high fluence only (figure 8). Similar experiments from our laboratory suggest that this approach is only effective at high doses of cisplatin with increased resistance to low doses (Bulmer and Rainbow, unpublished results). The increase in resistance at low doses likely reflects the induction of protective functions of p53. Taken together, the net effect of modulation of p53pathways can have different effects

on resistance to DNA damaging agents, suggesting that it may be difficult to predict the net effect of protective and cytotoxic functions of p53 in combined cancer therapies.

Figure 7. UV fluence dependent expression of $p21^{waf1}$. NDF, XP-C, CS-A and XP-A fibroblasts were UV irradiated with 5 (circles), 10 (triangles) or 30 J/m^2 (inverted triangles) and $p21^{waf1}$ expression was assessed at several times following irradiation using a quantitative elisa assay. The mean ($\pm$ standard error) presented was determied from a minimum of 3 experiments.

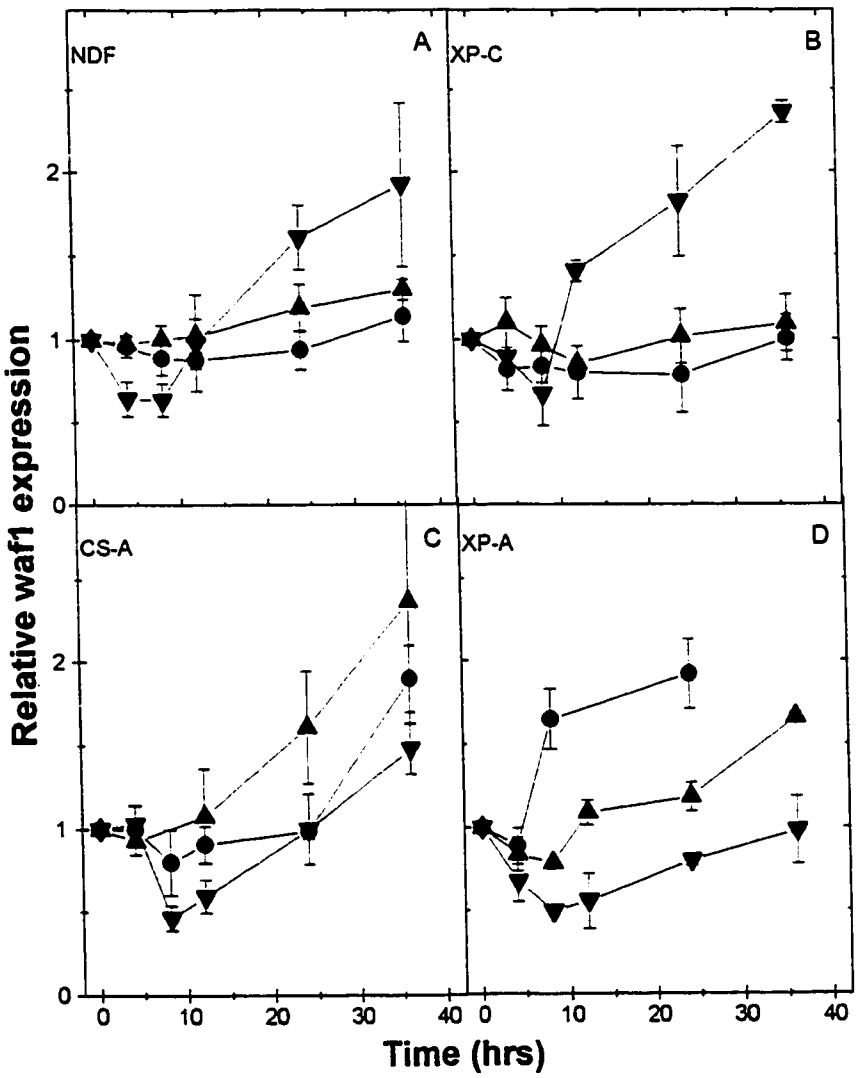
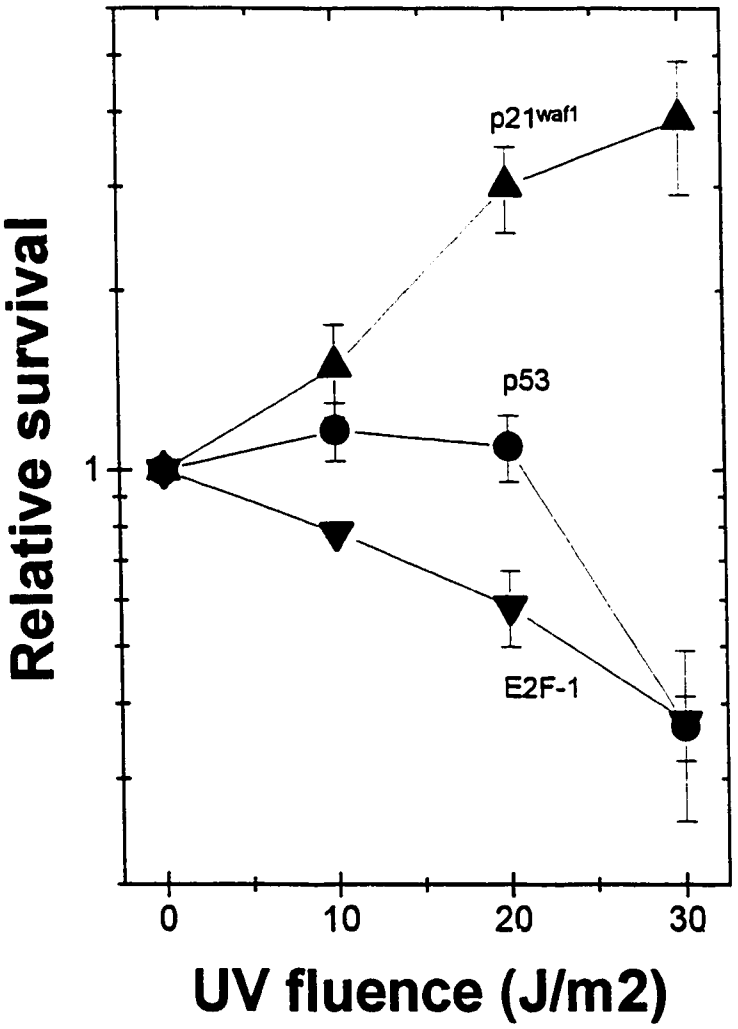

