THE REPAIR OF CISPLATIN-DAMAGED DNA IN HUMAN CELLS

AN EXAMINATION OF THE REPAIR OF CISPLATIN-DAMAGED DNA IN HUMAN CELLS USING ADENOVIRUS AS A PROBE

By

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ABSTRACT

The repair of DNA damage is vital to the health and survival of organisms and their cells. In humans, there exist several disorders that involve the inefficient processing or repair of DNA damage. Cellular sensitivity to DNA-damaging agents is a hallmark of repair-deficient syndromes which are often associated with an increased risk of cancer. In this work, I have investigated the repair of cisplatin-damaged DNA by utilizing host cell reactivation and cellular capacity assays that assess DNA repair using adenovirus (Ad) as a probe. Cisplatin is a widely used chemotherapeutic drug that induces both intrastrand and interstrand crosslinks in DNA. The host cell reactivation (HCR) assay examines the ability of host cells to repair and hence, replicate cisplatin-damaged Ad DNA. This assay is believed to primarily be a measure of bulk nucleotide excision DNA repair. The cellular capacity assay examines the ability of cisplatin-damaged cells to support the replication of undamaged Ad DNA, and is thought to reflect the repair of the active cellular genes necessary for Ad replication. The repair of cisplatin-damaged DNA was studied in three human genetic syndromes - Roberts syndrome (RS), xeroderma pigmentosum (XP) and Li-Fraumeni syndrome (LFS). Fanconi's anemia (FA) cells were also used as a control strain.

RS is characterized by growth retardation, limb reductions and craniofacial abnormalities. Cells from a subset of RS patients, termed RS+, are hypersensitive to several DNA-damaging agents, and it has been suggested that this hypersensitivity may result from a deficiency in the DNA repair capacity of these cells. XP patients are sensitive to ultraviolet light and are prone to the development of skin cancers. Cells from these patients are deficient in the nucleotide excision repair (NER) pathway responsible for repair of UV-induced lesions. Patients with FA have a variety of congenital abnormalities, including a high susceptibility to leukemia. FA cells are sensitive to DNA-crosslinking agents such as mitomycin C (MMC) and cisplatin. Using the HCR and cellular capacity assays, deficiencies in DNA repair were detected in the XP and FA

fibroblasts but not in the RS+ fibroblasts when compared to normal strains. The NER-deficient XP cells showed a significant reduction in both HCR of cisplatin-damaged Ad and in their capacity to support Ad replication following cellular cisplatin damage suggesting that cisplatin damage is repaired at least in part by the NER pathway. The normal HCR and capacity response of the RS+ cells compared to the XP cells suggests that the hypersensitivity of RS+ cells to DNA damage is not due to a deficiency in NER. The FA cells had normal HCR of cisplatin-damaged Ad but were significantly reduced in their capacity to support Ad replication following cisplatin treatment which was attributed to a deficiency in the repair of DNA interstrand crosslinks. RS+ cells were not reduced in their capacity to support Ad DNA replication, suggesting that the RS+ cellular hypersensitivity does not result from a deficiency in interstrand crosslink repair as seen with FA cells.

LFS is a cancer prone syndrome that involves mutations in the p53 tumour suppressor gene. It was found that HCR of cisplatin-damaged Ad was normal in both p53-heterozygous and -hemizygous LFS cells, whereas the NER-deficient XP cells had significantly reduced HCR. The capacity of cisplatin-damaged, p53-heterozygous LFS fibroblasts was significantly reduced compared to normal cells. This suggests that although the LFS fibroblasts appear to have normal bulk NER, as shown by HCR, they appear to be deficient in the repair of the actively transcribed cellular genes necessary for viral replication. These results suggest a role for p53 in the repair of cisplatin damage of active genes.

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Finally, special thanks go to the guys of the Rainbow lab - Murray, Bruce and Todd, for their valued assistance and friendship.

PREFACE

This thesis is presented in five chapters. The introduction is the first section which provides background information concerning the work presented. Chapter two is a summary of data from initial experiments conducted to determine the appropriate parameters of the experimental assays used for all subsequent work. Chapters three, four and five have been written as papers in preparation for submission and publication. Chapter three presents the results of the examination of the DNA repair capacity of cisplatin damage in Roberts syndrome cells. All work presented in this chapter was conducted by the author. Chapter four examines the repair of cisplatin-damaged virus in repair-deficient Chinese hamster ovary cells, xeroderma pigmentosum fibroblasts, and human tumour cell lines. This paper was written by J. Todd Bulmer. My contribution to this work includes most of the data for the xeroderma pigmentosum cells with the exception of the XP-C and XP-F cell strains. Experiments with the CHO and tumour cells were carried out by J.T. Bulmer. The last chapter of the thesis presents the results of the examination of the response of Li-Fraumeni cells to cisplatin-damaged DNA. All work presented in chapter five was carried out by the author with the exception of a single host cell reactivation experiment which was done by J.T. Bulmer. Finally, a brief summary of all data is presented including the major implications of this work.

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INTRODUCTION

INTRODUCTION

DNA REPAIR

A. DNA Damage and its Potential Consequences

The integrity of a cell's DNA is vital to its survival and proper functioning. Genomic damage can have potentially severe consequences as the code contained within it is the basis for all protein synthesis, and hence, all cellular functions. The need for mechanisms capable of removing and repairing DNA damage is ever-present both due to intrinsic damage that results from normal cellular metabolism and to cope with the genotoxic agents that are present in the environment.

The alterations of DNA that arise from within the cell include mismatched bases following DNA replication, tautomeric base shifts, deamination of bases, depurination and depyrimidination, and oxidative damage by free radicals (Friedberg et al., 1995). The physical factors that can alter DNA include ionizing radiation and ultraviolet (UV) radiation. These agents have been around since life began. It has been suggested that mechanisms of UV lesion repair must have evolved very early in order for organisms to have survived the damaging effects of UV light. There also exist numerous chemical agents that can react with and damage DNA. The study of chemically-induced damage has become important in the field of cancer chemotherapy, which takes advantage of the ability of certain drugs to kill tumour cells by damaging their DNA. As well, the abundance of environmental toxins that surrounds us also merits study of these chemicals to determine their potential effects on our cells and our health. Some of the major types of DNA-damaging chemicals include: alkylating agents, crosslinking agents such as cisplatin and photoreacted psoralens, and others such as aflatoxins which are metabolically activated by the body to more reactive forms. Xenobiotics, man-made molecules not naturally present in the environment, could pose the greatest threat because they may not be recognized by the evolutionary established repair mechanisms present in our cells.

The consequences of DNA damage on the cell vary depending on the nature of the damage and the region of the genome that is affected. (Some damage, such as double strand DNA breaks, may be lethal while other types of lesions may lead to mutagenesis and cancer) Oxidative damage is the most abundant type of damage seen by the cell every day and is believed to be a contributing factor of aging and cancer (Ames and Gold, 1991).

B. DNA Repair Mechanisms

In mammalian cells, many different types of DNA lesions are removed by excision repair in which the damaged bases or nucleotides are excised from the DNA strand and replaced by the appropriate sequence. There are three distinct biochemical mechanisms that involve excision repair. Base excision repair is mediated by DNA glycosylases and AP endonucleases, mismatch repair deals with mispaired bases resulting from replication, and nucleotide excision repair is responsible for removing more complex lesions. Other forms of repair include simple ligation of strand breaks and recombinational repair which involves both nucleotide excision and recombination.

i. Base Excision Repair

Damaged, mispaired, modified and inappropriate bases are all recognized by DNA glycosylases. Several different DNA glycosylases have been identified, and they all have specificity for certain bases with particular modifications. This process of repair begins with the removal of the damaged base from the nucleotide by the catalytic action of the glycosylase (Lindahl, 1976). This results in an apurinic or apyrimidinic (AP) site depending on the nature of the base removed. The second enzyme involved is a 5' AP endonuclease which incises the DNA strand by hydrolysis of the phosphodiester bond (Lindahl, 1979). The third step is excision of the deoxyribose-phosphate group (from which the base was removed) by DNA deoxyribophosphodiesterase (dRpase). The

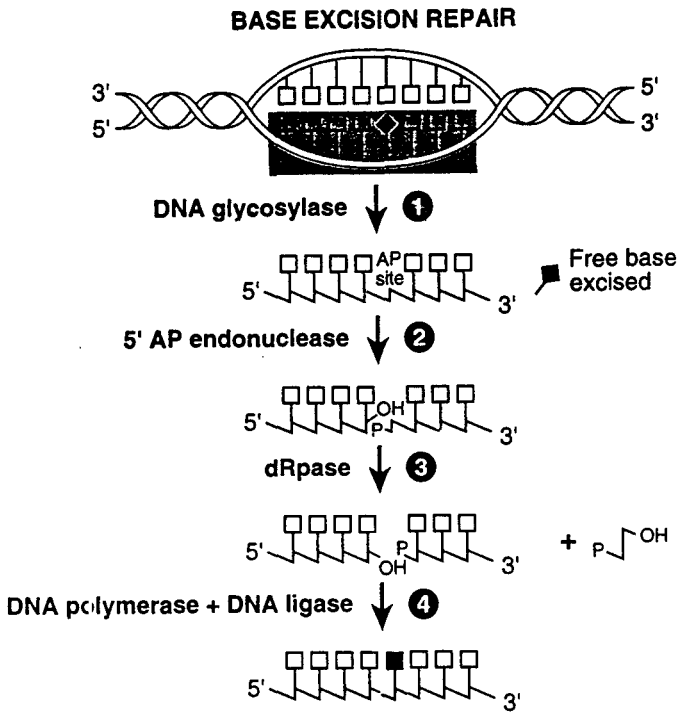

Figure 1. **Base Excision Repair**

Figure 1 is a schematic representation of the process of base excision repair. The modified base is excised by DNA glycosylase leaving an AP site. A 5' AP endonuclease then incises the DNA strand resulting in a terminal deoxyribose-phosphate group which is excised by dRpase. The resulting nucleotide gap is filled by DNA pol and the strand is sealed by DNA ligase. (from Friedberg et al., 1995)

resulting gap is then filled by DNA polymerase (DNA pol) and sealed by DNA ligase. This process is shown in Figure 1.

DNA glycosylase repair, or base excision repair, is the mechanism by which most oxidative DNA damage is repaired. This type of repair is very critical considering that the human genome is bombarded with thousands of oxidative hits every day.

ii. Mismatch Repair

Mismatch repair is the mechanism responsible for correcting mispaired bases in the DNA sequence that can arise by several processes, mostly by replication errors that occur during normal DNA replication. An interesting facet of this type of repair is that the incorrect base on the daughter strand must somehow be distinguished from the correct base in the mispair given that both bases are normal components of DNA. A distinction is made between long-patch and short-patch mismatch repair based on the length of tracts excised by each.

Long-patch repair in mammalian cells has not been well characterized but has been shown to be similar to that of *Escherichia coli*, having both mispair specificity and bidirectional excision capacity. In the *E. coli* system, discrimination of the parental and daughter strands in newly synthesized DNA is based on the state of methylation of GATC sites (Radman and Wagner, 1986; Modrich, 1989). Three proteins - MutS, MutL and MutH, are required for recognition and incision of the strand containing the mismatch (Modrich, 1991). In *Streptococcus pneumoniae*, it is thought that strand breaks in the lagging strand (due to Okazaki fragments) are the marker used to identify the daughter strands (Guild and Shoemaker, 1976). This is probably the mechanism for mammalian cells. Long-patch repairs a variety of mismatches, in particular G-T and A-C and is a highly conserved mechanism of repair. A defect of long-patch repair has been associated with hereditary nonpolyposis colorectal cancer (HNPCC) in humans (Fishel et al., 1993). Four human genes necessary for mismatch repair have been identified so far. hMSH2 is

the human equivalent of the MutS gene in *E. coli* while hMLH1, hPMS1 and hPMS2 are all homologs of the MutL gene (as reviewed in Marx, 1994).

Short-patch mismatch repair is characterized by shorter repair tracts, usually 10 nucleotides or less, and appears to have restricted sequence specificity (Modrich, 1991). The correction of G-T to G-C is carried out by short-patch repair, occuring preferentially at CpG sites (Sibghat-Ullah and Day, 1993).

iii. Nucleotide Excision Repair

Nucleotide excision repair (NER) is the system that recognizes and repairs DNA lesions induced by UV, cisplatin and many other DNA-damaging agents. The process involves recognition of the lesion by a damage-specific endonuclease (or endonuclease complex) that incises the DNA on both sides of the damage. The oligonucleotide fragment containing the lesion is then excised and the resulting gap is filled in by synthesis of new nucleotides by DNA pol. DNA ligase seals the strand. Figure 2 illustrates the NER process.

The mammalian system of NER repair is more complex than that found in lower organisms. The cloning of human genes that are responsible for NER has been accomplished by transfection of human DNA into UV-sensitive, NER-deficient CHO cells of different complementation groups (Hoeijmakers et al., 1987; Weeda and Hoeijmakers, 1993) as well as NER-deficient XP cells (Schultz et al., 1985). This led to the identification of many human genes named excision repair cross complementing (ERCC) genes for their ability to correct the NER deficiency in rodent cells from specific complementation groups. Since then, some of these ERCC genes have been shown to be responsible for human DNA repair disorders such as xeroderma pigmentosum (XP) and Cockayne syndrome (CS), and have therefore been renamed on this basis. The list of cloned genes that are associated with NER include: ERCC1, XPD (ERCC2), XPB (ERCC3), XPF (ERCC4), XPG (ERCC5), CSB (ERCC6), CSA, XPA and XPC. A UV-damage recognition protein found in XPE cells has been termed the XPE binding factor

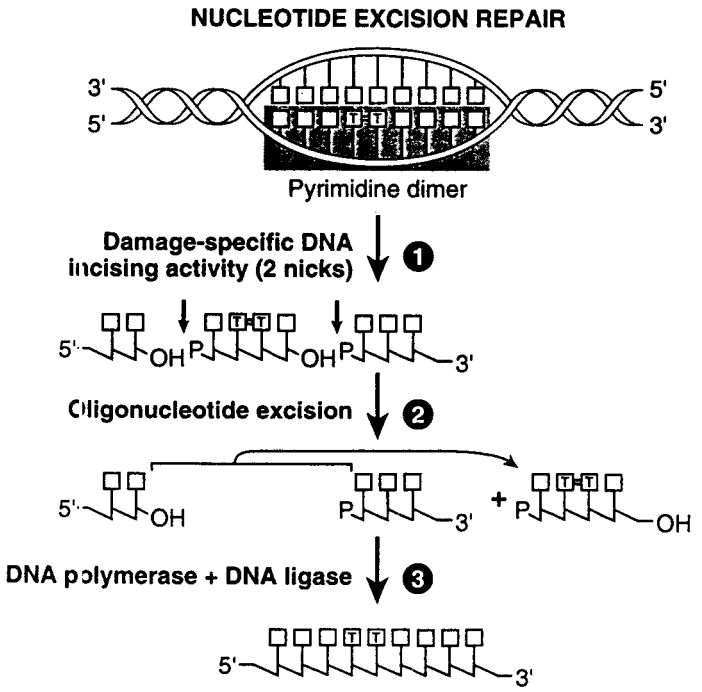

Figure 2. **Nucleotide Excision Repair**

Figure 2 is a schematic representation of the process of nucleotide excision repair. A damage-specific endonuclease incises the DNA strand on both sides of the lesion. The resulting oligonucleotide fragment is excised and the gap is filled by DNA pol and sealed by DNA ligase as for base excision repair. (from Friedberg et al., 1995)

(Keeney et al., 1993), however, this protein is not defective in all XPE patients (Keeney et al., 1992). The proteins involved in the recognition step of mammalian NER include XPA and XPE. XPB and XPD are helicases that unwind the DNA around the site of the lesion for incision by XPG, downstream of the damage, and incision by the XPF/ERCC1 complex upstream of the damage.

The repair of damage in mammalian chromatin poses a problem of accessibility of the repair machinery to the DNA. Recently, the discovery that the repair of actively transcribed genes is often preferential to the repair of silent regions of the genome has led to the suggestion that transcription-coupled repair may be a way for the repair enzymes to gain access to exposed DNA during transcription. The basal transcription factor of RNA polymerase II (TFIIH) has been shown to contain the XPB and XPD proteins that are also required for NER in addition to transcription. This, along with the fact that preferential repair of active genes is limited to genes transcribed by RNA pol II and not RNA pol I, has provided an interesting link between repair and transcription. In addition, many researchers have demonstrated that there is a bias of repair of the transcribed strand of an active gene over the nontranscribed strand. Both these observations of preferential repair may be the cell's way of urgently dealing with potentially harmful or lethal damage in genes that are critical for cell survival. Repair of the bulk genome is also important in order to reduce the mutational load of the cell but probably does not pose an immediate threat to cell survival (as reviewed in Friedberg et al., 1995).

iv. Recombinational Repair

The removal of interstrand crosslinks from DNA, like those induced by the antitumour drug cisplatin, requires another mechanism of repair in addition to NER. The proposed model involves incision on both sides of the lesion on one DNA strand which generates an oligonucleotide fragment that is not removed but remains attached via the crosslink. This generates a gap which may be extended by the 5'-3' exonuclease activity of Pol 1 in the *E. coli* system. A homologous DNA strand then displaces the crosslinked

fragment during recombination with the incised strand. The crosslinked fragment is then removed by incision of the other DNA strand and the gap is repaired by conventional NER (Cole, 1973). This process is illustrated in Figure 3. In yeast, the RAD1/RAD10 complex functions both in NER and recombinational repair pathways (Tomkinson et al., 1993). The human equivalents of the yeast genes, XPF and ERCC1, also form a complex that is involved in NER and their homology to the yeast system suggests a role for the XPF/ERCC1 complex in recombinational repair.

