

Recombinant adenoviruses as expression vectors and as probes for DNA repair in human cells

Review Article

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Abbreviations: adenovirus, (Ad); Cockayne syndrome, (CS); cytomegalovirus, (CMV); genomic repair, (GGR); Host cell reactivation, (HCR); human papilloma virus, (HPV); immediate early, (IE); Li-Fraumeni syndrome, (LFS); nucleotide excision repair, (NBR); nucleotide excision repair, (NER); phosphate buffered saline, (PBS); retinoblastoma, (pRb); retinoblastoma, (Rb); RNA polymerase II, (RNAPII); SV40 large T antigen, (SV40LT); transcription coupled repair, (TCR); transcription coupled repair, (TCR); ultraviolet, (UV); xeroderma pigmentosum, (XP)

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Summary

There is widespread interest in the use of recombinant adenovirus (Ad) vectors for gene therapy of cancer and as tools in molecular biology research. There are also potential benefits to be gained by combining strategies for Ad-based gene therapy of cancer with radiotherapy and chemotherapy. However, there is limited information available on the effects of cytotoxic agents on transgene expression which would allow a rational approach to combining these modalities. We have used recombinant nonreplicating Ad expressing the lacZ gene under the control of the cytomegalovirus (CMV) immediate early promoter to assess the effects of cytotoxic agents on the expression of a reporter gene in human cells. Using this approach we are able to examine both constitutive and inducible expression of the reporter gene. Pretreatment of normal human cells with low UV fluence results in an enhanced expression of the reporter gene. The enhanced expression occurs at lower doses of the DNA damaging agent in cells deficient in the transcription coupled repair (TCR) pathway of nucleotide excision repair (NER) suggesting that the enhancement in human cells is triggered by persistent damage in actively transcribed genes. The enhancement is also reduced or absent in SV40-transformed cells and cells expressing the human papilloma virus ('WV) E7 gene, but not in Li-Fraumeni syndrome (LFS) cells or cells expressing the 'WV E6 gene. Since SV40-transformation and HPV E7 expression both abrogate the retinoblastoma (pRb) family of proteins, whereas 'WV E6 abrogates p53 and LFS cells express mutant p53, these results indicate that the enhanced expression depends on one or more of the pRb family of proteins, but not on p53. We have also used recombinant Ad expressing a lacZ reporter gene as a probe for DNA repair in human cells. Using this approach we have examined constitutive as well as inducible DNA repair of a UV-damaged reporter gene in human cells. We detected enhanced host cell reactivation (HCR) of a UV-damaged reporter gene in pre-heat-shock treated or pre-UV treated TCR proficient but not in pretreated TCR deficient human fibroblasts or LFS cells. These results suggest the existence of an inducible repair response for UV-damaged DNA in human cells which is dependent on the TCR pathway of NER and the wild type p53 tumour suppressor. These results have important implications for the use of recombinant Ad-based expression vectors under the control of the CMV promoter in gene therapy for cancer when used in combination with DNA damaging agents.

I. Introduction

Adenovirus (Ad) vectors are a very efficient method for delivering foreign genes into mammalian cells both *in vitro* and *in vivo* (Hitt et al, 1997) and show great promise for gene therapy of cancer (Boulikas, 1998; Stewart et al, 1999). Ad infects both dividing and non-dividing cells in a wide variety of tissues and cell types of many different species. The Ad genome is relatively easy to manipulate using standard molecular biology techniques such that both replication proficient and replication deficient vectors can be easily produced and purified on a large scale (Graham and Prevec 1991; Hitt et al, 1995). Replication proficient Ad vectors with only the early region 3 (E3) deleted can accommodate up to about 4 kb of foreign DNA, whereas replication deficient Ad vectors deleted in both E3 and E1 can accommodate up to about 8 kb.

Several reports have proposed the use of Ad transgene delivery in combination with radiation therapy and chemotherapy (Stackhouse and Buchsbaum, 2000). To combine gene therapy and radiation therapy or chemotherapy into an effective combination of modalities for the treatment of cancer it is essential to understand the effects of radiation treatment and chemotherapy treatment of cells and transgenes on transgene expression. We have used a recombinant nonreplicating human Ad, either Ad5HCMVsp1lacZ (Morsy et al, 1993) or AdCA35 (Addison et al, 1997), expressing the lacZ gene under the control of the cytomegalovirus (CMV) immediate early promoter to assess the effects of cytotoxic agents on the expression of a reporter gene in mammalian cells. Using this approach we are able to examine both constitutive and inducible expression of the reporter gene in cells treated

with DNA damaging agents (Figure 1, 2).

In addition we have used the same non-replicating Ad expressing the lacZ reporter gene as a probe for DNA repair in mammalian cells. Using this approach we are able to examine both constitutive and inducible DNA repair of a UV-damaged reporter gene in several different mammalian cell types including normal human fibroblasts, repair deficient human fibroblasts and several different human tumour cells (Figures 3, 5).

II. Recombinant adenovirus expression vectors for gene transfer into mammalian cells

A. Adenovirus based transgene expression levels in mammalian cells

The level of expression in cells infected by Ad vectors is greatly influenced by the promoter controlling expression of the transgene. Xu et al, (1995) have demonstrated that the human CMV immediate early promoter directs the highest level of expression in the widest variety of mammalian cell types *in vitro* when compared to that directed by the human b-actin, Ad major late, and SV40 early and late promoters. In addition, Addison et al, (1997) showed that the murine CMV immediate early promoter (Dorsch-Hasler et al, 1985) is also an extremely strong promoter and rivals the human CMV promoter for *in vitro* expression in both murine and human cells. *In vivo*, the highest level of expression reported to date occurred following intravenous delivery