NDF
A
XP-C
B
CS-A
C
XP-A
D
Relative waf1 expression
Time (hrs)
0
1
2
0
10
20
30
40

Figure 8. Transient expression of p53 affects UV survival in a dose dependent manner. Clonogenic survival was assessed in NDF expressing the *lacZ*, p53, p21wafl or E2F-1 gene from recombinant Ad vectors. Survival determined for each treatment was normalized to the level of survival for *lacZ* expressing cells and is referred to as relative survival. Mean relative survival determined from several independent experiments performed in triplicate for p53 (circles), $p21^{wafl}$ (triangles) and E2F-1 (inverted triangles) is plotted with respect to UV fluence. Whereas p21wafl confers resistance to UV and E2F-1 confers sensitivity to UV, p53 expression increases sensitivity to high doses only.


Relative survival
1
p21waf1
p53
E2F-1
0
10
20
30
UV fluence (J/m2)

Chapter 9

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Appendix

The use of the polymerase chain reaction to assess repair of transcribed and non-transcribed regions of a replication defective adenovirus.

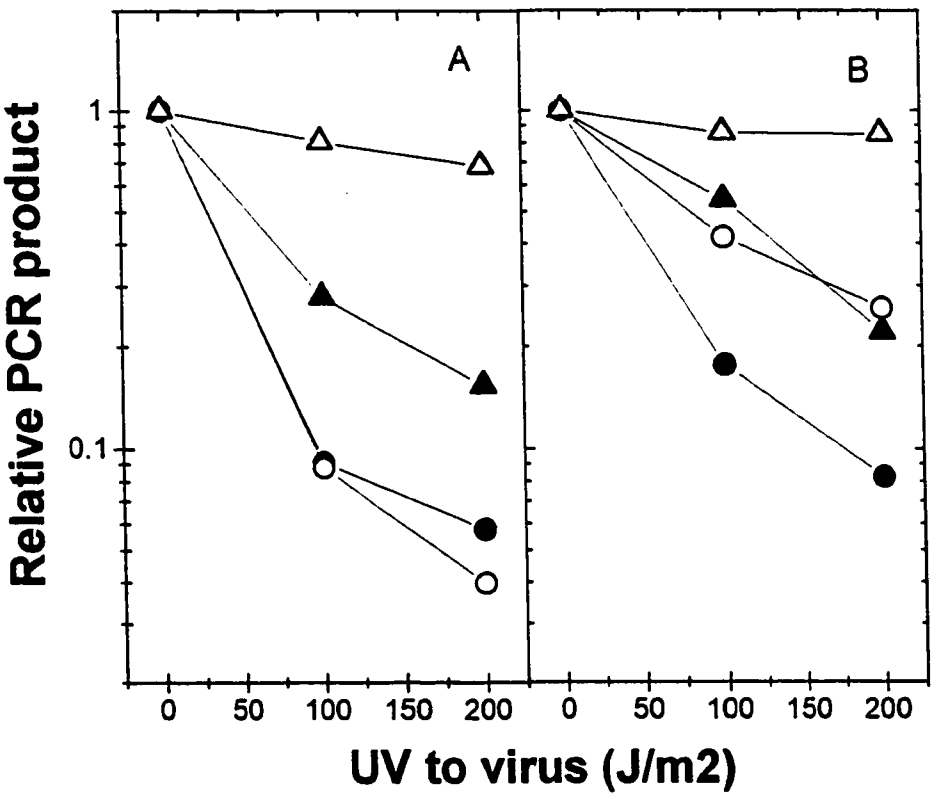
Bruce C. McKay and Andrew J. Rainbow

Our lab been able to assess constitutive and inducible levels of DNA repair within the transcribed strand of a UV damaged reporter gene in human cells. As reporter gene activity is the end point of these inducible DNA repair assays, repair of the template strand of the reporter gene is being assessed and thus inducible repair of inactive genes or the non template strand of active genes has not been evaluated. In an attempt to address this issue, we have developped a PCR based assay to assess repair of both the *lacZ* gene and the inactive E4 region of a recombinant replication defective Ad5 derived virus. In wildtype Ad5, the E1A gene product is the first viral gene product expressed and the expression of all other Ad genes is dependent on E1A expression (reviewed in Horwitz, 1990). The *lacZ* reporter gene has been inserted into the deleted E1 region of Ad5 making this recombinant deficient in the transcription of viral genes.