C. p53 Response to DNA Damage and its Involvement in DNA Repair

p53 is a tumour suppressor gene implicated in many human malignancies (Hollstein et al., 1991). Mutations in p53 alleles are the genetic basis of the cancer-prone Li-Fraumeni syndrome (Malkin et al., 1990). The p53 protein binds to DNA as a tetramer and acts as a transcriptional activator (Vogelstein and Kinzler, 1992; Zambetti and Levine, 1993) of genes such as WAF1/CIP1 (El-Deiry et al., 1993), MDM-2 (Juven et al., 1993) and GADD45 (Fornace et al., 1989). Normal p53 is required for G_1 cell cycle arrest following DNA damage (Kastan et al., 1991). This occurs by p53-activation of growth-inhibiting genes, especially WAF1/CIP1 which is an inhibitor of cyclin-dependent kinases that regulate progression through the cell cycle. The arrest of damaged cells in G_1 is thought to prevent the entry of these cells into S phase, giving them more time for removal of DNA damage. Loss of the G_1 checkpoint control would then permit replication of unrepaired DNA and would perpetuate genomic mutations. This is the way in which loss of functional p53 is thought to attribute to carcinogenesis. Levels of p53 protein accumulate in cells following DNA damage, attributed to increased post-translational stability. Experiments have indicated that the induction of p53 depends on DNA strand breaks which can be directly caused by ionizing radiation, or indirectly by the incision step of NER (Nelson and Kastan, 1994). The association of p53 and DNA damage repair has been strengthened by studies showing that p53 binds via its C-terminal

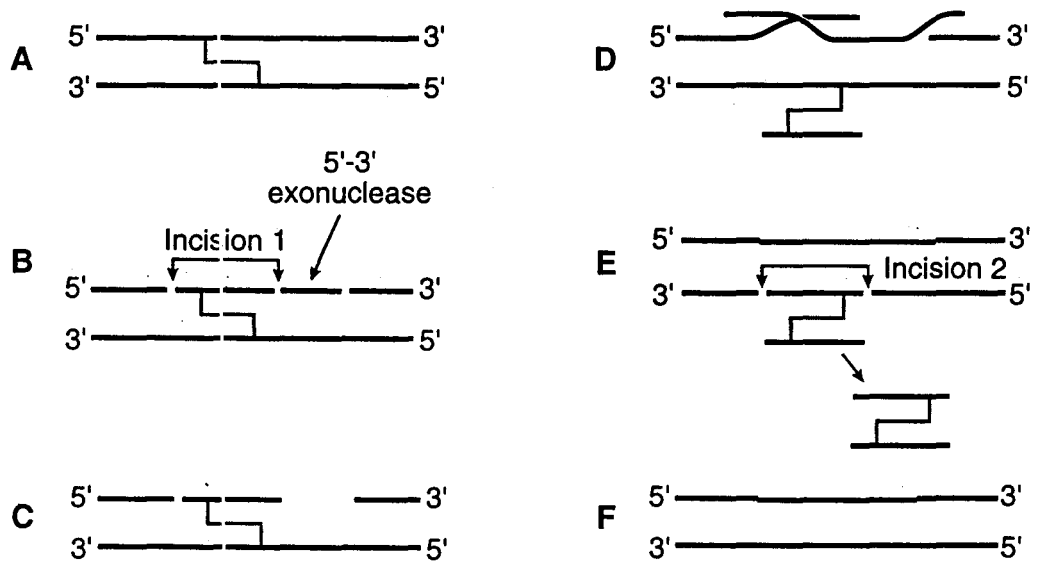

Figure 3. **Recombinational Repair of Interstrand Crosslinks**

Figure 3 is a schematic representation of the process of recombinational repair. One strand of the DNA is incised on both sides of the lesion. In *E. coli*, it is proposed that the 5'-3' exonuclease generates a gap 3' to the incised fragment. A homologous DNA strand then displaces the crosslinked oligonucleotide fragment and recombination takes place. The crosslink is then completely removed by incision of the second DNA strand and the gap is filled by repair synthesis and ligation. (from Friedberg et al., 1995)

domain to several TFIIH transcription repair factors including XPD, XPB, and CSB (Wang et al., 1995). As well, the UV-induction of p53 has been linked to damage of actively transcribed genes. In UV-sensitive CS cells, which have deficient repair of active genes, the minimum required dose (MRD) required for induction of p53 accumulation is eightfold lower than that for normal cells. In XP-C cells, which are normal for repair of actively transcribed genes, the MRD is as high as as normal (Yamaizumi and Sugano, 1994). This suggests that the damage within active genes is the signal for p53 induction. Cells which are deficient in p53 function have been shown to be deficient in global repair and repair of the nontranscribed strand of genes; however, they exhibit normal transcription-coupled repair of the transcribed strand (Ford and Hanawalt, 1995). In contrast, Wang et al. (1995) showed that Li-Fraumeni fibroblasts, heterozygous for a p53 mutation at codon 245, were significantly reduced in their gene-specific repair of UV-induced lesions.

p53 is also a regulator of apoptosis or programmed cell death (Lowe et al., 1993a; Williams and Smith, 1993; Yonish-Rouach et al., 1991). Cells that sustain high levels of DNA insult undergo apoptosis which is believed to prevent replication of genetically altered cells. The signal transduction pathway leading to p53-mediated apoptosis is as yet unknown but may involve DNA-dependent protein kinases (DNA-PK). p53 is currently thought to be activated by upstream factors, possibly DNA-PK, that recognize damaged DNA and initiate a cascade of kinase activity that phosphorylates several transcription factors including p53, myc, and AP-1 (Anderson, 1993). Activation of these factors would then lead to enhanced expression of genes that are DNA-damage inducible, and subsequently, to cell cycle arrest. It is believed that transcriptional activation by p53 is not necessary for induction of apoptosis, but that another function of the protein leads to cell death (Caelles et al., 1994).

D. DNA Repair-Deficient Syndromes

With all the genes that are required for proper functioning of the various mechanisms of human DNA repair pathways, it is not surprising that several genetic syndromes have been attributed to defects in DNA repair. Xeroderma pigmentosum (XP) is probably the most widely studied DNA repair-deficient syndrome and examination of the various complementation groups has been fundamental to the current level of understanding of nucleotide excision repair. Other human syndromes known to be associated with DNA repair defects or abnormalities in the processing of DNA damage include Cockayne syndrome, trichothiodystrophy, Fanconi anemia (FA), Bloom syndrome and ataxia telangiectasia. The occurrence of these diseases is rare as they are transmitted by autosomal recessive inheritance. All but trichothiodystrophy and Cockayne syndrome exhibit an increased risk for certain cancers.

i. Fanconi Anemia

This disease is characterized by intrauterine growth retardation, short stature, congenital abnormalities and pancytopenia (Fanconi, 1967). Patients exhibit increased incidence of leukemias and malignant neoplasms (German, 1972; Swift, 1971; Glantz and Fraser, 1982). FA cells undergo spontaneous chromosome breakage (Schroeder et al., 1976) and have increased sensitivity to crosslinking agents manifesting as both chromosomal aberrations (Sasaki and Tonomura, 1973; Auerbach and Wolman, 1976) and cell death (Weksberg et al., 1979; Averbeck et al., 1988; Diatloff-Zito et al., 1986). Many studies have demonstrated a deficiency in the repair of DNA crosslinks for FA cells (Matsumoto et al., 1989; Gruenert and Cleaver, 1985; Ishida and Buchwald, 1982). In particular, the repair of cisplatin damage has been shown to be greatly reduced in FA cells (Plooy et al., 1985; Ishida and Buchwald, 1982). Gene-specific repair of cisplatin-induced interstand crosslinks and intrastrand adducts is also deficient in FA cells (Zhen et al., 1993).

Four complementation groups (A-D) have been identified for FA (Strathdee et al., 1992a) which all differ in their sensitivity to DNA-crosslinking agents (Moustacchi et al., 1987; Matsumoto et al., 1989). The gene responsible for FA-C has been cloned (Strathdee et al., 1992b) but the function of the protein is unknown. Immunoprecipitation and immunofluorescence studies have localized the FAC gene product to the cytoplasm (Yamashita et al., 1994; Youssoufian, 1994). In FA-A cells, there is a defect in a chromatin-associated protein complex that recognizes and incises DNA containing interstrand crosslinks (Lambert et al., 1992). It is not yet known whether the recognition protein or the endonuclease is the abnormal component of the complex. Introduction of the normal functioning complex corrects the DNA-repair defect of the FA-A cells. Because of the complex phenotype of FA, it is thought that defective DNA repair is not the primary consideration of the syndrome. This is supported by more recently observed cellular characteristics of FA which include decreased production of interleukin 6 (Roselli et al., 1992; Bagnara et al., 1993), overproduction of tumour necrosis factor (TNF) α (Roselli et al., 1994) and abnormal p53 response to γ-radiation (Roselli et al., 1995). In fact, antibody inactivation of TNFα has shown to result in correction of some FA phenotypes *in vitro*, including deficient repair of MMC lesions (Roselli et al., 1994). As well, it has been shown that FA cells are sensitive to the damaging effects of oxygen (Schindler et al., 1988) and therefore, they may be defective in repair of oxidative damage. A link between p53 and Fanconi anemia has also been made although the relationship remains to be clarified. FA cells from complementation groups A, C and D are highly susceptible to MMC-dependent p53 accumulation suggesting that these FA gene products normally act to suppress p53 activation in response to MMC (Kruyt et al., 1996). However, because apotosis of the FA cells was not affected by a dominant-negative p53 mutation, the FA proteins may function in a p53-independent apoptosis pathway.

ii. Xeroderma Pigmentosum

XP is characterized primarily by photosensitivity of exposed regions of the skin and a very high incidence of skin cancers. Patients from some XP complementation groups also display neurological abnormalities. XP cells are defective in the nucleotide excision repair of DNA. Seven NER-deficient, XP complementation groups (A-G) have been identified, with a further distinction of an atypical variant (XP-V) form that appears to have normal NER but some deficiency in a post-replication recovery process for UV-damaged DNA (Lehmann et al., 1975). The various genes responsible for XP are involved in the early steps of NER - the recognition and incision of damaged DNA (Cleaver, 1969). The XPB and XPD gene products also participate in transcription, forming part of the basal transcription factor TFIIH (Schaeffer et al., 1993 and 1994).

The reduced host cell reactivation of UV-damaged viruses, as well as reduced cellular capacity for viral infection, and reduced clonogenic survival of UV-irradiated XP cells have been well documented (Day, 1974a and 1974b; Lytle et al., 1976; Rainbow, 1980). XP cells also display deficient repair of psoralen-induced (Gruenert and Cleaver, 1985) and cisplatin-induced (Plooy et al., 1985; Zhen et al., 1993) DNA crosslinks.

CISPLATIN

A. History and Clinical Significance

Cisplatin (*cis*-diamminedichloroplatinum(II)) was first described over one hundred years ago. It was not until 1965 that it was accidentally discovered to have the ability to suppress cell division. During studies on the effects of electric current on *E. coli*, it was found that cell division of the bacterium was inhibited by an electrolysis product from the platinum electrodes (Rosenberg et al., 1965). Today, cisplatin is widely used as a chemotherapeutic drug in the treatment of many cancers. It has shown success with head, neck, bladder, small cell lung, cervical, and most notably, testicular and ovarian tumours. The mechanism of cisplatin cytotoxicity is believed to be through its

interaction with DNA. Cisplatin-induced DNA damage can result in inhibition of DNA synthesis although apoptosis is thought to be the actual cause of cell death. The clinical use of cisplatin as a chemotherapeutic agent in cancer treatment is limited by its toxicity. Target organs include the kidneys and ears, as well as possible suppression of bone marrow. In an effort to reduce the severe side effects associated with clinical use of cisplatin, several derivatives have been developed. Carboplatin (diammine(1,1-cyclobutanedicarboxylato)platinum(II)) is one such analogue that is effective against ovarian cancer but with reduced side effects (Barnard et al., 1986). Another important clinical feature of cisplatin is the development of resistance in some tumours. Studies on the mechanisms of resistance will hopefully lead to strategies to counteract this phenomenon.

B. Structure and Formation of Active Cisplatin Species

As the name suggests, the structure of cisplatin includes two ammine groups and two chlorine atoms bonded to a divalent platinum atom in the *cis* configuration. The chloride ligands act as leaving groups while the ammine ligands are inert to substititution under biological conditions (as reviewed in Sherman and Lippard., 1987). This holds true for the *trans* isomer as well, however, only the *cis* isomer displays antitumour activity. Monofunctional platinum complexes with a single labile chloride ligand are also therapeutically inactive. In aqueous solution the reactive species of cisplatin are formed by the displacement of the chloride ions (Lippard, 1987). This allows the formation of the aquated species of the molecule as shown in Figure 4. As would be expected, the chloride ion concentration of the solvent influences the reactivity of the drug. In the plasma and extracellular spaces of the body the chloride concentration is about 100 mM, whereas inside the cells it drops to about 4 mM, which permits formation of the active species. The positively charged complexes act as electrophiles and are electrostatically attracted to nucleophiles within the cell such as DNA, RNA and some proteins. The binding of cisplatin to DNA is kinetically, not thermodynamically, controlled with the

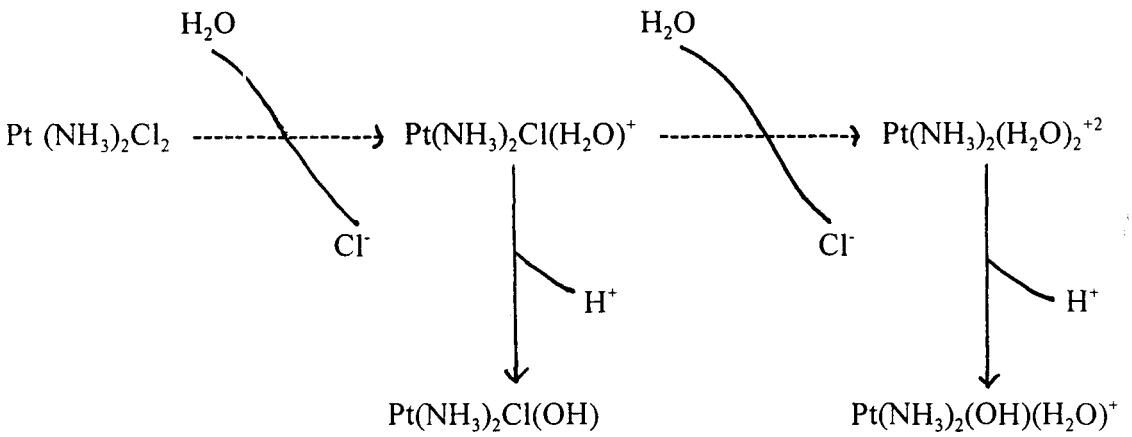

Figure 4. **Formation of Active Cisplatin Species**

Figure 4 illustrates the reactions involved in the conversion of cisplatin from an inert substance to its reactive species. In aqueous solution, the chloride ligands are displaced by water giving rise to the positively charged cisplatin molecules which react with DNA and other cellular nucleophiles. (from Chu, 1994)

rate-limiting step being hydrolysis of the drug to form the reactive species (Johnson et al., 1980; Ushay et al., 1981).

C. Cisplatin-Mediated DNA Damage

It is generally accepted that DNA is the main cellular target of cisplatin damage and that the biological effects seen with cisplatin-treated cells are due to the covalent bonding of cisplatin molecules to the DNA. Many studies have been conducted in an attempt to characterize the types of lesions formed, their relative abundance and their contribution to the cytotoxicity of the drug. Figure 5 illustrates the four main classes of cisplatin adducts which include DNA monoadducts, intrastrand and interstrand crosslinks, and DNA-protein crosslinks. From this point on, the intrastrand crosslink will often be referred to as the intrastrand adduct (IA) to distinguish it from the interstrand crosslink (ICL).

i. DNA Monoadducts

Monoadducts represent the first step in bifunctional adduct formation and have been shown by Fichtinger-Schepman et al. (1995) to undergo conversion to bifunctional lesions in Chinese Hamster Ovary (CHO) cells within 4-6 hrs. The authors also showed that the greatest decrease in the amount of monoadducts present in DNA corresponded to the highest increase in the levels of bifunctional adducts. Since the monofunctional platinum complex $[Pt(dien)Cl]^+$ (chlorodiethylenetriamineplatinum(II)) is inactive as an antitumour drug, the monofunctional adduct has been ruled out as the main cytotoxic lesion formed by cisplatin.

ii. DNA-Protein Crosslinks

DNA-protein crosslinks constitute a very small proportion of the total adducts formed in the cell. One study has estimated their relative abundance at 0.15% of total

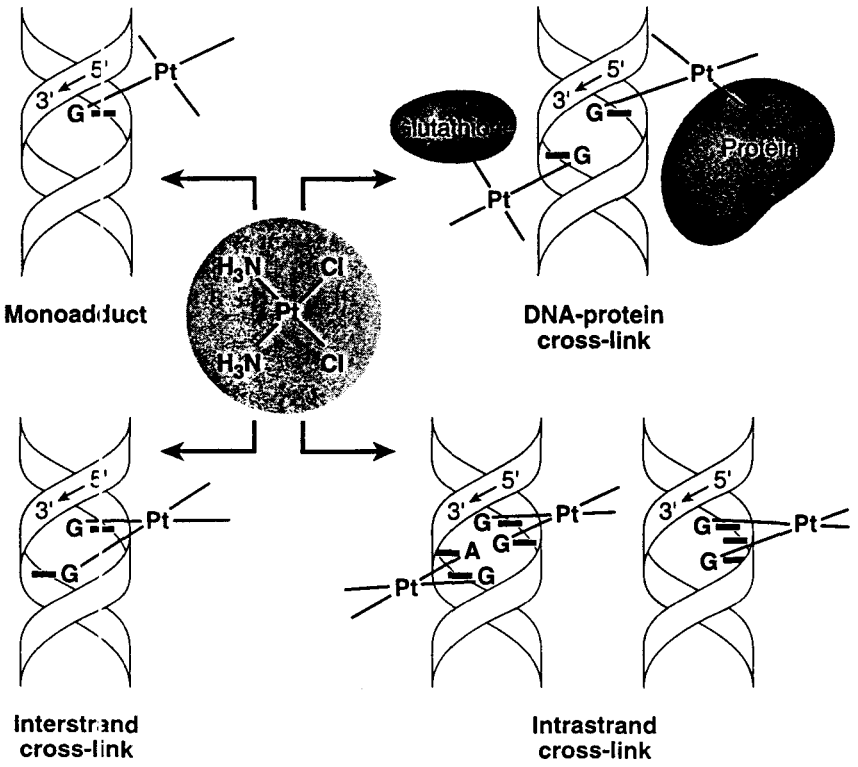

Figure 5. **Types of Cisplatin Adducts**

Figure 5 is a schematic representation of the four types of adducts formed by cisplatin. Monoadducts link cisplatin to a single base of one DNA strand. DNA-protein crosslinks involve glutathione or other cellular proteins. The interstrand crosslink links two bases on opposite strands of the DNA duplex. Intrastrand crosslinks link adjoining guanines or a guanine and adenine of the same strand, or can link two guanines separated by an intervening base. (from Friedberg et al., 1995)

lesions (Plooy et al., 1984), making them unlikely as the major cause of cell death. Studies have revealed that cisplatin is capable of linking DNA to non-histone chromosomal proteins (Ciccarelli et al., 1985; Banjar et al., 1984). Nitrogen atoms of histidine residues and sulphur atoms of cysteine and methionine residues are likely candidates involved in DNA-protein crosslinks due to their nucleophilic properties.

iii. Intrastrand Crosslinks

The intrastrand crosslink is believed to be the main cytotoxic lesion formed by cisplatin, accounting for up to 90% of total platinum adducts. There are three possible forms that the intrastrand adduct can take. The most prevalent form is a 1,2 d(GpG) crosslink between adjacent guanines which represents 60-65% of all lesions. The 1,2 d(ApG) adduct between adjacent adenine and guanine bases accounts for 20-25% of lesions, and the 1,3 d(GpXpG) adduct that links two guanines separated by an intervening base represents 5-6% of total cisplatin lesions (Fichtinger-Schepman et al., 1985; Eastman, 1986). Because the *trans* isomer of the drug is ineffective in treating tumours and is known to be incapable of forming the 1,2 intrastrand crosslinks, these lesions have been implicated as the major cause of cytotoxicity, however, the most effective of the two has not yet been determined. All three intrastrand lesions are described below in more detail.