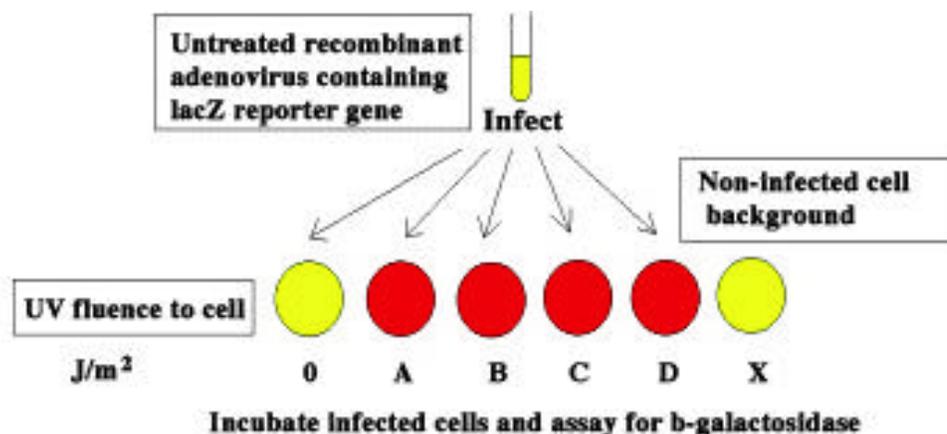


Figure 1. Enhanced expression of a recombinant adenovirus based reporter gene following pretreatment of human cells with UV. Cells were seeded in 96 well microtitre plates at a density of 2×10^4 cells/well, 18-24 h to treatment. For UV treatment of cells, the growth medium was aspirated and replaced with 40 ml phosphate buffered saline (PBS) and the cell monolayers were either left untreated or UV-irradiated with a range (A to D) of fluences. UV irradiation of cells was performed using a germicidal lamp (General Electric model G8T5) emitting predominantly 254 nm at a fluence rate of $1 \text{ J/m}^2/\text{s}$. After UV treatment, cells were either infected or at mock infected for 90 min at 37°C with untreated Ad5HCMVSP1lacZ in a total volume of 40 ml and the infected cells were incubated for a period of time (usually 12 - 48 h) before harvesting and scoring for β -galactosidase activity as reported previously (Francis and Rainbow 2000). Lysates from mock infected wells served as a measure of background levels for β -galactosidase activity.

to the mouse of an Ad vector under the control of the human CMV promoter (Kolls et al, 1994).

B. Enhanced expression of a lacZ reporter gene driven by the CMV immediate early promoter following pretreatment of human cells with DNA damaging agents

The CMV immediate early (IE) promoter is one of the most commonly used promoters in eukaryotic expression vectors, due primarily to its ability to yield high expression levels in many different mammalian cell types. Both stress-activated MAP protein kinases (Bruening et al, 1998) and ionizing radiation (Tang et al, 1997) can each up regulate expression of transgenes driven by the CMV promoter. Our protocol for assessing transgene expression in cells treated with DNA damaging agents is outlined in **Figure 1**. We report that up regulation of transgene expression from the CMV promoter in human cells also results from pretreatment of cells with UV light (Francis and Rainbow, 1997, 2000, **Figure 2**) or cisplatin (Francis 2000). UV radiation results in damage to DNA and the activation of cell surface receptors and their downstream signalling pathways. Although UV-induced DNA damage acts to directly block transcription, and cellular RNA levels immediately decrease following UV exposure (Mayne and Lehmann 1982), the expression of several cellular and viral genes are enhanced following UV exposure (Herrlich et al, 1994; Bender et al, 1997). These UV-inducible genes can be divided into those which respond immediately after UV exposure and those which have a response which is delayed and not observed until several hours after UV exposure (Herrlich et al, 1994; Bender et al, 1997). While the extent of the immediate response appears to depend on the magnitude of the initial UV insult, the strength and duration of the delayed response appears to be affected by the cells ability to repair UV induced DNA damage, particularly to its DNA (reviewed in Bender et al, 1997). Evidence for this comes from studies using cell lines which are deficient in repair of UV-damaged DNA (Miskin and Ben-Ishai, 1981; Blattner et al, 1998).

UV-induced up regulation of the CMV driven transgene in human cells appears to be a delayed response and expression from CMV-driven constructs is enhanced following lower UV exposures to TCR deficient compared to TCR proficient human fibroblasts (Francis and Rainbow, 1997, 2000; **Figure 2**). In addition, pre-infection of human fibroblasts with a UV-damaged Ad construct containing an actively transcribing gene can induce expression from a CMV driven transgene, while pre-infection with a UVdamaged Ad control vector which does not contain an actively transcribing gene (which presumably has minimal transcription activity) does not (Francis and Rainbow, 2000). Taken together, these data strongly suggest that persistent and unrepaired damage in active genes plays a direct role in eliciting enhanced

expression from CMV promoters. It is possible that it is the persistent stalling of RNA pol II at sites of unrepaired damage which acts as a trigger for this response following UV exposure as has been suggested for other UV-induced cellular responses (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996; Ljungman et al, 1999).

The p53 protein has been implicated in numerous cellular responses to UV, including DNA repair, and can regulate the expression of a large number of cellular genes (reviewed in McKay et al, 1999, Lakin and Jackson 1999, elDeiry 1998). We have examined UV-induced expression of a CMV-driven reporter construct in HeLa cells, 5V40-transformed fibroblasts, LFS fibroblasts, and spontaneously immortalised LFS sublines (Francis and Rainbow 2000). These cells have impaired p53 function as a result of human papilloma virus (HPV) E6-expression (Seedoif, 1987; Scheffner et al, 1990; Mietz et al, 1992), SV40 large T antigen (SV40LT) expression (Segawa et al, 1993), geamime transmission of a mutant p53 allele