We have generated primers specific for both the *lacZ* reporter gene and the E4 region of Ad5. The *lacZ* primers (ATA CTG TCG TCG TCC CCT CAA A and GTT GTG GGC CAT AAT TCA ATT C) and the E4 primers (TAC TGG AGC CTT GGG TTT TGA T and GGT TGA AGG TGC TGG AAT GTT T) can be used to amplify 2.6 kpb and 2.8 kbp DNA fragments by the polymerase chain reaction (PCR). As the taq DNA polymerase used in PCR, is inhibited by UV irradiation, quantitative PCR has been used to assess the extent of UV induced DNA damage and repair in human cells (Kalinowski et al, 1992). The presence of transcribed and non-transcribed regions of the Ad genome allows a means of assessing constitutive and stress induced repair of transcriptionally inactive and active Ad DNA. An advantage of this approach is that it permits assessment of repair of both regions of the genome from a single sample.

PCR amplification of unirradiated and UV-irradiated Ad DNA yields a single PCR product from either primer pair. DNA obtained from PCR reactions was slotted blotted to nitrocellulose and probed with either ^{32}P labelled *lacZ* or E4 specific PCR product. Radioactivity was quantified with a phosphorimaging system. Preliminary experiments for HeLa and L132 cells are presented in figure 1). UV irradiation of viral DNA resulted in a fluence dependent reduction in the amount of PCR product relative to unirradiated controls. A partial recovery in the relative amount of PCR product was observed within 4 hrs (figure 1). In both L132 and HeLa cells, repair of the nontranscribed region (E4 region) of the genome appears to be more rapid than the transcribed (*lacZ*) region suggesting that transcription may impede NER in this system. This technique should be useful in assessing the constitutive and inducible levels of repair of UV-induced lesions from both inactive and active genes. In addition, this approach may prove useful in evaluating the effect of transcription on NER.

Figure 1. Repair of UV induced DNA damage can be detected in UV irradiated adenovirus using the polymerase chain reaction. Total DNA was extracted from L132 (A) and HeLa (B) cells at the time of infection (closed symbols) or 4 hrs (open symbols) following infection with UV-irradiated or unirradiated Ad5HCMVsp1*lacZ*. PCR amplification of the *lacZ* gene (circles) and the E4 region of Ad5 (triangles) was performed as previously reported (Kalinowski et al, 1992). Each panel represents a single experiment.


A
B
Relative PCR product
1
0.1
0
50
100
150
200
UV to virus (J/m2)