Many studies which have analyzed the platination products of cisplatin have shown the 1,2 d(GpG) adduct to be the most prevalent (Fichtinger-Schepman et al., 1985; Eastman, 1986). Covalent bonding of the platinum to the DNA occurs via the 7th position nitrogen atoms (N7) of both guanine bases. Structural alterations resulting from this type of lesion include unwinding of the DNA duplex by ~21° and kinking (~58°) of the duplex at the d(GpG) site toward the major groove of the helix, with the minor groove being significantly widened (Yang et al., 1995). The covalent bonding of cisplatin to DNA is not a totally random event. It has been shown that cisplatin is selective for DNA sequences containing two or more consecutive guanosines (Stone et al., 1976; Roberts and Friedlos, 1981), which explains why the d(GpG) adduct occurs with the greatest

frequency. If there was no binding selectivity then one would expect a much broader range of adducts than those that are actually seen. It has been suggested that saturation of all the available oligo d(G) sequences leads to an increase in the formation of the secondary intrastrand crosslink - the 1,2 d(ApG) adduct.

The 1,2 d(ApG) adduct covalently links platinum to the N7 atom of adenosine and guanosine, with the adenosine being 5' and the guanosine 3'. Structural distortions resulting from this lesion are similar to those resulting from the 1,2 d(GpG) lesion. Monoclonal antibodies specific to the d(GpG) adduct also appear to recognize the d(ApG) adduct suggesting that distortions from these two lesions are immunochemically similar (as reviewed in Sherman and Lippard, 1987). Based on several studies using enzymatic digestion of platinated DNA to identify platinum-DNA adducts, regions of DNA containing the d(ApG) lesion have been shown to be more resistant to digestion than those containing other adducts (Fichtinger-Schepman et al., 1985; Eastman, 1986). This suggests that repair enzymes in the cell may experience greater difficulty in removing these particular lesions and leads to the question of which 1,2 intrastrand lesion makes the greatest contribution to cytotoxicity. The different steric properties of the two lesions have been postulated as the reason for the rarity of the d(ApG) relative to the d(GpG), and therefore, could also play a role in enzyme interaction. In fact, a recent report has indicated the d(ApG) adduct as the lesion responsible for the cytotoxic action of the drug based on comparison studies between cisplatin and its antitumour derivative carboplatin in which d(ApG) was the only lesion formed at comparable levels by both drugs (Fichtinger-Schepman et al., 1995).

The third and final intrastrand crosslink involves two guanosines separated by an intervening nucleotide. Covalent bonding of the 1,3 d(GpXpG) adduct also involves the N7 atoms of the bases. The structural alterations resulting from the 1,3 intrastrand adduct are thought to be more severe than those caused by the 1,2 intrastrand adducts. Studies indicate a nonhelical structure of the DNA duplex in the region of the platinum lesion (as reviewed in Sherman and Lippard, 1987). This adduct represents a small

proportion of the total cisplatin adducts (5-6%) formed in DNA and has been disregarded as the main cytotoxic lesion because it can be formed by both cisplatin and its therapeutically inactive isomer transplatin.

iv. Interstrand Crosslinks

Interstrand crosslinks differ from intrastrand adducts in that they covalently bond together both strands of the DNA double helix. It has been demonstrated that bonding occurs predominantly between the N7 atoms of two guanines on opposite strands (Eastman, 1985). The structure of this adduct has been resolved by nuclear magnetic resonance (NMR) techniques. The cisplatin bridge was determined to reside in the minor groove of the DNA helix with a localized shift from a right-handed helix to a left-handed, Z-DNA-like form in the vicinity of the crosslink (Huang et al., 1995). This results in a local unwinding of 87° and bends the axis of the helix towards the minor groove by a magnitude of 20° (Huang et al., 1995). This localized distortion of the DNA double helix is a result of the constraints imposed by the N7-Pt-N7 linkage.

Early estimates of the relative abundance of the interstrand crosslink in the overall genome were values of approximately 1% (Plooy et al., 1984; Roberts and Friedlos, 1981) which was considered too low to account for the cytotoxicity of cisplatin. More recently, the relevance of ICL in gene-specific repair of cisplain lesions has received attention. Studies have shown that the ICL may account for up to 5% of the total cisplatin lesions in the hamster DHFR gene (Jones et al., 1991) and approximately 3% in human DHFR and rRNA genes (Zhen et al., 1993). The higher preponderance of ICL within these active gene regions suggests that there may be a preferential distribution of ICL within the genome. The ICL may be more important than originally thought due to its persistence in some cells. In FA cells, a continued increase in the formation of ICL is observed in comparison to decreasing ICL levels in normal and XP cells at several time points after cisplatin treatment (Plooy et al., 1985). It has been postulated that the critical lesion in cytotoxicity may vary from cell to cell depending on the intrinsic

properties of the cells being studied. The very different structure and local distortions imposed by the ICL compared to the intrastrand adducts could result in significant contributions of the ICL to cytotoxicity despite its relatively low abundance, most likely resulting from differential repair of the two lesions.

D. Conseqences of Cisplatin Damage

In the past it was thought that the cytotoxic action of cisplatin resulted from the inhibition of DNA synthesis due to the presence of unrepaired lesions. Several pieces of evidence negate this. First of all, repair-deficient CHO cells have been shown to die at cisplatin concentrations that do not inhibit DNA synthesis (Sorenson and Eastman, 1988). As well, DNA-repair-proficient cells survive concentrations of the drug that are sufficiently high to inhibit DNA synthesis. Therefore, the lack of correlation between inhibition of DNA synthesis and cell death points to another mediator of cytotoxicity.

Apoptosis is now believed to be the major cause of cell death in cisplatin-treated cells. It has been demonstrated that cells treated with lethal doses of cisplatin reveal the DNA ladder fragmentation pattern of 180 bp multimers that is consistent with the internucleosomal cleavage of chromatin in the first step of apoptosis (Sorenson et al., 1990; Barry et al., 1990). This is followed by loss of membrane integrity and cell shrinkage. Induction of accumulation of the tumour suppressor p53 protein in reponse to several DNA-damaging agents has been shown. These include UV (Maltzman and Czyzk, 1984), cisplatin, MMC and gamma radiation (Fritsche et al., 1991). The cisplatin-induced accummulation of p53 is followed by apoptosis in the majority of the treated cells (Fritsche et al., 1993). Therefore, it seems that cells incapable of sufficient removal of cisplatin lesions will respond by signaling programmed cell death. p53-dependent apoptosis has been shown to modulate the cytotoxicity of other anticancer agents besides cisplatin (Lowe et al., 1993b) and the inhibition of apoptosis is known to reduce cell sensitivity to many chemotherapeutic agents (Lowe et al., 1993b; Miyashita and Reed, 1992). This suggests that cisplatin damage in resistant cells may no longer act to signal

cell death and is in keeping with the idea that cisplatin-resistant cells can tolerate much higher levels of platinum lesions than nonresistant counterparts. The actual signal transduction pathway controlling cisplatin-induced apoptosis is not known.

E. Repair of Cisplatin-Damaged DNA

There have been numerous studies conducted on the repair of cisplatin-induced DNA damage in various mammalian cells; however, a detailed mechanism for the recognition and repair of cisplatin damage has not yet been postulated. It is only recently that the potential proteins involved in the specific repair of cisplatin-induced DNA lesions are being uncovered. In particular, some researchers have concentrated on the role of gene-specific repair of cisplatin lesions, revealing new levels of repair specificity within cells.

i. Intrastrand and Interstrand Crosslink Repair Pathways

As was already indicated, the inefficient repair of the intrastrand adduct is believed to be the major contributing factor to both the cytotoxicity and antitumour activity of cisplatin. The repair of cisplatin adducts is believed to occur mainly via the nucleotide excision repair (NER) pathway. NER requires several different proteins for proper functioning. The steps involved include incision on both sides of the lesion, excision of the damaged sequence, synthesis of new DNA to fill in the resulting gap, and ligation to join the strand together. (see Figure 2 of introduction)

The unique properties of each of the three different cisplatin-induced intrastrand adducts causes slightly different distortions in the normal structure of the DNA double helix for each lesion. These minor differences could lead to distinct responses by the enzymatic complexes that recognize and repair these crosslinks. This has been shown to be the case for the UvrABC endonuclease system in *E. coli*. The stages of repair by UvrABC are very similar to the NER process in mammalian cells and the system is capable of recognizing and repairing several structurally different types of DNA lesions

including UV (Visse et al., 1994), cisplatin (Beck et al., 1985), HMT (Sancar et al., 1985) and N-AAAF (Seeberg and Fuchs, 1990). It has been shown that the 1,2 d(GpG) intrastrand adduct is incised by the UvrABC complex approximately 3.5 times more efficiently than the 1,3 d(GpCpG) intrastrand adduct and that the local structure of the two preincision complexes are different (Visse et al., 1994). The ability of the UvrABC endonuclease system to recognize such varying kinds of lesions is believed to be based on the recognition of the distorted helical DNA structures that result from the lesions, and not the actual lesions themselves.

The repair of interstrand crosslinks requires additional mechanisms in order to completely remove them from the DNA due to the more complex nature of this type of lesion. Early proposed models of ICL repair by Fujiwara et al. (1977) and Cleaver (1978) indicate that the first step would involve the unhooking of one arm of the crosslink by a specific enzyme that is not required for IA repair. A chromatin-associated nuclease has been identified that is essential for the repair of ICL (Lambert et al., 1988) and may represent such an enzyme. The next step would be repair of the remaining monofunctional adduct by the NER pathway. Two more recent models of ICL repair have also been proposed. The first involves incision and removal of the affected nucleotides on one DNA strand by NER enzymes, synthesis to fill in the gap, and then removal of the crosslink from the other strand by recombination via homologous strand exchange (van Houten et al., 1986). This model of recombinational repair is illustrated in Figure 3 of the introduction. The second model involves unhooking the crosslink from one strand followed by translesion synthesis which bypasses the remaining lesion on the other DNA strand. Evidently, the repair of cisplatin lesions requires further study to better understand the particular processes of cisplatin-DNA adduct removal.

ii. Gene-Specific Repair of Cisplatin Damage

Historically, DNA-repair processes have been examined at an overall genomic level. More recently, gene-specific repair is being established as an important parameter of the DNA-repair capabilities of cells. The preferential repair of UV-induced lesions in transcriptionally active genes has been shown to occur in many organisms (Bohr et al., 1985; Madhani et al., 1986). This preferential repair can even be strand specific, with lesions in the transcribed strand being removed faster than those in the nontranscribed strand of an active gene (Mellon et al., 1987; Ford and Hanawalt, 1995). Preferential repair can be linked to the transcription of the active genes in what is called transcription-coupled repair, but it can also be independent of transcription (Bohr, 1991). Deficiencies in both global and preferential repair can result in clinical syndromes. Cockayne's syndrome is a DNA repair-deficient syndrome which is defective only in the preferential repair of transcriptionally active genes, having normal bulk repair (Venema et al., 1990). Conversely, XP-C cells, which are defective in the overall bulk repair of DNA, show a normal level of preferential repair of pyrimidine dimers in active genes (Venema et al., 1991; Evans et al., 1993). The efficient removal of DNA lesions from active genes may be critical for cellular survival (Bohr, 1991). As well, specific removal of damage from the transcribed strand of genes plays a role in the mutational spectrum of the genome (Vrieling et al., 1991; Carothers et al., 1992). Thus, gene-specific repair may be a better predictor of cell survival than overall bulk repair.

Zhen et al. (1993) have shown a greater efficiency for repair of cisplatin lesions in the DHFR gene transcribed by DNA polymerase II compared to rRNA genes transcribed by DNA polymerase I. They also found more efficient repair of ICL than IA in normal cells for both the DHFR and rRNA genes. These findings have been supported by other researchers who found faster removal of ICL than IA from the DHFR gene as well as a preferential repair of crosslinks from active genes compared to the overall genome (Jones et al., 1991).

Strand bias has also been demonstrated in the repair of cisplatin lesions, but only for the removal of IA. It has been shown that IA are preferentially repaired from the transcribed strand at a two fold greater level than from the nontranscribed strand of the DHFR gene in CHO cells (May et al., 1993). In contrast, repair of ICL does not show any bias for the transcribed strand (May et al., 1993; Jones et al., 1991).

iii. Potential Proteins Involved in Repair of Cisplatin Damage

The function of true damage recognition proteins (DRP) in the cell is to facilitate repair of DNA lesions. Several proteins have been shown to bind to cisplatin-damaged DNA but their actual role in the repair of cisplatin lesions is uncertain, especially for those proteins known to have other important functions within the cell. The cisplatin-damage recognition proteins that have been identified so far include the UV-damage recognition protein (also called XP-E binding factor), ERCC1, several of the HMG box family of proteins, and yeast photolyase I.

The UV-damage recognition protein (UV-DRP) has an affinity for binding 6-4 photoproducts induced by UV (Hirschfeld et al., 1990; Treiber et al., 1992; Keeney et al., 1993; Reardon et al., 1993). It can also recognize several other types of damage including those induced by cisplatin, nitrogen mustard, denaturation and depurination. Because some XP-E patients lack this protein, it has also been called the XP-E binding factor (XPE-BF), although most XP-E patients do have normal levels of UV-DRP Kataoka and Fujiwara 1991; Keeney et al., 1992). It was previously postulated that this protein may be the damage recognition unit of the NER system. More recent evidence, however, suggests that this is not the case although the UV-DRP could still play a minor part in NER recognition of DNA lesions. Although the constitutive levels of UV-DRP do not correlate with cell survival following cisplatin treatment, the induction of the protein by cisplatin is observed in human cells. This induction of the protein requires *de novo* synthesis and is believed to be a consequence of the formation of platinum-DNA adducts or due to the inhibition of DNA synthesis caused by cisplatin treatment (Vaisman and

Chaney, 1995). Because UV-DRP actually has a very low affinity for binding cisplatin lesions, the biological function of the protein in cisplatin repair remains to be identified.

Some of the major human genes involved in excision repair have been discovered by correction of various repair deficiencies in mutant rodent cells (Hoeijmakers et al., 1987; Weeda and Hoeijmakers, 1993). These genes have been designated excision repair cross complementing (ERCC) genes. ERCC1 is a gene that restores normal resistance to UV and MMC damage in the CHO group 1 mutants that are hypersensitive to UV and bulky adducts like MMC (Wood and Burki, 1982). The ERCC1 protein has a putative nuclear localization signal and a helix-turn-helix motif characteristic of a DNA-binding protein (van Duin et al., 1986). The majority of the amino acid sequence is highly homologous to the yeast RAD10 protein which is required for NER and mitotic recombination in yeast cells (van Duin et al., 1986). ERCC1 has also been shown to be required for the incision of UV-damaged plasmid DNA in a cell free system (Biggerstaff and Wood, 1992) and has been suggested to complex with the XPF protein (Biggerstaff et al., 1993; van Vuuren et al., 1993). This functional protein complex is similar to the one in yeast which includes RAD1 and RAD10 and functions in NER and recombinational repair pathways (Tomkinson et al., 1993). Transfection of ERCC1 into mutant CHO cells restores cisplatin ICL gene-specific repair to normal levels; however, repair of IA was restored to only one third of normal levels in the same cells (Larminat and Bohr, 1994). This suggests that the ERCC1 gene may have a more significant role in the repair of ICL. This finding is in accordance with another report proposing that ERCC1 is more important in repairing MMC-induced lesions than those induced by UV (Belt et al., 1991). This is not surprising since ICL repair probably involves some form of recombination which has been shown as a function of the yeast counterpart of ERCC1 (RAD10), both by itself and as part of a complex with RAD1(XPF).

There is a family of cisplatin damage-binding proteins that are related due to the presence of a conserved region called the high mobility group (HMG) box (Bruhn et al., 1992; Pil and Lippard, 1992; Hughes et al., 1992; Brown et al., 1993b). The HMG box has a structural motif that represents a new class of DNA-binding proteins (Jantzen et al., 1990; Lilley, 1992; Bianchi et al., 1992). The recognition signal for the binding of HMG proteins to cisplatin adducts is believed to be the local unwinding and bending of the DNA duplex (Bruhn et al., 1992; Pil and Lippard, 1992). HMG proteins do not bind UV lesions. The HMG domain has known affinity for unusual DNA structures. For example, two HMG proteins, HMG1 and HMG2, bind cruciform structures and unwind negatively supercoiled DNA (Bianchi et al., 1989). Only adducts that are formed by the active isomer cisplatin are recognized by the HMG box proteins, whereas transplatin adducts are not (Pil and Lippard, 1992; Toney et al., 1989). The normal functions of the HMG box proteins do not seem to include DNA repair. More likely, they function in transcriptional regulation and maintenance of chromatin structure (Lilley, 1992). The HMG box proteins that bind cisplatin adducts include the structure specific recognition protein SSRP1, HMG1, HMG2, human upstream binding factor (hUBF), and the yeast intrastrand crosslink recognition protein IXR1.