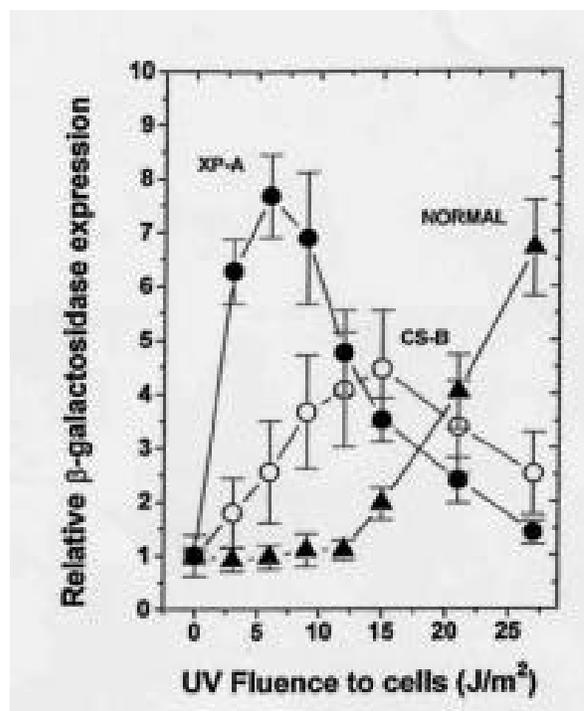


Figure 2. Enhanced expression of the CMV-driven β -galactosidase transgene in Ad5HCMVSp1lacZ following pretreatment of human fibroblasts with UV light. β -galactosidase reporter activity was quantitated 24 h following UV irradiation and subsequent infection at 10-20 plaque forming units per cell of normal (GM 38 (▲)), XP-A (GM XP12BE (●)), and CS-B (CS IAN (○)) fibroblasts with a highly purified preparation of AdHCMVsp1lacZ. Values were normalised to unirradiated controls. Each point is the average of 2 independent experiments (\pm SEM), each performed in 6 replicates. Adapted from Rainbow and Francis, 2000

(Malkin et al, 1990), and spontaneous loss of the wild type p53 allele from LFS fibroblasts (Yin et al, 1992), respectively. Although no UV induced expression of the CMV-driven lacZ gene from Ad5HCMVsp1lacZ was observed in any SV40transformed line examined, a significant UV-enhancement of reporter expression was observed in both ReLa and all LFS cell strains (Francis and Rainbow, 2000). These data suggest that p53 does not play an essential role in the UV-induced expression from CMV promoters, whereas some protein or pathway altered by SV40-transformation does play an essential role in this response. Candidate proteins altered by SV40-transformation include members of the retinoblastoma (Rb) family, which are known to play important roles in stress signalling, repair, and transcription (along with several other pathways). Since the pRb family of proteins are also altered in HeLa cells due to expression of the HPV E7 gene, the results of UV-enhanced expression of the reporter in HeLa cells might suggest that pRb does not play an essential role. However, it has been reported that, although HPV E7 binds pRb and its family members p107 and p130, only pRb is targeted for degradation, while the levels of the two other proteins are not significantly altered by E7 expression (Berezutskaya et al, 1997) Furthermore, even pRb remains at significant levels and accumulates still higher in ReLa cells following UV exposure (Pedley et al, 1996). Thus it is possible that sufficient levels of one or more of the pRb family of proteins remain in HeLa cells to induce expression of reporter activity. In addition, more recent experiments using pRb-null or p53-null mouse embryo fibroblasts (Francis and Rainbow, 1999a; Francis, 2000) and human fibroblasts transformed with the HPV E7 or HPV E6 gene (Francis and Rainbow, unpublished data) support our earlier data indicating that p53 does not play an essential role and suggest a role for the pRb protein(s) in UV-enhanced expression from a CMV-driven reporter. Since the pRb proteins and other pathways involving stress-activated MAP protein kinases (Bruening et al, 1998) are frequently altered in human tumour cells, the outcome of protocols combining gene therapy and radiotherapy or chemotherapy using CMV-driven transgenes may be tumour cell type specific.

Other reports have suggested the use of ionizing radiation-activated gene therapy vectors for combined gene therapy and radiotherapy (Joki et al, 1995; Seung et al, 1995; Mauceri et al, 1996; Takahashi et al, 1997; Tang et al, 1997; Marples et al, 2000; Scott et al, 2000; Stackhouse and Buchsbaum 2000). -ray enhanced expression from a reporter gene is both cell-type specific and promoter specific (Tang et al, 1997; Marples et al, 2000) and -ray enhanced expression from the CMV promoter was only detected when cells were irradiated and the transgenes were not. No amplification of the transgene was detected when both host cells and transgene were subjected to irradiation (Tang et al, 1997). In contrast, -ray induced expression of a plasmid born reporter gene

under the control of a synthetic radio-responsive transcriptional enhancer could be repeated by additional radiation treatments in human tumour cells (Marples et al, 2000). These results suggest that depending on the promoter of the transgene, the timing sequence of gene-therapy and radiotherapy or chemotherapy may be an important determinant of clinical outcome. It thus appears likely that with additional information on the various parameters controlling the up regulation of transgene expression, adenovirus-mediated gene therapy and radiotherapy or chemotherapy can potentially be formulated into synergistic protocols for the treatment of cancer.

III. Recombinant adenovirus as a probe for DNA repair in mammalian cells

A. Nucleotide excision repair of damaged DNA

The integrity of the human genome is constantly being compromised by alterations induced by a wide variety of exogenous physical and chemical agents, as well as by products of cellular metabolism. Several highly conserved repair pathways have evolved to remove damage from cellular DNA and disruption of each of these DNA repair pathways is associated with carcinogenesis (as reviewed in Friedberg et al, 1995). The nucleotide excision repair (NER) pathway repairs a wide range of bulky DNA adducts induced by numerous carcinogenic and antineoplastic compounds, including ultraviolet (UV) irradiation from the sun. NER can be divided into two interrelated subpathways: (1) transcription coupled repair (TCR) which preferentially removes DNA damage at a faster rate from the transcribed strand of actively transcribed genes, and (2) global genomic repair (GGR) which removes damage from throughout the entire genome and from the non-transcribed strand of active genes (Mellon et al, 1986, 1987). Individuals with the genetic diseases xeroderma pigmentosum (XP) have some deficiency in NER and show an increased incidence of a variety of skin cancers (Wei et al, 1993, Kraemer et al, 1994). Several additional links between NER and carcinogenesis have been reported. Mutations in the p53 tumour suppressor gene, the most commonly altered gene in malignancy (Hollstein et al, 1991), have also been shown to result in reduced NER (Ford and Hanawalt 1995; Smith et al, 1995; Wang et al, 1995; McKay et al, 1997; McKay et al, 1999; Therrien et al, 1999). Mismatch repair proteins have also been implicated in NER (Mellon et al, 1996) and mutations within genes encoding these proteins are associated with hereditary non-polyposis colorectal cancer (Fishel et al, 1993). Decreased NER has also been observed in a variety of tumour and transformed cell lines (Squires et al, 1982; Rainbow 1989). These reports suggest that DNA repair mechanisms are disrupted