The identification of the SSRP1 protein came about by screening a human cDNA expression library using a cisplatin-crosslinked DNA probe. The structure specific recognition protein (SSRP) is an 81 kD protein that binds cisplatin-crosslinked DNA (Toney et al., 1989). The mouse homolog of SSRP1 has been shown to be involved in the V(D)J recombination in B cells (Shirakata et al., 1991) that gives rise to all the antibodies produced by the immune system.

HMG1 and HMG2 are high mobility group box proteins of low molecular weight. They are highly conserved and are present in abundance in the nucleus and cytoplasm of cells but have undetermined function. HMG1 consists of two tandem HMG boxes in the N-terminal portion of the protein. An interesting facet of these HMG proteins is their selective binding to the 1,2 intrastrand d(GpG) and d(ApG) adducts,

induced only by cisplatin, with no specificity for 1,3 intrastrand adducts (Pil and Lippard, 1992) that are induced by both the *cis* and *trans* isomers of the drug. The relevance of binding only to clinically effective lesions may be significant; however, there is no evidence that these proteins are involved in the repair of cisplatin damage.

The human upstream binding factor (hUBF) is a ribosomal RNA transcription factor containing an HMG box (Jantzen et al., 1990). This protein has been shown to bind with high affinity to cisplatin intrastrand d(GpG) adducts (Treiber et al., 1994). A specific protein-DNA interaction occurs within a 14 bp region flanking the adduct, as shown by DNase I protection patterns (Treiber et al., 1994). The comparable affinity of hUBF for rRNA promoter sequences and cisplatin adducts implies that the adducts may act as decoys for hUBF, disrupting the positive regulation of rRNA genes and hence rRNA synthesis. Because of its high affinity for the d(GpG) lesions, hUBF may shield these lesions from DNA repair machinery such as the XPA protein (involved in incision of damaged DNA) which has a relatively low affinity for d(GpG) lesions (Jones and Wood, 1993) and presumably would not be able to displace hUBF.

The last HMG box protein to be discussed is the intrastrand crosslink recognition (IXR) protein that has been isolated from yeast. The IXR1 protein actually confers sensitivity to cisplatin. When this gene is disrupted, the yeast mutants grow normally but display a 2-3 fold increase in resistance to cisplatin (Brown et al., 1993b).

Although not an HMG protein, the yeast photolyase protein PHR1 has also been shown to bind to cisplatin damage (Patterson and Chu, 1989). PHR1 is a DNA-repair protein involved in photoreactivation of cyclobutane dimers induced by UV (Sancar, 1990). Deletions of this gene also lead to a cisplatin-resistant phenotype (Fox and Feldman, 1994). The IXR1 and PHR1 gene products may influence cellular cisplatin sensitivity by binding to cisplatin lesions and blocking access of the repair enzymes. This explains how their disruption can lead to an increased resistance to cisplatin.

The mechanisms by which these cisplatin damage recognition proteins exert their effects, if any, on repair is not clear. Sensitivity or resistance to cisplatin may be

determined by competition between the proteins that repair DNA, those that may interfere with repair by shielding damaged regions, and those that signal apoptosis.

F. Cisplatin Resistance

The use of cisplatin as an antitumour agent has been very successful for treating human malignancies, especially those of testicular and ovarian origin. As is the case for other cell-killing drugs, such as antibiotics, the development of drug resistance poses a problem. Recurrent ovarian tumours often exhibit cisplatin resistance resulting from the initial chemotherapy with cisplatin. Understanding the mechanisms of resistance is desirable in order to possibly prevent it or to determine methods to circumvent it. Several ideas of how cells may develop resistance have been postulated. The most generally accepted are decreased drug accumulation, intervention by intracellular thiols and increased DNA repair.

i. Drug Accumulation

Cellular uptake of cisplatin was first believed to occur by passive diffusion through the lipid membrane (Gale et al., 1973) even though the solubility of cisplatin in water and the membrane content and fluidity were shown to be inconsistent with this concept (Mann et al., 1988). The idea of protein-mediated uptake has been supported by the identification of two possible cisplatin transport proteins. One is a 48 kDa protein that has reduced expression in resistant cells (Bernal et al., 1990), presumably involved in drug uptake, and the other is a 200 kDa glycoprotein with increased expression in resistant cells (Kawai et al., 1990), presumably an efflux protein. Both are distinct from the glycoprotein encoded by the multidrug resistance (MDR) gene which is not involved in cisplatin resistance (Deuchars and Ling, 1989). Regardless of the mechanism, decreased drug accumulation is one of the most consistent observations associated with cisplatin resistance. Most studies have shown reduced accumulation in the range of 2-3 fold but this does not account for the often large increases in resistance. It has also been

shown that intracellular cyclic AMP increases can increase cisplatin uptake into the cell (Mann et al., 1989), suggesting an energy-dependent process. Calcium levels can also have effects on the accumulation of cisplatin (Ikeda et al., 1987; Vassilev et al., 1987).

ii. Intracellular Thiols

Two of the intracellular thiols possibly involved in cisplatin resistance are glutathione and metallothionein. It has been shown that cisplatin has an affinity for sulphur (Cotton and Wilkinson, 1980) and therefore, sulphur-rich proteins. Increased levels of glutathione and metallothionein have been postulated as a mechanism of cisplatin resistance but their levels in cells do not always correlate with resistance. The glutathione tripeptide is the most abundant thiol in the cell consisting of gly-cys-gly residues. It functions as a free radical scavenger, reducing oxidative stress induced by foreign agents including xenobiotics (Meister and Anderson, 1983). It can react with and inactivate hydrogen peroxide to prevent the formation of peroxide free radicals. Glutathione-platinum complexes are eliminated from the cell by an ATP-dependent glutathione S-conjugate export pump (Ishikawa and Ali-Osman, 1993) which reduces the amount of cisplatin within the cell. Glutathione may also act to protect the DNA by sequestering active cisplatin species away from the DNA, or it may also quench monofunctional adducts, preventing their conversion to bifunctional lesions.

Metallothionein is a small protein (61-62 amino acids) that has 20 cysteine residues (Hamer, 1986) and has been shown to bind to cisplatin (Zelazowski et al., 1984). It plays a role in heavy metal detoxification, and therefore, can prevent free radical formation in the cell by its sequestering of metal ions. The contribution of these thiols to cisplatin resistance probably depends on the other resistance mechanisms at work and the cell strain or line being studied.

iii. DNA Repair

Many researchers have shown increased DNA repair capabilities in cisplatin-resistant cells. This includes cells that have been made resistant in culture (Johnson et al., 1994a and 1994b), tumour cells with intrinsic cisplatin resistance (Zeng-Rong et al., 1995), and tumour cells that have developed resistance by previous treatment with cisplatin chemotherapy (Dabholkar et al., 1992). Increased DNA repair seems to be the first mechanism activated in the process of cisplatin resistance; however, the degree of enhanced repair often does not account for the large increase in resistance. Therefore, other mechanisms must play a role in the overall degree of cellular resistance. The repair of ICL seems to be more significant than IA in gene-specific repair. Zhen et al. (1992) have shown that resistant ovarian cell lines repair ICL much more efficiently than their sensitive counterparts. This held true for the repair of DHFR, MDR1 and γ-globin genes. No difference in IA repair was seen between the cisplatin sensitive and resistant cells. The ERCC1 protein has been suggested as a possible mediator of enhanced DNA repair. The possible involvement of ERCC1 in recombinational repair (based on yeast homologs) suggests that it would be important for repair of ICL. CHO cells expressing the ERCC1 gene are five fold more resistant to cisplatin than cells that do not express the gene (Lee et al., 1993). As well, ERCC1 levels were shown to be 2.6 times higher in clinically cisplatin-resistant tumours than in sensitive tumours (Dabholkar et al., 1992). Treatment of tumours with the less toxic derivative carboplatin has also been observed to elevate the levels of ERCC1, making the cells 10 fold less sensitive to the drug.

The increase in DNA repair observed in cisplatin resistant cells does not fully account for their tolerance to the high levels of DNA-Pt adducts that are found in these cells (Johnson et al., 1994). It has been suggested that resistant cells exhibit damage tolerance by translesion bypass of unrepaired cisplatin lesions. This has been demonstrated in cisplatin-resistant murine leukemia cells which can synthesize DNA past platinum adducts 3-4 fold moreso than their cisplatin-sensitive counterparts (Gibbons et al., 1991). Translesion synthesis has also been observed in monkey cells in which a

cisplatin d(GpG) intrastrand adduct within a human RAS gene vector was bypassed during replication (Pillaire et al., 1994). DNA pol β is the only major mammalian replication enzyme that has been shown capable of *in vitro* translesional synthesis of cisplatin adducts (Hoffmann et al., 1995).

iv. Miscellaneous Resistance Mechanisms

As the study of cisplatin resistance continues, other possible mechanisms that may account for the development of resistance are being discovered. Increased constitutive levels of p53 have been demonstrated in ovarian tumour cell lines. Transfection of a mutant p53 gene into these resistant cells significantly increased their sensitivity to cisplatin (Brown et al., 1993a) suggesting that p53 is a determinant of cisplatin resistance in this cell line. In addition, mitochondrial defects (Andrews and Albright, 1992), damage tolerance by translesion synthesis, sequestering of active cisplatin molecules by ribonucleotides (Seki et al., 1993), and the shielding of DNA damage from repair enzymes by other cisplatin adduct-recognition proteins like the HMG box family have all been named as potential contributors to cisplatin resistance.

VIRUSES AS PROBES FOR DNA REPAIR

Viruses have been used extensively as probes in the study of DNA repair. Double-stranded DNA viruses such as simian virus 40 (SV40), herpes simplex virus (HSV) and the adenoviruses (Ad) have proven useful as they require host cell machinery for viral replication which takes place in the nucleus of infected cells (Flint and Broker, 1980). The utilization of viral probes is probably best exemplified by the host cell reactivation (HCR) assay which examines the ability of infected host cells to repair and replicate DNA-damaged virus. The cellular capacity assay also makes use of viral replication as an indicator of DNA repair capacity. In this case, DNA-damaged cells are examined for the ability to replicate undamaged virus. These assays most often take

advantage of adenoviruses as the probe of choice. I have utilized adenovirus, serotype 5 (Ad5) in HCR and capacity assays to assess the DNA-repair capacity of cells from several human genetic syndromes.

A. Adenovirus

Adenoviruses (Ad) have been used in HCR and capacity assays by a number of researchers. All adenoviruses are nonenveloped viruses with an icosahedral structure. The Ad genome is transcribed and replicated within the nucleus of the infected host cell and therefore makes use of host cell enzymes that are available to it. The virus encodes its own DNA polymerase. Adenoviruses have numerous features which afford several advantages over the use of other viral systems. The Ad genome is double-stranded DNA which is closely associated with the host cell histones during infection. The virus replicates in both proliferating and nonproliferating human cells and relies heavily on the host cellular machinery for replication. As well, I have specifically used Ad serotype 5 (Ad5) which has low pathogenicity in humans (as reviewed in Horwitz, 1990).

The successful replication of Ad requires at least three viral gene products and four host factors. Therefore, damage to these genes, as well as others, can reduce the synthesis of new virions. The necessary viral proteins for viral replication are: the single-stranded, DNA-binding protein (DBP), the precursor to the terminal protein (pTP) which is cleaved during viral assembly to the actual terminal protein present at each 5' end of the viral genome, and the Ad DNA polymerase (Ad DNA pol) all of which are necessary for adenoviral DNA replication. The cellular host factors required for adenoviral replication include three nuclear factors and the ORP A protein, all of which complement initiation and elongation of replication of viral DNA by increasing the activity of the viral origin of DNA replication. Nuclear factor I (NFI) and NFIII recognize and bind specific sequences near the origins of replication at both ends of the

viral genome. NFII is a topoisomerase that is required for full elongation of the transcribed viral DNA. The ORP A factor also appears to act near the origin of viral replication (as reviewed in Horwitz, 1990).

B. Host Cell Reactivation Assay

The host cell reactivation (HCR) assay is a widely used test of DNA repair. HCR examines the ability of infected host cells to repair, and hence, replicate damaged viral DNA. Viral inactivation arises from unrepaired lesions in the viral DNA and reflects constitutive levels of repair of the host cell. HCR can be assessed by several different endpoints including viral antigen production (Rainbow, 1980; Maynard et al., 1989; Eady et al., 1992), plaque forming ability (Aaronson and Lytle, 1970; Day, 1974a), reactivation of a reporter gene within a recombinant vector (Francis and Rainbow, 1995; Valerie and Singhal, 1995), and by the amount of viral DNA synthesized by the host cells (Arnold and Rainbow, 1996).

HCR in repair-deficient cells compared to normal controls has been effectively shown to reflect differences in repair capabilities (Day, 1974a and 1974b; Rainbow and Howes, 1979; Rainbow, 1980; Rainbow, 1989). The reactivation of UV- or γ-irradiated Ad has been shown to be significantly reduced in NER-deficient XP cells (Rainbow, 1980; Rainbow, 1989; Rainbow and Howes, 1979) as well as in CS cells (Rainbow, 1989). HCR of adenovirus can be used as a sensitive measure of DNA repair capacity for damage induced by both physical and chemical agents. HCR of cisplatin-damaged DNA has been shown to be greatly reduced in both Chinese hamster ovary (CHO) mutants (Sheibani et al., 1989; Collins, 1993) and human repair-deficient XP cells (Hansson et al., 1991; Poll et al., 1984) compared to their repair-proficient counterparts. HCR is also useful to determine if cellular resistance to cisplatin is arising from increased DNA repair. Enhanced DNA repair capacity has been shown to exist in cisplatin-resistance cells that exhibit increased reactivation of a cisplatin-damaged plasmid (Sheibani et al., 1989).

A modified version of the HCR assay is carried out by pretreating host cells with a DNA-damaging agent prior to infection with the DNA-damaged virus. This can result in an enhanced reactivation (ER) of the virus. UVER and γ-ray ER of adenovirus have been detected in XP cells (Lytle et al., 1976), as well as CS cells (Jeeves and Rainbow, 1983a and 1983b). The pretreatment of cells with DNA-damaging agents is thought to elicit an inducible DNA repair response.

There are limitations of the HCR assay as demonstrated by repair studies of cisplatin damage in cells that are known to be crosslink-repair deficient. The testicular teratoma cell line (SuSa), previously shown to be deficient in the repair of d(GpG), d(ApG) and interstrand crosslinks, was found to reactivate cisplatin-treated virus to a similar level as a repair-proficient control line (Maynard et al., 1989). As well, the examination of HCR in extracts from crosslink-sensitive Fanconi anemia cells revealed no deficiency in the repair of cisplatin-damaged plasmid, while a significant reduction was observed in the XP cell extracts (Hansson et al., 1991). From these studies it appears that the HCR assay is not a reliable test for the detection of crosslink repair defects. Studies on the repair of interstrand crosslinks in the Ad genome suggest that these lesions are not repaired in repair-proficient normal cells (Day, 1975), and therefore, we would not expect to see any decrease in their repair by ICL repair-deficient cells such as FA.

C. Cellular Capacity Assay

This assay has been described by Coohill et al. (1977) as the ability of UV-treated cells to replicate untreated herpes simplex virus. They termed it viral capacity. Since then, other viruses and damaging agents have been used in cellular capacity assays to examine the sensitivity of various cells. Physical and chemical treatment of cells reduces their ability to support viral infection (Rainbow, 1981; Defais et al., 1983). The task of synthesizing viral DNA and proteins depends on the metabolic activities of the cells and on the integrity of cellular genes that produce the host proteins required for viral replication. Therefore, a reduced cellular capacity is thought to result from unrepaired

damage within the cellular genes that must be transcribed for viral replication and hence reflects the repair of transcriptionally active genes. Like HCR, the capacity assay has utilized viral probes as a means of measuring the DNA repair capabilities of cells. The capcity of UV-irradiated cells to support viral infection has been shown to be a cellular, DNA-dependent function (Coohill et al., 1977). Capacity studies have shown that DNA repair-deficient cells from XP and CS patients exhibit a reduced capacity to support adenoviral replication following UV treatment (Rainbow et al., 1993; Ryan and Rainbow, 1986; Lytle et al., 1976). Parsons et al. (1986) have proposed that the cellular capacity assay may be a potential predictor of drug sensitivity in tumour cells in order to better plan chemotherapeutic strategies for cancer patients.

Delaying the viral infection of cells following treatment permits cellular recovery and can increase capacity curves. This recovery has been shown for several types of cells and is thought to occur as a result of DNA repair during the delay period before infection. Lytle et al. (1976) observed a recovery in the capacity of normal fibroblasts following a 96 hr. delay of infection after cell treatment with UV; however, no recovery was seen in XP fibroblasts which showed a further decrease in capacity. Therefore, delayed cellular capacities can enhance the detection of repair defects that may not be seen with immediate infection.

ROBERTS SYNDROME

Roberts syndrome (RS) was first described by Dr. John Roberts in 1919 in a child with cleft lip and palate, protrusion of the intermaxillary part of the upper jaw and abnormal development of the limb bones (Roberts, 1919). It is a rare, autosomal recessive disease with only about 100 cases identified worldwide. The clinical diagnostic features include pre- and post-natal growth retardation, symmetric limb reductions and craniofacial deformities like cleft lip and cleft palate (Freeman et al., 1974). Because of the similarities of the presenting features, RS has been associated with other genetic

syndromes, most notably SC-phocomelia syndrome (Herrmann et al., 1969). There exists a vast range in the severity of symptoms with many cases resulting in spontaneous abortion or stillbirth of markedly malformed fetuses. Mildly afflicted patients may reach adulthood and lead normal lives, often benefitting from surgeries to correct club feet, facial clefts and to lengthen limbs (Holden et al., 1992). RS is an interesting disorder due to the division of affected individuals into two subgroups that are clinically indistinguishable but that exhibit striking differences at the cellular level. Chromosomes from RS+ individuals display a distinct abnormality of the constitutive heterochromatin which manifests as premature separation of the centromeric and nucleolar organizing regions of the chromosomes, as well as the Yqh region in males (Freeman et al., 1974; German, 1979; Tomkins et al., 1979; Louie and German, 1981). The remaining patients, denoted RS-, have normal chromosome morphology (Burns and Tomkins, 1989). Recent reports approximate that 79% of RS patients are positive for the heterochromatin repulsion (Van Den Berg and Francke, 1993a).

RS+ cells are peculiar in other ways, aside from the heterochromatic puffing. Studies have demonstrated abnormal growth characteristics in RS+ fibroblasts including prolonged mitosis, abnormal cytokinesis, abnormal cellular morphology, reduced cell growth, reduced plating efficiency and low cell density at confluence (Tomkins and Sisken, 1984). The nuclei of RS+ cells often contain blebs and lobules and there is an increased frequency of micronucleation that has been suggested to result from lagging chromosomes which do not successfully complete the anaphase step of cell division (Jabs et al., 1991). Due to these observations, it has been postulated that RS may be a mitotic mutant, however, analysis of the kinetochore structure did not reveal any anomalies (Jabs et al., 1991).

Another characteristic that is exhibited solely by the RS+ cells is a hypersensitivity to many mutagenic and carcinogenic agents, most notably to DNA-crosslinking agents (Gentner et al., 1986). Mitomycin C (MMC) sensitivity has been demonstrated in both lymphoblast and fibroblast cells (Allingham-Hawkins and Tomkins,

1991; Gentner et al., 1985; Burns and Tomkins, 1989; Van Den Berg and Francke, 1993b). Treatment of fibroblasts with cisplatin has revealed a dose reduction factor of four compared to normal controls (Gentner et al., 1986). In light of this data, it has been suggested that RS may represent a syndrome that is deficient in DNA repair, particularly DNA crosslink repair.

LI-FRAUMENI SYNDROME

The Li-Fraumeni syndrome (LFS) is a rare, autosomal dominant disease that is characterized by a very high incidence and early onset of several types of cancer. These include soft tissue sarcomas, breast carcinomas, acute leukemias, osteosarcomas, brain tumours and adrenocortical carcinomas (Li and Fraumeni, 1969). The predisposing factor in LFS patients is an inherited germline mutation in one p53 allele (Malkin et al., 1990). The most common mutations involve transversions and transitions that lead to amino acid substitutions, with a few resulting in stop codons. Frameshift mutations also occur as a result of base pair insertions and deletions. The identification of abnormally spliced transcripts has been found in rare cases, leading to significant disruptions of the gene (as reviewed in Malkin, 1994). As well, there is a case of a trinucleotide repeat mutation that suggests a defect in DNA repair can lead to LFS (Strauss et al., 1995).

Normal functioning of the p53 tumour suppressor gene is a complex matter. The p53 protein appears to function in several ways. It is involved in the regulation of the cell cycle (Kuerbitz et al., 1992; Yin et al., 1992), in mediating cellular response to DNA damage (Kastan et al., 1991), in controlling p53-dependent apoptosis (Lowe et al., 1993a and 1993b), and in the repair of DNA damage (Wang et al., 1995; Yamaizumi and Sugano, 1994; Ford and Hanawalt, 1995). It is believed that p53 arrests damaged cells in G_1 in order to allow sufficient time for DNA repair before DNA replication. If the cell

incurs too much damage, then apoptosis is triggered. Therefore, loss of proper p53 function, as in LFS, may faciliatate the onset of cancer by permitting the replication and/or survival of genetically damaged cells.

The response of Li-Fraumeni cells to DNA-damaging agents is interesting because of their altered p53 genotype, and because of the association of cancer susceptibility and hypersensitivity to DNA-damaging agents. Some researchers have found no significant difference in the colony survival of p53-heterozygous Li-Fraumeni fibroblasts compared to normal controls when treated with x-rays, UV irradiation, MNNG and mitomycin C (Little et al., 1987), whereas immortalized LFS cells that have lost their wt p53 allele exhibit enhanced UV resistance compared to normal and p53-heterozygous cells (Ford and Hanawalt, 1995). This UV-enhanced resistance has been attributed to decreased apoptosis in the irradiated p53-deficient LFS cells (Ford and Hanawalt, 1995). The DNA repair capacity of the immortalized, p53-deficient LFS cells was shown to be deficient in global repair and in repair of the nontranscribed strand of active genes, but was normal for transcription-coupled repair of the transcribed strand (Ford and Hanawalt, 1995). Wang et al. (1995) have observed that p53-heterozygous LFS fibroblasts were significantly reduced in their gene-specific repair of UV-induced lesions. The nuclear accumulation of wt p53 has also been linked to DNA repair capacity. Cockayne syndrome cells, which are deficient only in the preferential NER of actively transcribed genes, had an eightfold lower minimum required dose of UV in order to induce accumulation of p53. In contrast, XP-C cells , which have normal preferential repair but are deficient in bulk genomic repair, exhibited a minimun required dose similar to that for normal control cells (Yamaizumi and Sugano, 1994). These results suggest that the signal for p53 induction is the presence of unrepaired DNA lesions in actively transcribed regions of the genome.

PROPOSED STUDY

Previous studies on the cellular sensitivity of RS+ cells to DNA-damaging agents revealed a hypersensitivity of these cells to such agents, especially to DNA crosslinkers. This led to the suggestion of a possible DNA-repair defect in these cells. I set out to examine the repair capacity for cisplatin-damaged DNA in RS+ cells using adenovirus as a probe in HCR and cellular capacity assays and observed no deficiency in the repair of DNA in these cells. XP and FA cells were also studied for their response to cisplatin damage in order to provide comparative results for these syndromes with known repair deficiencies. XP cells were shown to have significantly reduced HCR of cisplatin-damaged Ad as well as significantly reduced capacity to replicate Ad DNA following cellular cisplatin damage. Normal HCR was observed for FA cells; however, cisplatin-treated FA cells were reduced in their capacity to support Ad replication reflecting their inability to repair DNA interstrand crosslinks.

Work with Li-Fraumeni syndrome cells was also conducted to try and elucidate the repair capacity of these cells and to examine the effect of p53 status on DNA repair. It was observed that LFS cells show normal host cell reactivation of cisplatin-damaged virus regardless of the presence of wt p53 in the cells; however, the p53-heterozygous LFS fibroblasts were observed to have reduced capacity to support Ad replication after cisplatin damage to the cells. This suggests that p53 status is a factor in the repair of cellular DNA damage and supports a role for p53 in the repair of actively transcribed cellular genes.

CHAPTER TWO

DETERMINATION OF OPTIMAL EXPERIMENTAL PROTOCOLS TO EXAMINE DNA REPAIR IN ROBERTS SYNDROME CELLS USING ADENOVIRUS AS A PROBE

DETERMINATION OF OPTIMAL EXPERIMENTAL PROTOCOLS TO EXAMINE DNA REPAIR IN ROBERTS SYNDROME CELLS USING ADENOVIRUS AS A PROBE

ABSTRACT

Roberts syndrome cells that display the heterochromatin separation (RS+) are hypersensitive to several DNA-damaging agents, most notably to DNA-crosslinking agents. It has been suggested that this sensitivity to DNA-damaging agents results from some deficiency in the repair of DNA damage. This work was conducted in order to determine the best experimental parameters for investigating the DNA repair abilities of RS+ cells using adenovirus (Ad) as a probe. Such parameters included the origin of cells being studied (lymphoblasts or fibroblasts), the agent used to damage the DNA (UV or cisplatin), and the methods used to analyze repair capacity. The host cell reactivation (HCR) of UV-irradiated and cisplatin-treated Ad as well as the capacity of UV-irradiated cells for Ad DNA synthesis were examined. Protocols were developed to examine HCR for Ad DNA synthesis as well as for reporter gene expression using a recombinant Ad containing the β-galactosidase (lacZ) gene, in either human lymphoblasts or skin fibroblasts. Results indicate that both normal and RS+ cells showed a similar HCR for UV- and cisplatin-treated Ad. This was true for both lymphoblast and fibroblast cell types. The reduction in viral DNA synthesis was about 10 to 20 times greater than the reduction in expression of the β-gal reporter gene following either UV or cisplatin treatment of the virus. In all the experiments conducted, there was no evidence for a DNA-repair defect in RS+ cells. The cellular capacity of UV-irradiated RS+ fibroblasts was not reduced compared to that of normal cells, and both the normal and RS+ cells showed similar HCR of UV- and cisplatin-damaged virus when measured by either Ad DNA synthesis or β-gal activity. These initial observations were the basis for more extensive studies on the DNA-repair capacity of Roberts syndrome which also included

xeroderma pigmentosum (XP) and Fanconi anemia (FA) cells, with protocols developed here also being used for the later study of Li-Fraumeni syndrome.

INTRODUCTION

There are several studies which report an increased sensitivity to a number of DNA-damaging agents for cells from a subgroup of Roberts syndrome patients termed RS+ (Gentner et al., 1985 and 1986; Van den Berg and Francke, 1993; Burns and Tomkins, 1989). It has been suggested that the increased sensitivity of RS+ cells results from a DNA-repair deficiency. A major objective of the present work was to examine the DNA-repair capacity of RS+ cells, using adenovirus (Ad) as a probe.

Previous work in our laboratory has examined the host cell reactivation (HCR) of DNA-damaged Ad in human fibroblasts which have been reported to be sensitive to various DNA-damaging agents. Using this approach, our laboratory has detected a repair deficiency for UV-irradiated Ad in fibroblasts from patients with xeroderma pigmentosum (XP) and Cockayne syndrome (CS) (Rainbow, 1989). Since RS+ cells have been reported to be approximately two-fold more sensitive to UV than normal cells (Gentner et al., 1985), it was considered of interest to firstly examine the HCR of UV-irradiated Ad in RS+ cells. Both RS+ fibroblasts and RS+ lymphoblastoid cell lines were available to us and initial work was carried out to examine the optimum experimental protocols for HCR studies using these cells. HCR of UV-irradiated virus was examined using either survival of Ad DNA synthesis (Arnold and Rainbow, 1996) or survival of expression of the β-gal reporter gene encoded by a recombinant Ad (McKay and Rainbow, 1996; Francis and Rainbow, 1996). The expression of the β-gal reporter gene is a measure of the amount of transcription of this gene.

Another assay that can be used to detect cellular, DNA-repair deficiencies is the cellular capacity assay which measures the capacity of cells treated with various DNA-damaging agents to support viral replication. Using this approach, nucleotide excision

repair (NER)-deficient xeroderma pigmentosum (XP) cells have been shown to have greatly reduced capacity for viral replication following UV treatment (Lytle et al., 1976). Therefore, it was of value to examine UV-irradiated RS+ cells for their capacity to replicate Ad DNA.

The hypersensitivity of RS+ cells to several DNA-crosslinking agents (Gentner et al., 1986; Van den Berg and Francke, 1993; Burns and Tomkins, 1989; Tomkins, personal communication) suggests the possibility of a defect in the repair of DNA crosslinks for RS+ cells. Mean dose reduction factors for cisplatin treatment of RS+ cells have been reported to be in the range of 4-5 (Gentner et al., 1986; Tomkins, personal communication). For this reason, the HCR of cisplatin-treated Ad was also examined in RS+ cells.

The first RS+ cells that were available in our laboratory were lymphoblastoid cells immortalized with Epstein-Barr virus. It was of interest to determine whether these cells could be readily used in the HCR assays, as well as assess any differences in the response of lymphoblasts compared to fibroblasts in the HCR assays. It is possible that an HCR assay for DNA-damaged Ad in lymphoblasts could be extended to primary human lymphocyte cultures and be of value in population screening for DNA-repair capacity and in predicting patient response to chemotherapeutic agents.

MATERIALS AND METHODS

Cell Strains

Roberts Syndrome Cells: The R22 and S6012 Roberts syndrome fibroblasts and the Epstein-Barr-immortalized R-20 and LB-1 RS lymphoblastoid cell lines were kindly provided by Dr. Darrell Tomkins, McMaster University, Hamilton, Ont. All RS strains have been shown to exhibit the RS+ phenotype by Giemsa staining of metaphase chromosomes. *Normal Cells*: Normal fibroblast strains GM969, GM37e, GM8399 and GM3440B were all obtained from the NIGMS Human Genetic Mutant Cell Repository

(Coriell Institute, Camden, NJ). The normal 423 strain was kindly provided by Dr. Patricia Chang, McMaster University, Hamilton, Ont. The EBV-immortalized normal lymphoblastoid cell line HSC-3TO was provided by Dr. Manuel Buchwald, Hospital for Sick Children, Toronto, Ont. *Xeroderma Pigmentosum Cells*: The xeroderma pigmentosum strains XP2BI (GM3021) and XPCS1BA (GM13025) were obtained from the NIGMS Human Genetic Mutant Cell Repository and belong to the XP complementation groups G and B respectively.

All fibroblast cells were grown in 75 cm^2 tissue culture flasks (Corning Incorporated, Corning, NY) in α-minimal essential medium (α-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO) and 1% (v/v) antibiotic/antimycotic containing 10 000 U/mL penicillin G sodium, 10 000 ug/mL streptomycin sulfate and 25 ug/mL amphotericin B as Fungizone® in 0.85% saline (Gibco BRL, Life Technologies Inc., Grand Island, NY). Cells were incubated in a 5% CO_2, 100% humidified, water-jacketed incubator (Forma Scientific Inc., Marietta, OH) at 37°C. At confluency, cells were passaged by aspirating spent growth medium, rinsing in 1x phosphate buffered saline (PBS) and by the addition of 2x trypsin-EDTA containing 0.5% trypsin and 5.3 mM EDTA•4Na (Gibco BRL, Life Technologies Inc., Grand Island, NY). Once the cells rounded up, they were resuspended in growth medium and subcultured at appropriate dilutions. Lymphoblastoid cell lines were grown in suspension in 75 cm^2 flasks in RPMI 1640 medium supplemented with 15% FBS and 1% L-glutamine (Gibco BRL, Life Technologies Inc., Grand Island, NY) and incubated as above. At sufficient density, cells were collected by centrifugation and counted using Trypan blue exclusion (Gibco BRL, Life Technologies Inc., Grand Island, NY). For passaging, normal lymphoblasts were seeded at a density of $2.5X10^5$ cells/mL and RS+ lymphoblasts at $5x10^5$ cells/mL.

Cisplatin

Cisplatin (*cis*-diamminedichlorideplatinum(II)) was obtained from Sigma Chemical Company (St. Louis, MO). Stock solutions of 1 mM were made in 1x PBS (1.4 M chloride ion) for the treatment of cells or in 1x low chloride PBS (6.6 mM chloride ion) for the treatment of virus. Stock solutions were made fresh and filtered through a 0.45 μm disposable filter (Nalge Company, Rochester, NY) just prior to use.

Virus

Human adenovirus serotype 5 (Ad5) and the recombinant Ad5(lacZ) were the viruses used for infection of cells in the assay for host cell reactivation (HCR) of viral DNA synthesis. They are both replicating viruses and are used to assess amount of viral DNA synthesis. The Ad5(lacZ) virus contains the *E. coli* β-galactosidase (lacZ) gene inserted into the deleted, non-essential E3 region of the Ad viral genome and was generously provided by Dr. Frank Graham, Departments of Biology and Pathology, McMaster University, Hamilton, Ont. The recombinant virus used in the HCR assay for reporter gene expression, Ad5HCMVsp1lacZ, also contains the *E. coli* β-galactosidase (lacZ) gene but it is inserted into the E1 region of the Ad viral genome which prevents replication of the virus. Construction of this virus has been previously described (Morsy et al., 1993) and was also provided by Dr. Frank Graham. This virus is used to assess the amount of expression of the β-gal gene which is a reflection of its transcription. Preparation of viral stocks was carried out using 293 human embryonic kidney cells (Graham et al., 1977). Confluent cell monolayers were infected with virus at an MOI of 1-2 plaque forming units (pfu)/mL. Following a 90 min. incubation, cells were overlayed with complete growth medium and incubated for several days. When the cytopathic effect was evident (usually at 48 to 72 hours after infection), the cell suspension was collected and centrifuged to pellet the cells. The pellet was then resuspended in 10% glycerol in medium and was freeze-thawed three times in order to release the viral particles. The titre of the viral stocks was determined by infecting 293 cells with varying dilutions of

virus and counting the resulting plaques. Viral stocks generally contained titres of $5x10^8$ to $5x10^9$ pfu/mL.

Host Cell Reactivation of Cisplatin- and UV-Damaged Virus in Fibroblasts

Cells were seeded at a density of $1x10^5$ cells/well in 24-well tissue culture plates (Nunc, Denmark) and were incubated 24 hrs. prior to infection. Ad5wt, Ad5(lacZ), or AdHCMVsp1lacZ were treated with varying doses of cisplatin in serum-free medium containing 1% antibiotic/antimycotic for 24 hrs. at 37°C to damage the viral DNA. Because cisplatin molecules contain two chloride atoms as leaving groups, the chloride ion concentration of the treatment solution is an important factor to consider. For this reason, and in order to minimize variation between experiments, the chloride ion concentration was set to 50 mM for all HCR experiments. For UV treatment of virus, viral suspensions were diluted in serum-free α-MEM and were irradiated in 35 mm dishes at varying fluences with a germicidal UV light source (General Electric, Cleveland, OH). The medium was aspirated from the wells and cells were infected with 0.2 mL of viral suspension in serum-free α-MEM at a multiplicity of infection (MOI) of 20 pfu/cell. After a 90 min. incubation at 37°C, the infection was terminated by aspirating the virus and then adding 0.5 mL of fresh growth medium to the wells. Plates were then incubated for 48 hrs. (quantification by β-gal activity) or 72 hrs. (quantification by viral DNA synthesis) at 37°C to allow for repair and reporter gene expression, and repair and replication of viral DNA, respectively.

Quantification of Viral DNA Synthesis:

Samples were lysed by the addition of 0.2 mL 4x pronase solution (4 mg/mL pronase, 2.4% SDS, 4 mM Tris, 4 mM EDTA) to each well and were collected after a 3 hr. incubation at 37°C. DNA was extracted by the phenol:chloroform method and was precipitated by adding 5 M NaCl and 2 volumes of cold absolute ethanol. Samples were spun at 14 000 rpm for 30 mins. to pellet the DNA and were inverted to dry after pouring

off the ethanol. After resuspending in Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8), samples were denatured with 0.29 M NaOH, neutralized with 0.2x SSC, and then slot-blotted onto nylon membrane (Gene Screen Plus, NEN Research Products, Boston, MA) using the Minifold II Slot-Blot system (Schleicher and Schuell Inc., Keene, NH). The membranes were then prehybridized and probed with ^{32}-P labelled Ad DNA.