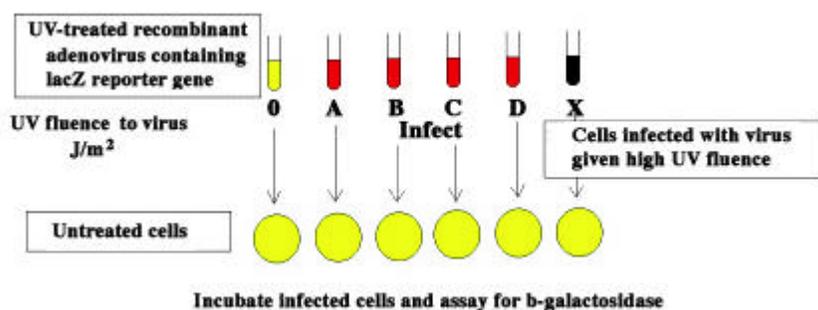


Figure 3. Host cell reactivation of a UV-damaged recombinant adenovirus based reporter gene in human cells. Cells were seeded in 96 well microtitre plates at a density of 2×10^4 cells/well and 18-24 h later infected for 90 min at 37°C with unirradiated or UV-irradiated AdS HCMVsp1lacZ in a total volume of 40 μl . AdSHCMVsp1lacZ was UV-irradiated with range of fluences (A to D) using a germicidal lamp (General Electric model G8T5) emitting predominantly at 254 nm at an incident fluence rate of $2 \text{ J/m}^2/\text{s}$. 12-48 h later, infected cells were harvested and scored for β -galactosidase activity as reported previously (Francis and Rainbow 1999). Lysates from wells infected with heavily irradiated AdSHCMVsp1lacZ ($10,000 \text{ J/m}^2$), X, served as a measure of background levels for β -galactosidase activity.

in tumour cells and that DNA repair contributes to resistance to neoplasia. XP is composed of a minimum of seven complementation groups (XP-A-G), each displaying a general deficiency in NER which compromises at least the GR subpathway and usually TCR as well. The exception is XP-C which retains viable TCR in spite of a severe deficiency in GGR (Venema et al, 1990, 1991). The genetic disease Cockayne syndrome (CS) is also associated with a deficiency in NER, although unlike

XP patients, CS patients do not display an increased risk of skin cancer. Two complementation groups of CS (CS-A and CS-B) have been identified, each of which exhibits a deficiency in TCR, while the GGR pathway appears to function normally (Venema et al, 1990a; van Hoffen et al, 1993). The XPB and XPD proteins are components of the transcription factor TFIIH (Schaeffer et al, 1993, Schaeffer et al, 1994) which plays a role in both NER and transcription by RNA polymerase II (RNAPII) (Drapkin et al, 1994). The CSA and CSB proteins co-immunoprecipitate (Henning et al, 1995) and are required for TCR. CSA interacts with TFIIH directly (Henning et al, 1995) whereas CSB does so via XPG (Iyer et al, 1996). TFIIH, XPG and the RPAIXPA/XPFIERCC1 complex (Park and Sancar 1994; Matsuda 1995) are required for both subpathways of NER, whereas the XPC/HHR23B complex (Masutani et al, 1994) appears to be required only for GGR (Venema et al, 1990a, 1991; Evans et al, 1993).

B. Host cell reactivation of DNA-damaged reporter genes in mammalian cells

Host cell reactivation (HCR) of reporter gene activity has been assessed in mammalian cells using either recombinant Ad or plasmid constructs by a number of different laboratories.

For plasmid constructs, this approach has typically involved the transfection of a DNA-damaged plasmid carrying a reporter gene into the cells of interest (Protic et al, 1988; Ganesan and Hanawalt 1994; Smith et al, 1995; Stevsner et al, 1995; Ganesan et al, 1999). Since primary human fibroblasts take up exogenous DNA 10-100 times less efficiently than either rodent or human cell lines derived from tumour tissue or transformed by viral antigens (Murnane et al, 1985; Canaani et al, 1986; Hojmakers et al, 1987), most experiments examining the reactivation of plasmid born UV-damaged reporters has been performed in tumour and transformed cell lines rather than primary human fibroblasts. However, many tumour and transformed cells have been reported to have a reduced DNA repair capacity for UV-damaged DNA (Squires et al, 1982; Rainbow 1989; McKay and Rainbow 1996; McKay 1997). Furthermore, the cellular response to DNA damage is stimulated by at least some transfection procedures (Renzing and Lane 1995; Seiget et al, 1995), leading to cell cycle arrest (Renzing and Lane 1995), suggesting that the transfection procedure itself may affect the outcome of DNA repair experiments. In contrast, recombinant non-replicating Ad reporter constructs have the ability to infect and express high levels of recombinant gene products in most cell types including primary human fibroblasts. Furthermore, non-replicating Ad reporter constructs do not appear to elicit the DNA damage response, or inhibit host DNA synthesis following infection (Blagoskionnay and el-Deity 1996).

Recombinant non-replicating Ad constructs have been used to introduce UV-damaged (Valerie and Singhal 1995; McKay and Rainbow 1996; McKay et al, 1997; Francis and Rainbow 1999) or cisplatin damaged (Moorehead et al, 1996) reporter genes into non-treated human and rodent cells in order to assess the repair of damaged DNA in the

absence of cellular stress using host cell reactivation (HCR) of reporter gene activity as an endpoint. UV-induced lesions in the template strand of active genes inhibit progression of RNA polymerase II (Donahue et al, 1994) and a single UV-induced cyclobutane pyrimidine dimer (CPD) is thought to be sufficient to inhibit reporter gene expression (Protic-Sabaji and Kraemer 1985; Francis and Rainbow 1999). UV-induced DNA lesions are removed from plasmid born (Ganesan and Hanawalt 1994, Ganesan et al, 1999) and recombinant adenovirus born (Boszko 2000, Boszko and Rainbow 2000) reporter genes when introduced into repair proficient human cells and the removal is reduced when the same reporter genes are introduced into NER deficient cells. Thus HCR of reporter gene activity is thought to require the repair of transcription blocking DNA lesions and reflect repair of DNA lesions in the transcribed strand.