Quantification of β-gal Activity:

Samples were lysed by the addition of 200 μL of a solution containing 250 mM Tris-HCl (pH 7.8), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.5% NP40. Cell lysates were scraped from the bottom of the wells and collected into eppendorf tubes. Tubes were vortexed briefly before adding 175 μL of the second solution containing 20 mM KCl, 2 mM $MgSO_4$, 200 mM Na_2PO_4, and 100 mM 2-mercaptoethanol and were then incubated for 5 mins. at 37°C. The next step involved the addition of 132 μL of the β-gal enzyme substrate, o-nitrophenol β-D-galactopyranoside (4 g/L in 100 mM Na_2PO_4, pH 7.5) to each tube, followed by incubation at 37°C until the colour reaction was evident. The reaction was terminated by the addition of 172 μL of 1 M Na_2CO_3. The absorbance of the samples was measured at 420 nm using a Beckman DU-7 spectrophotometer (Beckman Scientific Instruments, Irvine, CA).

Host Cell Reactivation of UV-Damaged Virus in Lymphoblast Cells

One million cells were placed in eppendorf tubes with equal volumes of serum-free medium. Viral suspensions of either Ad5 or Ad5(lacZ) were UV-irradiated as for the HCR of fibroblasts and 40 μL of the viral suspension was added to each tube containing the cells. Tubes were incubated for 60 mins. at 37°C with intermittent shaking. To terminate the infection, RPMI 1640 complete medium was added to the tubes and the infected cells were incubated for 48 hrs. (quantification by β-gal activity) or 72 hrs. (quantification by viral DNA synthesis).

Quantification of Viral DNA Synthesis:

The samples of infected cells were centrifuged for 10 mins. to pellet the cells. The supernatant was discarded and the cells were lysed by the addition of 0.5 mL of 1x pronase solution (1 mg/mL pronase, 1.2% SDS, 2 mM Tris, 2mM EDTA). Tubes were incubated for 2 hrs. at 37°C and the DNA was extracted and blotted as for the fibroblasts.

Quantification of β-gal Activity:

Samples were centrifuged for 10 mins. to pellet the cells. The supernatant was discarded and the cells were lysed by the addition of 200 μL of the same solution used for the fibroblasts. Samples were then treated in the identical manner as for the fibroblast experiments.

Cellular Capacity Experiments

Cells were seeded at a density of 1.9×10^4 cells/well in 96-well plates (Falcon, Becton Dickinson & Company, Lincoln Park, NJ). After a 24 hr. incubation, the growth medium was aspirated and 40 μL of 1x PBS was added to the wells before irradiation. Cells were exposed to varying fluences of UV light and the PBS was aspirated before infecting with 40 μL of Ad5(lacZ) viral suspension at an MOI of 100 pfu/cell. Plates were incubated for 90 mins. at 37°C and infection was terminated by aspiration of virus and addition of 200 μL growth medium. Plates were incubated for 48 hrs. at 37°C to allow for repair of cellular DNA and subsequent replication of virus. Samples were lysed by aspirating the growth medium from the wells and adding 50 μL of proteinase K solution (1 mg/mL proteinase K, 1% SDS, 0.2x SSC) for 1.5 hrs. at 37°C. To denature the viral DNA for blotting, 50 μL of NaOH solution (1 M NaOH, 1 mM EDTA), was added to the wells and plates were incubated another 1.5-2 hrs. before slot-blotting to nylon membrane. Blots were probed as for HCR experiments, in the manner discussed below.

Radiolabelling of Membranes

Prehybridization: To help prevent non-specific binding, dried membranes were sandwiched between mesh screens and briefly soaked in 2x SSC before being inserted into a hybridization tube (Hybaid, Bio/Can Scientific, Mississauga, Ont.) with 15 mL of prehybridization buffer (0.15 g bovine serum albumin, 7.5 mL 1 M NaH_2PO_4, 2.25 mL deionized formamide, 5.25 mL 20% SDS). Prehybridization was carried out for 2 hrs. at 60°C in a Hybaid mini hybridization oven (Bio/Can Scientific, Mississauga, Ont.).

Labelling of DNA Probe: Ad2 DNA was labelled with ^{32}P-dCTP by random primer extension. This was carried out by boiling 200 ng of Ad2 DNA in 8 µL of double distilled water in order to denature it. It was then quenched on ice for 3 mins. and centrifuged at 14 000 rpm for 10 secs. to collect the condensate. To this was added 2 µL of cocktail C trinucleotides, 5 µL of random primer, 5 µL of α-^{32}P-dCTP (DuPont, NEN Research Products, Boston, MA) and 2 µL of Klenow, the large fragment of DNA polymerase I. Following a 45 min. incubation at 37°C, the probe was diluted with approximately 200 µL of TE buffer (pH 8). The probe was then denatured by heating at 100°C for 10 mins. and further diluted with TE buffer before being added to the hybridization tube. Hybridization was carried out at 60°C for approximately 15 hrs.

Membrane Washes: Following hybridization, membranes were washed with 150 mM NaH_2PO_4, 1% SDS once for 15 mins. and twice for 30 mins. at 60°C. A final wash of 30 mM NaH_2PO_4, 0.1% SDS was carried out for 30 mins. at 50°C, after which time the membranes were removed from the tube and allowed to dry. To quantitate the amount of radioactivity, the blots were exposed to a phosphor screen and were analyzed using a Molecular Dynamics phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA) which provided numerical counts of radioactivity.

RESULTS

Survival of UV-irradiated Adenovirus in Lymphoblasts

The host cell reactivation of DNA-damaged Ad has been used extensively in our laboratory to examine DNA repair in fibroblasts from a number of different human syndromes. Since lymphoblastoid cell lines are available from patients with different human syndromes including lymphoblastoid lines from RS+ patients, is was considered of value to examine if HCR of DNA-damaged Ad could be used to examine the repair capacity of lymphoblast cell lines. Two endpoints were used to measure viral survival following treatment with DNA-damaging agents: the amount of viral DNA synthesized at 72 hrs. following infection with Ad, and the expression of a β-galactosidase (β-gal) reporter gene at 48 hrs. following infection with the non-replicating, recombinant virus Ad5HCMVsp1lacZ.

Figure 1 shows typical results for the survival of UV-treated Ad in normal and RS+ lymphoblasts, as measured by both Ad DNA synthesis and β-gal expression. Results indicate that the survival of viral DNA synthesis and β-gal expression for UV-irradiated Ad can both be used to examine HCR of Ad in lymphoblastoid cell lines. It can be seen also that following UV exposure to the virus, the reduction in viral DNA synthesis was substantially greater than the reduction in expression of the β-gal reporter gene. A comparison of D_0 values for UV survival using these two viral endpoints indicates that for normal lymphoblasts the DNA synthesis assay exhibits an 11 fold greater sensitivity, and the LB-1 RS+ lymphoblasts exhibit an eight fold greater sensitivity compared to D_0 values obtained in the reporter gene expression assay. Furthermore , it can be seen that the survival of UV-irradiated Ad following infection of RS+ cells was similar to that following infection of normal cells. When compared to the normal cell line, relative D_0 values for UV survival of Ad for the RS+ lymphoblast cells are 1.4 and 1.1 using the endpoint of β-gal activtiy and 0.8 for the one RS+ cell line used in the Ad DNA synthesis

assay. These results suggest that normal and RS+ lymphoblasts have similar repair of UV-irradiated Ad DNA.

Survival of β-gal Expression of UV-irradiated Adenovirus in Lymphoblasts Versus Fibroblasts

Due to the EBV-immortalization of the lymphoblast cells, and also because of their different cell type, the repair capacity of the lymphoblasts was compared to that of fibroblasts to determine if they exhibited a similar HCR response.

Figure 2 shows typical results for the survival of β-gal reporter gene expression for UV-irradiated Ad following the infection of both lymphoblasts (A) and fibroblasts (B). It can be seen that normal and RS+ cells have a similar HCR of UV-damaged virus for infection of both fibroblasts and lymphoblasts. Mean relative D_0 values for RS+ cells compared to normal were 0.80 for the lymphoblast line LB-1, and 0.75 and 0.97 for the fibroblast strains R22 and S6012, respectively. This indicates that a deficiency in repair of UV-damaged DNA could not be detected in either RS+ fibroblasts or lymphoblasts.

Survival of Cisplatin-damaged Adenovirus in Fibroblasts

Previous work has shown that RS+ fibroblasts are two-fold more sensitive to UV exposure compared to normal fibroblasts (Gentner et al., 1985) and substantially more sensitive compared to normal cells following exposure to DNA-crosslinking agents (Gentner et al., 1986). Dose reduction factors (DRF) for cisplatin-treated RS+ fibroblasts have been reported to be in the range of 4-8 (Gentner et al. 1986, Tomkins, personal communication). It is possible that the difference in the repair capacity of normal and RS+ cells may not be detectable when using UV-irradiated Ad due to the relatively small increase in sensitivity of RS+ cells to UV, and for this reason, the HCR of cisplatin-treated Ad was also examined. Figure 3 shows the survival of cisplatin-damaged Ad in fibroblasts for both Ad DNA synthesis and β-gal activity. It can be seen that the reduction in Ad DNA synthesis was considerably greater than the reduction in β–gal

activity for cisplatin-treated Ad, as found for UV-irradiated Ad following infection of lymphoblasts (Figure 1). The relative D_0 values for survival of cisplatin-treated Ad for the endpoint of Ad DNA synthesis compared to β-gal activity were 13-fold and 20-fold reduced for the normal 423 and the RS+ R22 cell strains, respectively. Results also show no significant difference in the survival of cisplatin-treated Ad following infection of normal and RS+ fibroblasts. For the survival of Ad DNA synthesis, the mean relative D_0 values for infection of the normal strains were 1.0, 1.2 and 0.80, and for the RS+ strains, 1.1 and 1.3. For survival of β-gal activity, the D_0 values were not obtainable for all the cell strains used because of the lack of reduction in survival over the dose range used in the experiment. However, the results are qualititively similar to those obtained using the survival of Ad DNA synthesis with no indication of a DNA-repair defect for the RS+ fibroblasts compared to the normal controls.

Survival of DNA Synthesis of Cisplatin-damaged Ad in Fibroblasts at Different Times Following Infection

RS+ cells are hypersensitive to several DNA-damaging agents, including UV and cisplatin, suggesting the possibility of a DNA-repair deficiency in RS+ cells. However, a reduction in HCR for either UV- or cisplatin-treated Ad was not detected. It was considered possible that a difference in repair rate for RS+ cells might not be detected in the HCR assay using an endpoint for Ad DNA synthesis at 72 hrs post infection. For this reason, HCR experiments were also performed by measuring survival of Ad DNA sythnthesis at an earlier timepoint after infection.

Figure 4 shows the survival of Ad DNA synthesis for cisplatin-treated Ad measured at 36 hrs. and 72 hrs. post-infection. It can be seen that there in an increase in the survival of Ad DNA synthesis with increasing incubation time; however, there was no difference in the survival of cisplatin-damaged Ad between the normal and RS+ cells at either timepoint. The relative D_0 values for survival of Ad DNA synthesis in the RS+

compared to normal cells were 1.0 and 1.1 when measured at 36 and 72 hrs. respectively. These results give further support for normal repair of cisplatin-damaged DNA in RS+ cells.

Cellular Capacity of UV-irradiated Fibroblasts for Ad DNA Synthesis

The cellular capacity assay has been used previously to show the sensitivity of cells to DNA-damaging agents as well as deficiencies in DNA repair. UV-treated, NER-deficient XP fibroblasts show a reduction in capacity for viral infection compared to UV-treated normal cells (Lytle et al., 1976; Rainbow, 1980; Rainbow, 1989). We therefore examined the capacity of UV-irradiated RS+ fibroblasts for Ad DNA synthesis. Figure 5 shows the cellular capacity for Ad DNA synthesis following UV-irradiation of RS+ fibroblasts as well as excision deficient XP and normal human fibroblasts. The D_{37} values for the survival of Ad DNA synthesis in UV-irradiated fibroblasts were calculated. Relative D_{37} values obtained for infection of the RS+ and and XP-B cell strains compared to that obtained for the normal fibroblast strain were 1.6 and 0.23, respectively. The capacity curve for UV-irradiated XP-G fibroblasts did not lend itself to a linear quadratic curve fit such that a D_{37} value for the capacity of UV-irradiated XP-G cells could not be obtained. Notwithstanding, it can be seen that the two XP strains are more sensitive than the normal strain in the cellular capacity assay as previously reported (Lytle et al., 1976). The RS+ strain did not exhibit an increased sensitivity in cellular capacity compared to the normal strain, and even appeared somewhat more resistant than the normal strain tested. The inclusion of other normal and RS+ cells will be necessary in order to determine if there is any significant difference in the cellular capacity of UV-irradiated normal and RS+ fibroblasts.

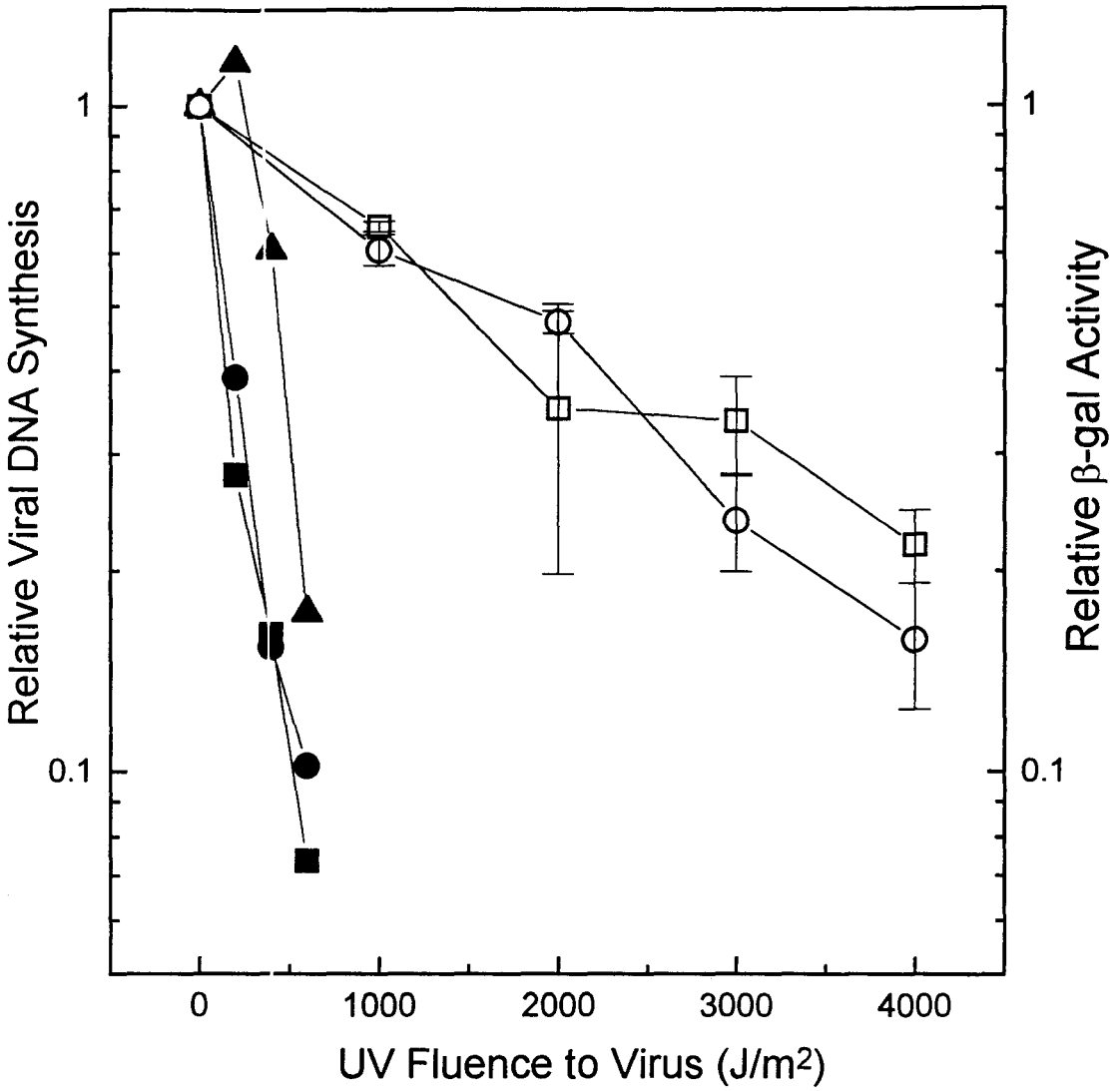

Figure 1. **Reactivation of UV-irradiated Ad in lymphoblasts.** Viral repair quantitated by DNA synthesis is represented by the left axis (closed symbols). Samples were collected 72 hrs. after infection. Data points are single determinations from one experiment. Viral repair quantitated by β-gal activity is represented by the right axis (open symbols). Samples were collected 48 hrs. after infection. Data points are the mean of single determinations from two experiments shown with standard error about the mean. ■HSC-3TO (normal); ●LB-1, ▲R-20 (RS+).

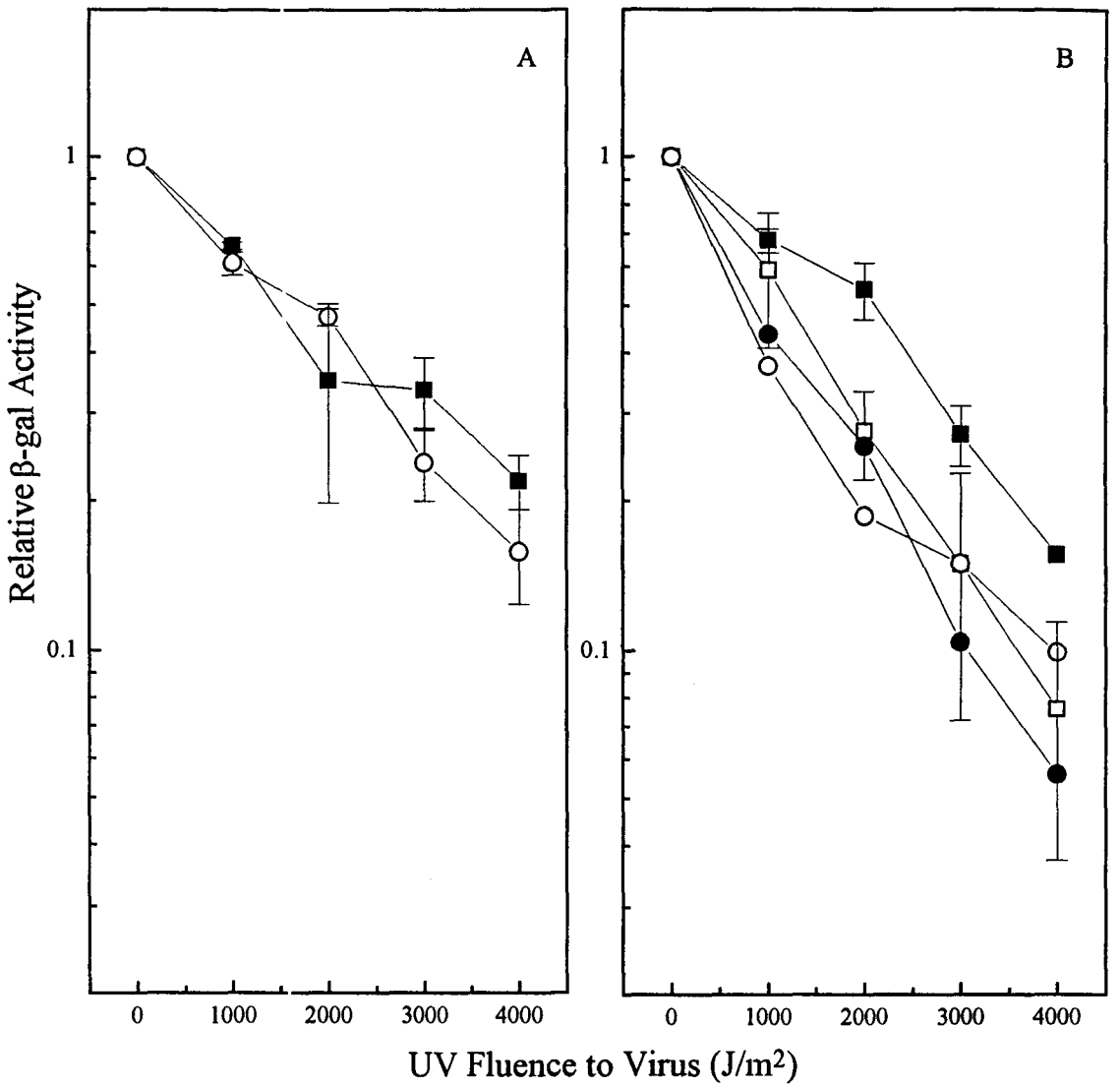

Figure 2. **Reactivation of UV-irradiated Ad in lymphoblasts and fibroblasts quantitated by β-gal activity**. Samples were collected 48 hrs. after infection. Data points are the mean of single determinations from two experiments shown with standard error about the mean except for R22 and S6012 points which are single determinations from one experiment. (A) Lymphoblasts: ■HSC-3TO (normal); ○LB-1 (RS+). (B) Fibroblasts: ■GM8399, □GM3440B (normal); ●R22, ○S6012 (RS+).

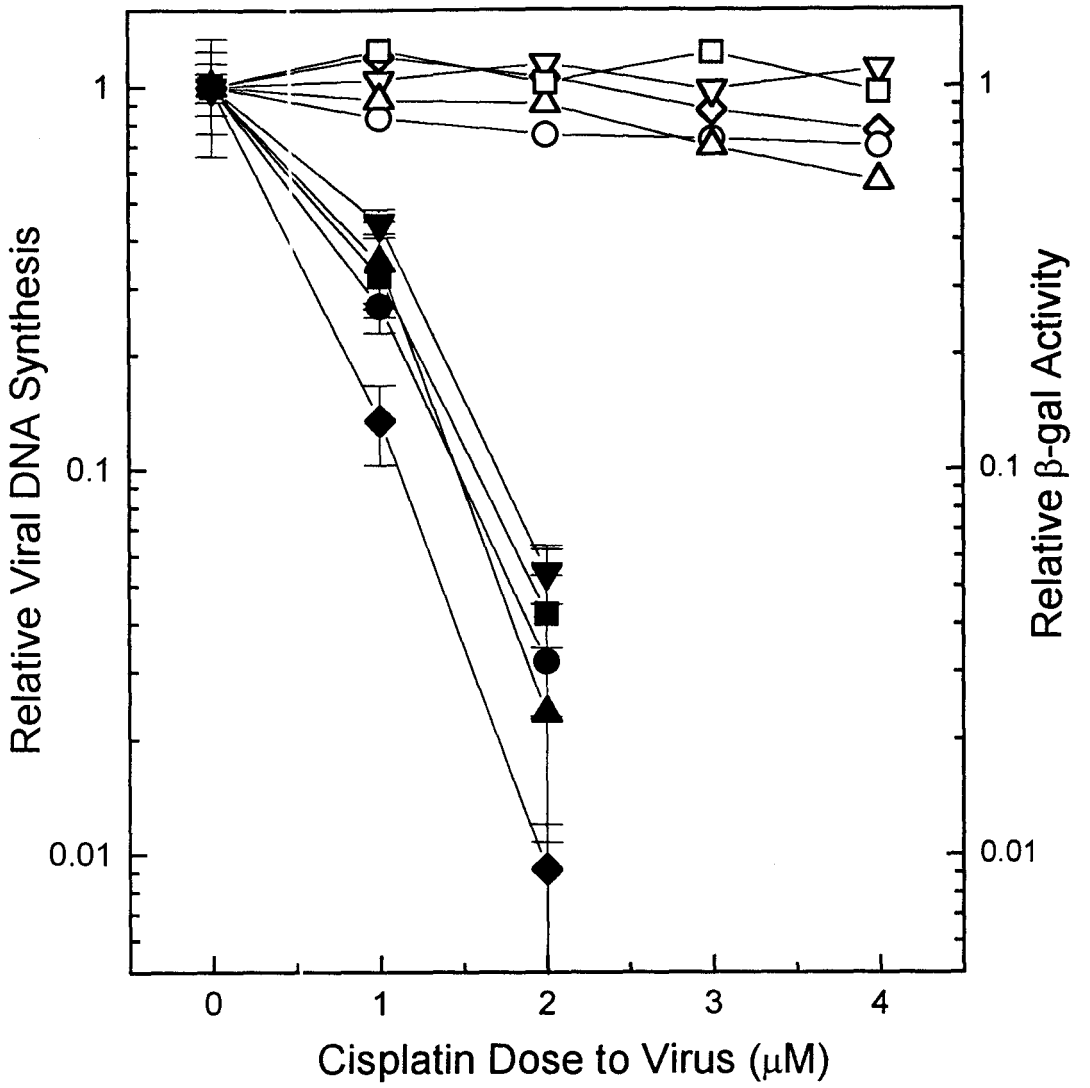

Figure 3. **Reactivation of cisplatin-damaged Ad in fibroblasts**. Viral repair quantitated by DNA synthesis is represented by the left axis (closed symbols). Samples were collected 72 hrs. after infection. Data points are the mean of duplicate determinations from a single experiment shown with standard error about the mean. Viral repair quantitated by β-gal activity is represented by the right axis (open symbols). Samples were collected 48 hrs. after infection. Data points are single determinations from a single experiment. ■GM969, ▲423, ◆GM37e (normal); ●R22, ▼S6012 (RS+).

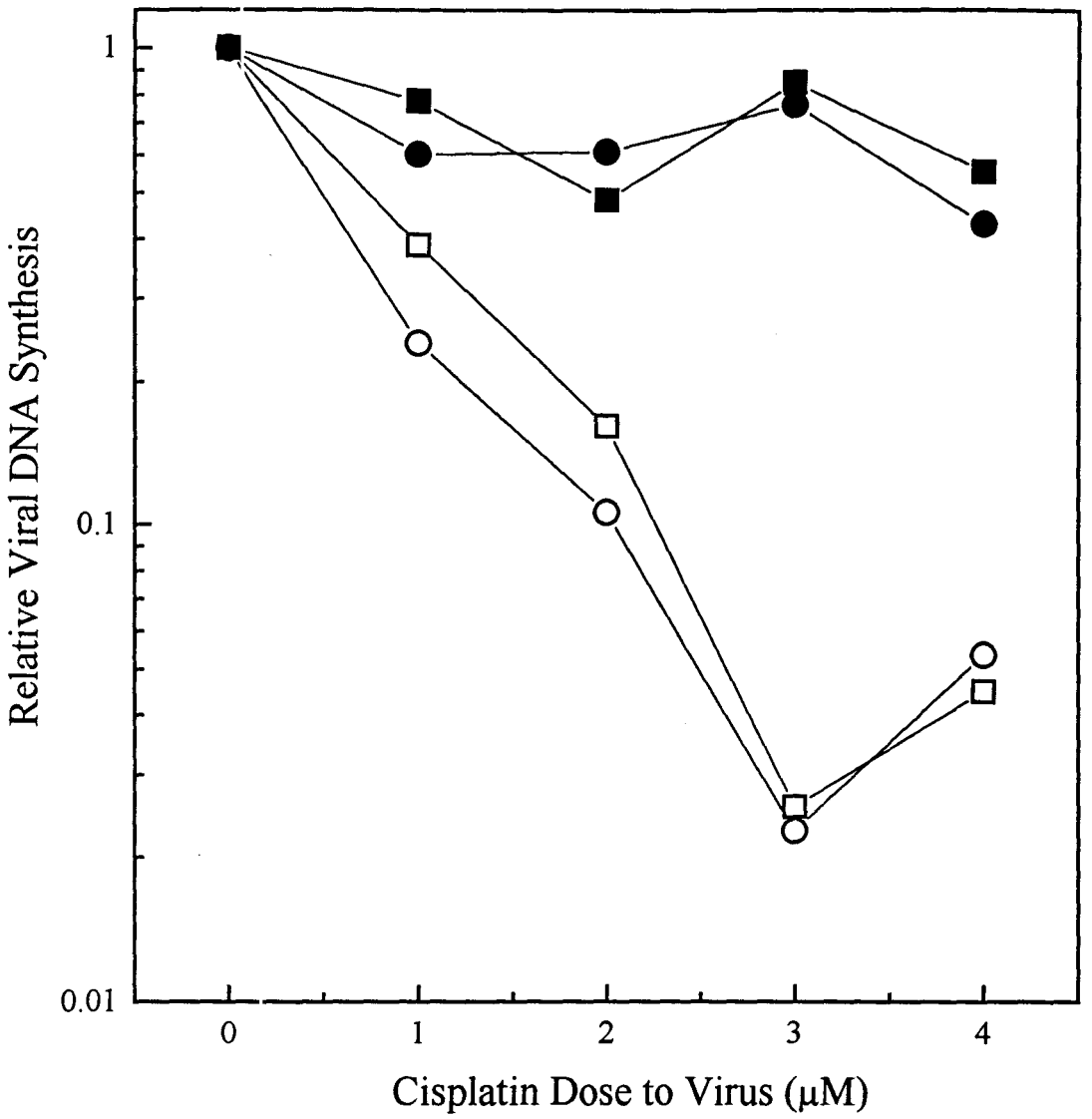

Figure 4. **Reactivation of cisplatin-damaged Ad in fibroblasts at different times post-infection**. Data points are single determinations from a single experiment. Open symbols represent collection of samples at 36 hrs. after infection, closed symbols represent collection of samples at 72 hrs. after infection. ■423 (normal); ●R22 (RS+).

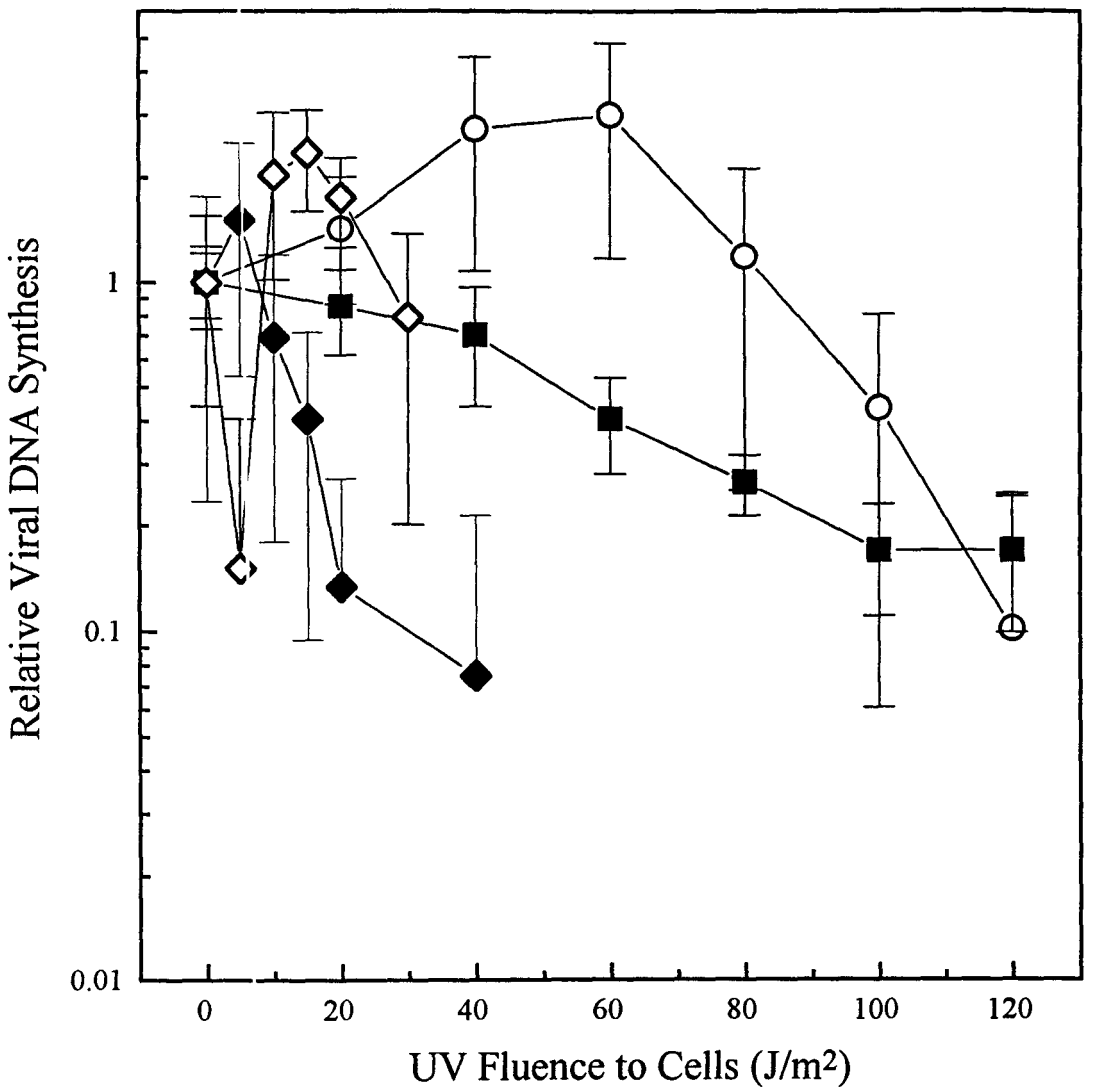

Figure 5. **Capacity of UV-irradiated fibroblasts to synthesize Ad DNA.** Samples were collected 48 hrs. after infection. Data points are the mean of quadruplicate determinations from a single experiment shown with standard error about the mean. ■GM969 (normal); ○S6012 (RS+); ◆XPCS1BA (XP-B), ◊XP2BI (XP-G).

DISCUSSION

By evaluating the preliminary data obtained, suggestions for improvement in the protocols for the HCR experiments were made and the parameters for future experiments were decided. Fibroblasts were chosen as the preferred cell type to be used, in part because of the more efficient and consistent infection and treatment of cells in monolayers rather than in suspension, and also because of the availability of many other fibroblast cell strains from different repair-deficient human syndromes that were currently available in our laboratory. In addition to the use of RS+ and normal cells, it was evident that cells of known repair deficiencies should be included in the HCR and cellular capacity experiments as controls. XP cells, deficient in nucleotide-excision repair (NER), and FA cells, deficient in DNA- crosslink repair, were selected for this purpose.

Although the lymphoblast work was not continued, the results obtained in the preliminary experiments suggest that the HCR assay for DNA-damaged Ad may prove useful for predicting drug sensitivity of cancer cells of blood origin. Parsons et al. (1986) have shown that Ad5 replication in HCR and capacity assays can be a predictor of sensitivity in fibroblast cells. Use of the β-gal method of analyzing repair of a damaged reporter gene in lymphoblasts suggests that this may be a possible way of testing drug sensitivity that would be faster and easier than other methods. The assay may also prove useful in screening the DNA-repair capacity for various DNA-damaging agents in the human population using lymphocyte samples.

Because of the suggestion made by Gentner et al. (1986) that RS+ fibroblasts may have a defect in the repair of interstrand crosslinks in DNA, and also because of the greater sensitivity of RS+ cells to cisplatin than to UV, it was decided that cisplatin would be the agent used to damage the viral and cellular DNA. Cisplatin causes both intrastrand and interstrand crosslinks in DNA as well as monofunctional lesions.

Comparison of the DNA synthesis and β-gal activity methods of quantitating survival in HCR assays showed that lower doses of the DNA-damaging agent were

necessary when using the endpoint of Ad DNA synthesis compared to the endpoint of β-gal expression of the lacZ reporter gene encoded in Ad5HCMVsp1lacZ. Replication of Ad DNA requires the expression of several viral-encoded genes such that replication of UV-irradiated Ad DNA is thought to require repair of DNA lesions in these viral-encoded genes as well as repair and/or bypass of lesions in the entire Ad genome. It is therefore thought that the HCR of Ad DNA synthesis of DNA-damaged Ad reflects the repair capacity of the infected cells for global DNA repair. In contrast, the expression of β-gal following infection of cells by Ad5HCMVsp1lacZ requires only removal of transcription-terminating lesions from the lacZ gene and is therefore thought to reflect the capacity of the infected cell for repair in an actively transcribed gene. It was thus thought that the HCR assay for survival of Ad DNA synthesis would be the more sensitive assay in detecting cellular repair deficiencies and was used in subsequent experiments to examine repair in RS+ and Li-Fraumeni cells.