A typical protocol to examine HCR of reporter gene activity for UV-irradiated recombinant Ad expressing the lacZ gene is shown in **Figure 3**. Using this approach we have shown that HCR of reporter gene activity for UV damaged DNA is reduced in several different nucleotide excision repair (NER) deficient cells of both human and rodent origin, including skin fibroblasts from patients with

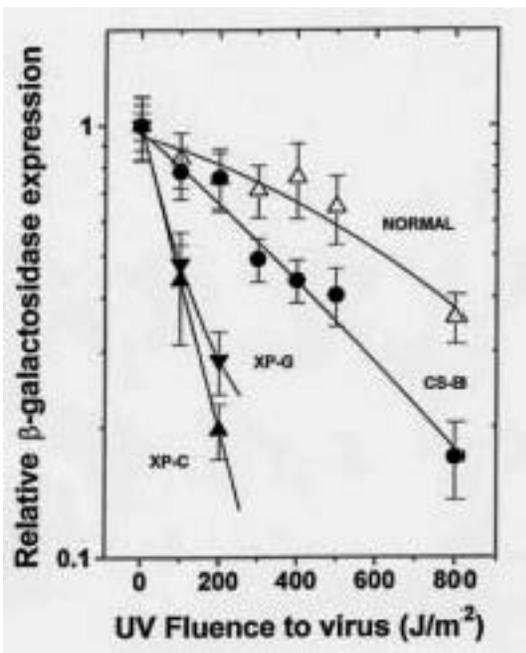


Figure 4. Nucleotide excision repair deficient cell strains show reduced HCR of UV-irradiated reporter activity. Lines represent repair proficient normal GM 3440 (○); and repair deficient XP-C (XP3BE (▲)), XP-G (XP2BJ (▼)) and CS-B (CS IAN (●)) primary human fibroblasts. Each point is the average of 4 replicates, error bars represent one standard error. Untreated cells were infected with unirradiated or UV-irradiated virus at 10-20 plaque forming units per cell and scored for β -galactosidase activity 40-44 h later. Adapted from Francis and Rainbow 1999.

xeroderma pigmentosum from complementation group C (XPC) which showed HCR levels ranging from 25-75% that obtained in NER proficient normal primary human fibroblasts (**Figure 4**, McKay and Rainbow 1996; Francis and Rainbow 1999). The result for XP-C is surprising since XP-C cells are reported to be proficient in transcription coupled repair (TCR) and thus would be expected to reactivate UV induced lesions in the transcribed strand of the reporter gene. Blockage of RNA polymerase II by UV induced DNA lesions does not appear to be sufficient to promote the preferential repair of these transcription blocking lesions in non-UV-treated XP-C cells (McKay and Rainbow 1996; Francis and Rainbow 1999). Also of interest was the finding that CS fibroblast strains retained a considerable ability to repair the UV-damaged reporter gene in non-treated cells (57-90% of normal levels) in spite of their being characterized as deficient in repair of the transcribed strand of active genes following UV irradiation of the cell (Francis and Rainbow 1999). It is therefore apparent that in the absence of UV exposure to the cell, damage in the transcribed strand of the recombinant Ad-based reporter gene is repaired to a large extent by the global genomic repair (GGR) pathway of NER in primary human fibroblasts, although significant TCR must also occur since HCR is also reduced in the CS strains. Since the Ad 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 following UV treatments used to examine repair in cellular DNA.

Several studies using plasmid born reporter genes have reported decreased HCR in NER deficient XP cells (Lehmann and Ooman, 1985; Protic-Sabljić and Kraemer, 1985; Barrett et al, 1991) and CS cells (Klocker et al, 1985; Barrett et al, 1991) compared to repair proficient human cells. These studies have generally used SV40-transformed repair deficient XP and CS cell lines and repair proficient "normal" cell lines derived from human tumours rather than primary fibroblast strains. SV40 transformed cells and many human tumours have alterations in the p53 tumour suppressor, the pRb tumour suppressor and other stress activated pathways which have been shown to affect the GGR and/or TCR pathway of NER (Ford and Hanawalt, 1995, 1997; McKay et al, 1997; 1999; Ford et al, 1998; Therrien et al, 1999). Therefore, a direct comparison of the relative contribution of TCR and GGR to repair in the transcribed strand of plasmid born reporter genes using SV40-transformed cells and tumour cells with that obtained using an Ad based reporter in primary human fibroblasts may not be appropriate.

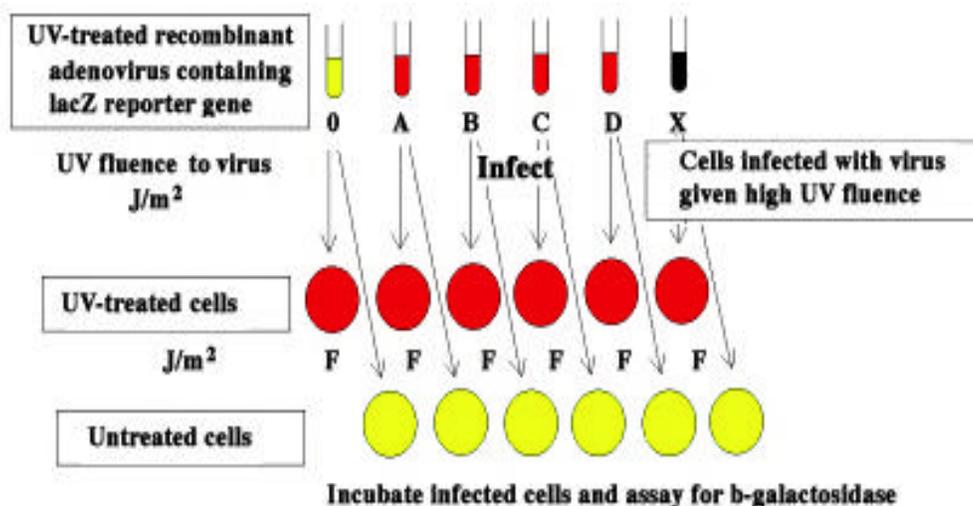


Figure 5. UV-enhanced host cell reactivation of a UV-damaged recombinant adenovirus based reporter gene in human cells. Cells were seeded in 96 well microtitre plates at a density of 2×10^4 cells/well, 18-24 h prior to UV-treatment of cells. The growth medium was then aspirated, replaced with 40 ml phosphate buffered saline (PBS) and sets of cell monolayers were either UV-irradiated with a fluence F or were mock-irradiated and received no UV. After treatment, both UV-irradiated and non-irradiated sets of cells were infected for 90 min at 37 °C in a total volume of 40 ml with unirradiated Ad5HCMVsp1lacZ or Ad5HCMVsp1lacZ which had been UV-irradiated with a range of fluences (A to D). Infected cells were incubated for a period of time (usually 12 - 48 h) before harvesting and scoring for b-galactosidase activity as reported previously (Francis and Rainbow 1999). Lysates from wells infected with heavily irradiated Ad5HCMVsp1lacZ ($10,000 J/m^2$), X, served as a measure of background levels for b-galactosidase activity. UV irradiation of cells and virus was as for Figures 1 and 3 and as described previously (Francis and Rainbow 1999). Enhanced HCR in UV-treated compared to non-treated cells suggests inducible repair of the UV-damaged reporter gene.

B. Enhanced host cell reactivation of a UV-damaged reporter gene following pretreatment of mammalian cells with DNA damaging agents

1. Evidence for inducible DNA repair.

Examination of DNA repair in mammalian cells generally requires that the cells are treated with a DNA damaging agent in some manner, which makes it difficult to determine if the repair pathways are constitutively active or induced by the DNA damaging agent. In contrast, the use of viral probes for DNA repair allows the virus and cell to be treated with a DNA damaging agent independently and thus allows an examination of both constitutive and inducible pathways affecting survival of the virus or expression of the reporter gene. There are many reports showing that pretreatment of a variety of different mammalian cells with chemical or physical DNA damaging agents results in an increased survival (or enhanced reactivation) for several nuclear replicating double stranded DNA viruses damaged by UV or ionising radiation (for a review see Rainbow 1981, Defais et al, 1983). It has been suggested that the enhanced reactivation of DNA-damaged viruses results, in part at least, from an induced DNA repair pathway (Jeeves and Rainbow 1983, 1983a, 1983h; Bennett and Rainbow 1988; Brown and Cenrutti 1989).

We and others have examined HCR of a UV-damaged reporter gene in pre-treated compared to non-treated cells (McKay et al, 1997; Li and Ho 1998; Francis and Rainbow 1999; Boszko and Rainbow 2000). A typical protocol for these enhanced HCR experiments is shown in **Figure 5**. Using this approach we show that pre-treatment of normal human fibroblasts with low UV fluences (McKay et al, 1997; Francis and Rainbow 1999) as well as heat shock (McKay and Rainbow 1996; McKay et al, 1997) results in enhanced HCR of the UV-damaged reporter suggesting the presence of inducible DNA repair in human cells. Prior exposure of cells to low UV fluences or heat shock resulted in enhanced HCR for expression of the UV-damaged reporter gene in normal and XP-C fibroblast strains, but not in TCR deficient XP and CS strains (**Figure 6**). These results suggest that UV or heat shock treatment results in an induced repair of UV-damaged DNA in the transcribed strand of the reporter gene in normal and XP-C cells through an enhancement of TCR or through a mechanism which involves the TCR pathway. More recently we have used a novel quantitative polymerase chain reaction (PCR) technique to examine direct removal of UV-induced photoproducts from lacZ reporter gene in AdHCMVsp1lacZ following infection of human fibroblasts. Using this technique we show a significant removal of UV photoproducts after infection

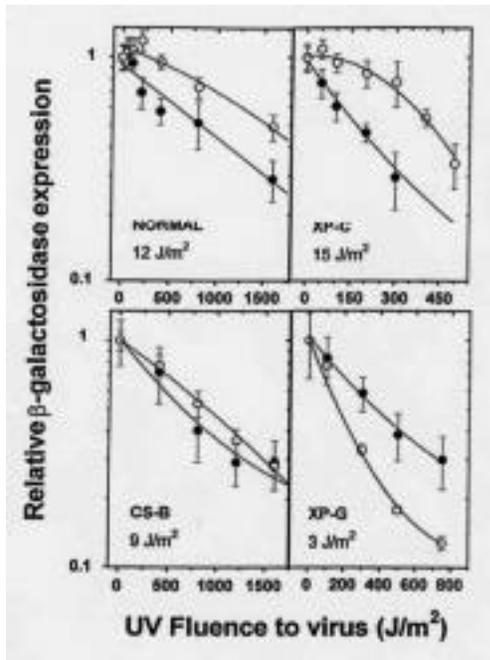


Figure 6. Pre-UV irradiation of cells results in enhanced HCR of UV-irradiated reporter activity in normal and XP-C but not in other TCR deficient cells. Results of typical experiments representing unirradiated (●) and UV-irradiated (○) primary human fibroblasts. UV exposures to cells are indicated on the figure and cell strains presented are GM 3440 (normal), XP3BE (XP-C) CS1BE (CS-B), and XP2BI (XP-G). Immediately following UV exposure cells were infected with unirradiated or UV-irradiated virus at 10-20 plaque forming units per cell and scored for β -galactosidase activity 40-44 h later. Each point is the average of 4-6 replicates, error bars represent one standard error. Adapted from Francis and Rainbow 1999.

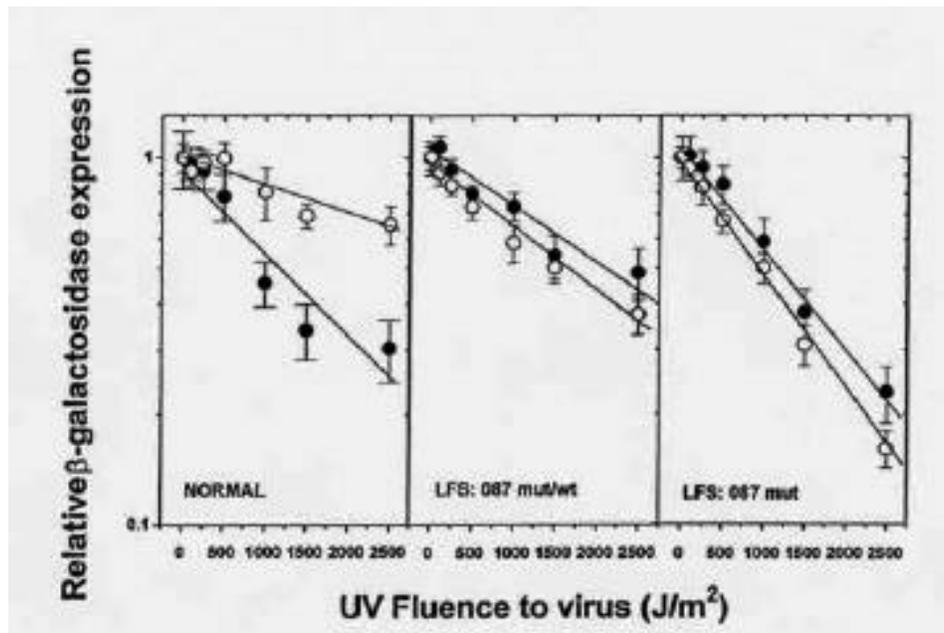


Figure 7. Pre-UV irradiation of cells results in enhanced HCR of UV-irradiated reporter activity in normal but not in Li-Fraumeni syndrome cells. Results of typical experiments representing unirradiated (●) and UV-irradiated with 15 J/m^2 (○) normal human fibroblasts and Li-Fraumeni syndrome (LFS) cells. Cell strains and cell lines presented are GM 9503 (normal), LFS: 087 mut/wt (heterozygous for a mutation in p53), LFS 087 mut (expressing only mutant p53). Immediately following UV exposure cells were infected with unirradiated or UV-irradiated virus at 10-20 plaque forming units per cell and scored for β -galactosidase activity 40-44 h later. Each point is the average of 3-6 replicates, error bars represent one standard error. Adapted from McKay et al, 1997 and Francis 2000.

of normal human fibroblasts, but a reduced removal of lesions, compared to normal fibroblasts, after infection of NER deficient XP and CS fibroblasts. In addition, we show that pre-UV exposure of normal human fibroblasts results in an enhanced rate of removal of photoproducts from the reporter gene, giving evidence that UV-enhanced HCR for expression of the UV-damaged reporter gene results from enhanced removal of UV-induced lesions from DNA (Boszko 2000, Boszko and Rainbow, 2000). Other evidence for damage-induced DNA repair pathways in mammalian cells comes from a number of studies including the enhanced DNA repair capacity of mammalian cells following carcinogen treatment (Protic et al, 1988), the p53-mediated enhancement of NER by the DNA damaged induced GADD45 gene (Smith et al, 1996, Smith et al, 1994) and the identification of a novel DNA repair response which is induced by irradiation of cells at the GuS border (Leadon et al, 1996). Pretreatment of normal human lung fibroblasts with the drug emodin enhances NER of UV and cisplatin damaged DNA (Chang et al, 1999) and pretreatment of cells with dinucleotides prior to UV irradiation increased the repair of UV-induced DNA damage as assessed by unscheduled DNA synthesis (Eller et al, 1997). Pre-treatment of human cells with quinacrine mustard resulted in an enhanced removal of UV-induced CPD from both the transcribed and the non-transcribed strand of the p53 gene (Ye et al, 1999), also giving evidence for an inducible NER response in human cells. Most of these studies provide evidence for an induction of the GGR rather than the TCR pathway of NER (McKay et al, 1999). Some mammalian cells exhibit a hypersensitivity to low doses of x-rays or cisplatin, but increased resistance following higher doses of these agents (Skov et al, 1994, Joiner et al, 1996, Caney et al, 2000). The increased radioresistance at higher x-ray doses is absent in some DNA repair deficient cell lines (Skov et al, 1994), and hypersensitivity at low doses of x-rays or cisplatin can be removed by pretreatment of cells with "priming doses" of a DNA damaging agent (Joiner et al, 1996, Caney et al, 2000), suggesting an inducible DNA repair response in mammalian cells. Pre-exposure of human cells with low "priming" doses of ionising radiation leads also to an enhanced removal of thymine glycols after higher doses (Le et al, 1998) providing evidence for an inducible repair of base damage in human cells.

2. Evidence for the involvement of p53 in NER

Over the past few years it has become clear that p53 and/or p53 regulated gene products contribute to NER of UV-induced DNA damage in mammalian cells (Smith et al, 1994, 1995, 1996; Ford and Hanawalt 1995, 1997; Wang et al, 1995; McKay et al, 1997, 1997a; Ford et al, 1998; Li and Ho 1998). We have reported that UV and heat shock enhanced HCR for expression of the UV-

damaged reporter gene was absent in Li-Fraumeni cells expressing mutant p53 (**Figure 7**, McKay et al, 1997, 1999) indicating a role for p53 in the induced DNA repair response. A similar p53 dependent enhancement in HCR for a CMV driven plasmid based and UV-damaged reporter gene has been reported in UVB-treated murine fibroblasts (Li and Ho 1998). In addition, thymine dinucleotides have been shown to induce the reactivation of a UV-damaged reporter gene under control of the SV40 early promoter, by a process which may also involve p53 (Eller et al, 1997). Furthermore, Huang et al 1998 report that transcription from a p53 driven promoter in the presence of wild-type p53 results in up regulation of both transcription and repair of a UV-damaged reporter gene, and that the enhanced DNA repair of the reporter gene is a separate and distinct activity of p53, but is dependent on p53 driven transcription. As discussed above, UV and heatshock enhanced HCR of the recombinant Ad-based and UV-damaged reporter gene are thought to reflect an induction of TCR or a repair process dependent on TCR. The absence of UV and heat-shock enhanced HCR of the UV-damaged reporter in LFS cells suggests further that either TCR or a repair process dependent on TCR requires functional p53.

Hwang et al. 1999 have reported that transcription from the p48 gene, which is mutated in GGR-deficient, damage-specific DNA binding (DDB) protein deficient, XP-E cells (Hwang et al, 1998), is up regulated (in a p53-dependent manner) in response to UV treatment in human cells. This provides a model for a UV-inducible GGR response in human cells which is dependent on p53 transcription. A UV-induced increase in p48 transcription would require removal of UV-induced lesions from the p48 gene and therefore be dependent on TCR as has been reported for other p53 responsive genes (McKay et al, 1999, McKay and Ljungman, 1999). Thus the UV-induced up regulation of p48 leading to enhanced GGR would be expected to be dependent on both wild-type p53 and TCR. It is thus possible that the p53 and TCR dependent UV-enhanced repair of the UV-damaged reporter gene results from an up regulation of GGR in the transcribed strand of the reporter gene mediated by a UV-induced up regulation of the p48 gene product. Previous reports have also suggested that the DDB protein is responsible for the enhanced repair of UV-damaged expression vectors (Protic et al, 1989). However, some recent reports suggest that TCR also may be up regulated by a p53 dependent mechanism. Pre-treatment of human cells with low doses of quinacrine mustard resulted in an enhanced rate of removal of CPD by NER (Ye et al, 1999). Although the enhanced rate of removal was greater for non-transcribed strand, an enhanced rate of removal also occurred for the transcribed strand of an exon 9 portion of the p53 gene, such that both GGR and TCR may be up regulated by pre-treatment. In addition, Therrien et al, 1999 showed that the rate of repair of UV-induced CPD was reduced along both the transcribed and the non-transcribed strands of the p53

and/or c-jun loci in LFS cells expressing mutant p53 and human fibroblasts expressing the human papilloma virus (HPV) E6 oncoprotein that functionally inactivates p53. The reduction in the rate of CPD repair for the LFS cells compared to normal cells was considerably greater in the transcribed (6 fold) compared to the non-transcribed strand (3 fold) providing evidence that both TCR and GGR are dependent on wild-type p53 in UV-irradiated human cells. Our results for UV-enhanced HCR of a UV-damaged reporter gene are therefore also consistent with a model in which pretreatment of cells with UV results in an up regulation of TCR through a p53 dependent mechanism. It is possible that a p53 dependent up regulation of both GGR and TCR can contribute to UV-enhanced HCR of a UV-damaged reporter gene.

3. Gene therapy using Ad vectors expressing p53 and p53 responsive genes

The p53 tumor suppressor and several p53 responsive genes also play a role in arresting the cell cycle at the G1 checkpoint in response to DNA damage and in inducing apoptosis in cells that have received extensive radiation damage (Hartwell and Kastan, 1994; Hinds and Weinberg, 1994). The p53 gene and other tumor suppressor genes have been found to be mutated in a variety of tumours and many of these mutations are thought to be responsible for the proliferative capacity and resistance of these cells to radiotherapy and chemotherapy. On this basis, both p53 and the p53 responsive gene p21^{waf1} have been proposed as gene therapy vectors to prevent replication of tumor cells. p21^{waf1} is a member of the family of cyclin-dependent kinase (CDK) inhibitors and plays a role in the maintenance of the cell cycle checkpoints and cell progression (Harper et al, 1993). Following infection of cells with Ad expressing a p53 transgene *in vitro*, the biological effects of p53 are readily detected, including the upregulation of p21^{waf1}, an overall growth suppression, and an increased number of cells undergoing apoptosis for a variety of tumour cell lines carrying p53 mutations (Bacrietti and Graham, 1993; Liu et al, 1994; Yang et al, 1995). Furthermore, administration of p53 expressing Ad vectors has been found to be efficacious in several tumor models (Fujiwara et al, 1994; Lui et al, 1994; Yang et al, 1995). *In vitro* infection of a variety of tumour cells with p21^{waf1} recombinant Ad vectors induces a growth arrest at the G₀/G₁ checkpoint without inducing apoptosis (Eastam et al, 1995; Katayose et al, 1995; Yang et al 1995) and p21^{waf1} expressing Ad vectors have been reported to suppress tumour growth *in vivo* (Eastam et al, 1995; Yang et al, 1995).

Ad constructs expressing p53 have been suggested as a means of sensitizing tumor cells to conventional radiotherapy and chemotherapy (Fujiwara et al, 1994). However, this approach may be detrimental in some situations. Down regulation of the p53 responsive GADD45 gene decreased DNA repair and sensitized cells

to UV irradiation and cisplatin (Smith et al, 1996) whereas upregulation of the p53 responsive p21^{waf1} gene by Ad-mediated transgene expression results in an increased resistance of cells to UV and cisplatin (McKay et al, 1998, Bulmer and Rainbow, unpublished data). Recently it has been reported that p53 expression protects against or confers sensitivity to UV-induced apoptosis depending on the timing of p53 expression relative to the genotoxic stress (McKay et al, 2000). Thus it is possible that upregulation of p53 and p53 responsive genes such as p21^{waf1} and GADD45 through the use of gene therapy vectors may result in the upregulation of p53 protective functions, including DNA repair, resulting in an enhanced resistance of tumor cells to radiation and chemotherapy. Furthermore, we have found that expression of p53 regulated gene products is both positively and negatively regulated by DNA damage depending on the cell type and the extent of such damage (McKay et al, 1998). Therefore, it may be difficult to predict the net effect of protective and cytotoxic functions of p53 in combined therapies.

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