In all the preliminary experiments conducted, the results obtained did not suggest that the RS+ cells are any less efficient at repairing DNA lesions than normal cells. This was true for both cell types (lymphoblasts and fibroblasts), for the two different DNA-damaging agents used (UV and cisplatin), for both methods of measuring HCR (DNA synthesis and β-gal activity), and also in both the HCR and cellular capacity assays. This somewhat unexpected result was the basis for furture work with RS+ cells, attempting to clarify the DNA-repair status of Roberts syndrome.

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CHAPTER THREE

ROBERTS SYNDROME FIBROBLASTS HAVE NORMAL HOST CELL REACTIVATION OF CISPLATIN-TREATED ADENOVIRUS AND NORMAL CAPACITY OF CISPLATIN-TREATED CELLS FOR ADENOVIRAL DNA SYNTHESIS

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ABSTRACT

Roberts syndrome (RS) is a rare, recessively inherited disorder characterized by growth retardation, limb reductions and craniofacial deformities. Cells from a subset of afflicted individuals, termed RS+, display unusual separation or puffing of the heterochromatic regions of their chromosomes and are hypersensitive to several DNA-damaging agents including mitomycin C (MMC) and cisplatin, both of which induce interstrand crosslinks in DNA. For this reason, we have investigated the ability of RS+ fibroblasts to repair cisplatin-induced DNA lesions using adenovirus as a probe. Host cell reactivation (HCR) of cisplatin-treated adenovirus (Ad) was significantly reduced in nucleotide excision repair (NER)-deficient xeroderma pigmetosum (XP) cells but was normal in the two RS+ fibroblast strains and the Fanconi anemia (FA) fibroblast strain tested. The capacity of cisplatin-treated cells for Ad DNA synthesis was reduced in XP and FA cells compared to normal human cells, but was not reduced in RS+ cells. These results indicate that the hypersensitivity of RS+ cells to cisplatin does not result from a deficiency in NER nor from a deficiency in the interstrand DNA crosslink repair pathway which is deficient in FA cells. This suggests that fibroblasts from RS+ patients are not deficient in repairing cisplatin damage from the transcriptionally active regions of the genome, and that abnormal DNA repair, *per se*, is not the cause of the RS+ mutagen hypersensitivity.

INTRODUCTION

Roberts syndrome (RS) is a rare, recessively inherited disease characterized by pre- and post-natal growth retardation, limb reductions (most often as tetraphocomelia), and craniofacial deformities such as cleft lip and palate (Freeman et al., 1974). Affected RS individuals are divided into two subgroups that are clinically indistinguishable but exhibit striking differences at the cellular level. RS+ patients display a distinct abnormality of the constitutive heterochromatin which appears as premature separation of the centromeric and nucleolar organizing regions of their chromosomes, as well as the Yqh region in males (German, 1979; Tomkins et al., 1979; Louie and German, 1981). Cells from RS+ individuals exhibit a hypersensitivity to a number of different DNA-damaging agents (Burns and Tomkins, 1989; Van Den Berg and Francke, 1993; Gentner et al., 1985 and 1986), whereas cells from the RS- subgroup of patients have normal chromosome morphology (Tomkins et al., 1979) and are not hypersensitive to DNA damaging agents (Burns and Tomkins, 1989). In addition to the heterochromatin separation, RS+ cells show a number of other abnormalities. RS+ fibroblasts take longer than normal human cells to complete mitosis, with a large proportion of cells failing to do so (Tomkins and Sisken, 1984). They also display a reduced rate of DNA synthesis, have reduced plating efficiencies (Tomkins and Sisken, 1984), contain nuclear blebs and lobules, and have an increased frequency of micronucleation which has been attributed to lagging chromosomes during cell division (Jabs et al., 1991).

RS+ fibroblasts have been shown to be sensitive to a wide spectrum of mutagenic agents including ultraviolet (UV) irradiation, gamma irradiation, methylnitrosurea, mitomycin C (MMC), 8-methoxypsoralen activated by UV-A light (8-MOP) and cisplatin (Gentner et al., 1985 and 1986; Van Den Berg and Francke, 1993; Burns and Tomkins, 1989). The greatest difference in sensitivity between RS+ and normal cells is observed with DNA-interstrand-crosslinking agents such as MMC and cisplatin. RS+ fibroblasts are 4-5 times more sensitive to MMC (Burns and Tomkins, 1989) and to cisplatin (Gentner et al., 1986; Tomkins, personal communication) than are

normal fibroblasts. Sensitivity to one or more DNA-damaging agents is a hallmark of a number of rare, recessively inherited diseases such as xeroderma pigmentosum (XP), Fanconi anemia (FA), ataxia telangiectasia (AT), Bloom syndrome (BS), and Cockayne syndrome (CS) some of which involve defective DNA-repair pathways (Arlett and Lehmann, 1978). It is thus possible that the increased sensitivity of RS+ cells to MMC and cisplatin could result from some deficiency in the repair of damaged DNA, in particular the repair of DNA-interstrand crosslinks.

Fanconi anemia (FA) is characterized by pancytopenia, growth retardation, various congential abnormalities (Fanconi, 1967) and also by a predisposition of affected individuals to cancer, especially leukemia (Swift, 1971; German, 1972). Cells from FA patients exhibit spontaneous chromosomal breakage (Schroeder and Kurth, 1971) and like RS+ cells, show an increased sensitivity to a number of DNA-crosslinking agents including cisplatin (Sasaki and Tonomura, 1973; Fujiwara et al., 1977; Ishida and Buchwald, 1982; Plooy et al., 1985). The repair of MMC-induced DNA lesions is significantly reduced in FA cells compared to that in normal human cells, with FA cells from different complementation groups exhibiting varying degrees of sensitivity (Weksberg et al., 1979; Matsumoto et al., 1989). The defect in FA group A (FA-A) cells has been localized to a deficiency in a protein complex that recognizes and incises interstrand crosslinks (Lambert et al., 1992; Hang et al., 1993). The interstrand crosslink recognition protein is defective or absent in FA-A cells which renders FA-A cells capable of incising on only one side of a DNA crosslink (Hang et al., 1993). Xeroderma pigmentosum (XP) is a DNA-repair-deficient syndrome in which the majority of affected individuals show a deficiency in the incision step of NER (Friedberg et al. 1995). XP patients are hypersensitive to sunlight, with a predisposition to skin cancer (Cleaver, 1983). Cells from XP individuals show increased sensitivity to UV irradiation (Zelle and Lohman, 1979; Mitchell et al., 1985) and several other DNA-damaging agents including cisplatin (Plooy et al., 1985).

Cisplatin is a chemotherapeutic drug used in the treatment of many types of cancer. It induces several types of lesions in DNA, the most significant being intrastrand adducts and interstrand crosslinks (Eastman, 1986). Intrastrand adducts (IA) involve a single strand of the DNA double helix and represent the majority of total lesions. IA either covalently link the N-7 atoms of adjoining purines (either two guanines or adenine/guanine), or may join two guanines separated by a single base (Fichtinger-Schepman et al., 1985; Eastman, 1983). These adducts are repaired by the NER pathway. The interstrand crosslink (ICL) involves both strands of the double helix and although it represents only 1-5% of the total cisplatin adducts, it has been implicated as a potentially lethal lesion (Plooy et al., 1985). Repair of the ICL is a complex process involving the NER pathway but also requires additional mechanisms that are not yet well understood.

In order to investigate the possibility of a DNA-repair defect in RS+ cells, we have used a host cell reactivation (HCR) assay to study the ability of RS+ fibroblasts to repair and replicate cisplatin-damaged adenovirus (Ad). We have also carried out cellular capacity experiments to examine the ability of the cisplatin-treated RS+ cells to replicate Ad DNA. Using these two viral assays, we were able detect repair deficiencies for cisplatin-damaged DNA in FA-A fibroblasts and NER-deficient XP fibroblasts, but not in the two RS+ fibroblast strains tested.

MATERIALS AND METHODS

Cell Strains

Roberts syndrome cells: The R22 and S6012 Roberts syndrome fibroblasts have both been shown to exhibit the RS+ phenotype by Giemsa staining of metaphase chromosomes. They were kindly provided by Dr. Darrell Tomkins, McMaster University, Hamilton, Ontario, Canada. The GM3913A fibroblast strain is the same as the R22 strain and was obtained from the NIGMS Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ). *Normal cells*: Normal strains GM969, GM8399 and GM3440B

were also obtained from the NIGMS Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ). The normal 423 strain was kindly provided by Dr. Patricia Chang, McMaster University, Hamilton, Ont. *Fanconi anemia cells*: The Fanconi anemia strain GM1309B, from complementation group A, was obtained from the NIGMS Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ). *Xeroderma pigmentosum cells*: The xeroderma pigmentosum strains XP12BE (GM5509B), XPCS2BA (GM13026), XP1BR (GM3615) and XP2BI (GM3021), from complementation groups A,B,D and G respectively, were also obtained from the NIGMS Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ).

All cells were grown in 75 cm^2 tissue culture flasks (Corning Incorporated, Corning, NY) in α-minimal essential medium (α-MEM) supplemented with 10% (v/v) fetal bovine serum (Sigma Chemical Co., St. Louis, MO) and 1% (v/v) antibiotic/antimycotic containing 10 000 U/mL penicillin G sodium, 10 000 ug/mL streptomycin sulfate and 25 ug/mL amphotericin B as Fungizone® in 0.85% saline (Gibco BRL, Life Technologies Inc., Grand Island, NY). Cells were incubated in a 5% CO_2, 100% humidified, water-jacketed incubator (Forma Scientific Inc., Marietta, OH) at 37°C. At confluency, cells were passaged by aspirating growth medium, rinsing in 1x phoshpate buffered saline (PBS) followed by the addition of 2x trypsin-EDTA containing 0.5% trypsin and 5.3 mM EDTA•4Na (Gibco BRL, Life Technologies Inc., Grand Island, NY). Once the cells rounded up, they were resuspended in complete growth medium and subcultured at appropriate dilutions.

Cisplatin

Cisplatin (*cis*-diamminedichloroplatinum(II)) was obtained from Sigma Chemical Company (St. Louis, MO). Stock solutions of 1 mM were made in 1x PBS (1.4 M chloride ion) for the treatment of cells or in 1x low chloride PBS (6.6 mM chloride ion) for the treatment of virus. Stock solutions were made fresh and filtered through a 0.45 μm disposable filter (Nalge Company, Rochester, NY) just prior to use.

Virus

Human adenovirus serotype 5 (Ad5) and the recombinant Ad5(lacZ) were the viruses used for infection of cells. The Ad5(lacZ) virus contains the *Escherichia coli* β-galactosidase (lacZ) gene inserted into the deleted, non-essential E3 region of the Ad viral genome and was generously provided by Dr. Frank Graham, Departments of Biology and Pathology, McMaster University, Hamilton, Ont. Viral stocks were grown on monolayers of human 293 cells and were titred by plaque assay as described previously (Hitt et al., 1995). Stock virus was suspended in PBS to a titre ranging from 10^8-10^{10} plaque forming units (pfu)/mL.

Host Cell Reactivation

Cells were seeded at a density of $1.9x10^4$ cells/well in 96-well Microtest III tissue culture plates (Falcon, Becton Dickinson & Company, Lincoln Park, NJ) and were incubated for 24 hrs. prior to infection. Ad5 or Ad5(lacZ) was treated with varying doses of cisplatin in serum-free medium containing 1% antibiotic/antimycotic for 24 hrs. at 37°C to damage the viral DNA. Because cisplatin molecules contain two chloride atoms as leaving groups, the chloride ion concentration of the treatment solution can affect the amount of damage induced in the viral DNA during the treatment. For this reason, the chloride ion concentration during treatment of the virus was set to 50 mM for all HCR experiments in order to maintain consistency from experiment to experiment. The medium was aspirated from the wells and cells were infected with 40 μL of viral suspension at a multiplicity of infection (MOI) of 20 pfu/cell. After a 90 min. incubation at 37°C, the infection was terminated by aspirating the virus and adding 200 μL of fresh growth medium to the wells. Plates were then incubated for 72 hrs. at 37°C to allow for repair and replication of viral DNA.

Cellular Capacity of Ad DNA Synthesis

Cells were seeded at a density of $1.9x10^4$ cells/well in 96-well plates. After a 24 hr. incubation, the growth medium was aspirated and cells were treated with varying concentrations of cisplatin in growth medium for 3 hrs. at 37°C. In this assay, chloride ion concentration was not set to a specified value as the environment inside the cells provides an appropriate chloride ion concentration for cisplatin activity. Following treatment, the cisplatin was aspirated and cells were rinsed with 1x PBS to remove any residual drug. For the immediate capacity assay, cells were then immediately (within 30 minutes) infected with 40 µL of either Ad5 or Ad5(lacZ) viral suspension at an MOI of 100 pfu/cell, and subsequently incubated for 90 mins. at 37°C. Infection was terminated by aspirating the virus and adding 200 µL of fresh growth medium to the wells. Plates were then incubated for 48 hrs. at 37°C to allow for repair of cellular DNA and subsequent replication of virus. The delayed cellular capacity experiments were carried out as described above for the immediate capacity assay, except that there was a 24 hr. delay period between the treatment of the cells and infection with virus

Quantification of Viral DNA Synthesized

Following the appropriate incubation time, the growth medium was aspirated from the wells and 50 µL of proteinase K solution (1 mg/mL proteinase K, 1% SDS, 0.2x SSC) was added to the wells for 1.5 hrs. at 37°C in order to lyse the cells. To denature the viral DNA for blotting, 50 µL of NaOH solution (1 M NaOH, 1 mM EDTA) was added to the wells and plates were incubated another 1.5-2 hrs. before slot-blotting to nylon membrane (GeneScreen Plus®, DuPont, NEN Research Products, Boston, MA) using the Minifold II Slot-Blot system (Schleicher and Schuell Inc., Keene, NH). The membrane was then air dried before probing with ^{32}P-labelled Ad2 DNA. To quantitate the amount of radioactivity, blots were exposed to a phosphor screen and were analyzed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Host Cell Reactivation of Cisplatin-treated Adenovirus

The survival of viral DNA synthesis for cisplatin-treated Ad was examined following the infection of normal and RS+ fibroblasts as well as FA and NER-deficient XP fibroblasts. The amount of viral DNA synthesized by each cell strain following infection with cisplatin-treated virus was expressed as a fraction of that synthesized in the same cell strain following infection with untreated virus. Results of a representative experiment for a normal fibroblast strain, two RS+ strains, an FA-A strain and two NER-deficient XP strains are presented in Fig. 1. It can been seen that both the RS+ and the FA-A strains show a similar host cell reactivation of cisplatin-damaged virus to that found for the normal strain, whereas the XP strains show a greatly reduced HCR with no overlap of error bars between normal and XP cells. These results indicate normal levels of repair for cisplatin-damaged Ad DNA in the RS+ and FA-A cells, whereas DNA repair appears significantly reduced in the XP cells.

Survival curves for viral DNA synthesis of cisplatin-treated Ad were fitted to a linear quadratic equation (ln relative amount viral DNA= $-(\alpha x+\beta x^2+\gamma)$) and the parameters α, β, γ were obtained by least squares analysis in order to calculate a D_{37} value for each survival curve. The D_{37} values obtained in normal fibroblasts ranged from 0.88-1.12 μM, but were substantially reduced in the XP strains (0.10-0.24 μM). A range of D_{37} values similar to that in normal fibroblasts was obtained in RS+ cells (0.72-1.88 μM) and the FA-A strain (0.92-1.06 μM). For each experiment, the D_{37} value obtained for each cell strain was expressed relative to the average D_{37} value obtained for the normal strains used in that experiment. The mean relative D_{37} values so obtained for a number of experiments are shown in Table 1. It can be seen that the mean relative D_{37} was greatly reduced compared to normal in the XP strains from complementation groups A,B,D and G, but not in the two RS+ strains or the FA-A strain.

Capacity of Cisplatin-treated Cells for Ad DNA Synthesis

The cellular capacity of various cisplatin-treated fibroblasts to support DNA replication of Ad is shown in Figs. 2 and 3. Fig. 2 shows representative results for infection of cells immediately (within 30 minutes) following cisplatin treatment. It can be seen that the capacity of cisplatin-treated cells for Ad DNA synthesis was similar to that in normal fibroblasts for the RS+ strains, but was greatly reduced compared to normal for the FA-A and XP strains.

Previous studies have shown that the cellular capacity assay can be used to examine the recovery and/or repair of mammalian cells following exposure to DNA-damaging agents (Lytle et al., 1976; Coppey et al., 1979; Rainbow, 1989). The restoration in the capacity of UV-irradiated normal human fibroblasts was greater compared to that in UV-irradiated NER-deficient XP cells when viral infection was delayed following UV exposure to cells (Lytle et al., 1976; Coppey et al., 1979). Since the capacity of UV-irradiated cells for viral infection is more sensitive to differences in the DNA-repair capacity of XP cells when viral infection is delayed, it was considered of interest to examine the capacity of cisplatin-treated RS+ fibroblasts using a delay between cisplatin treatment of cells and viral infection. Fig. 3 shows representative results for infection of cells 24 hrs. following cisplatin treatment. It can be seen that for the 24 hour delayed infection, the capacity of cisplatin-treated cells for Ad DNA synthesis was similar to that in normal fibroblasts for the RS+ strains, but was greatly reduced in the FA-A and XP strains tested with no overlap of error bars between normal, FA-A and XP cell. Cellular capacity curves for viral DNA synthesis following cisplatin treatment were fitted to a linear quadratic equation (ln relative amount of viral DNA= $-(\alpha x+\beta x^2+\gamma)$), and the parameters α, β, γ were obtained by least squares analysis in order to calculate a D_{37} value for each capacity curve. For each experiment, the D_{37} value obtained for each cell

strain was expressed relative to the average D_{37} value obtained for the normal strains used in that experiment. The mean relative D_{37} values obtained for a number of 24 hr.-delayed capacity experiments are shown in Table 2. It can be seen that the mean relative D_{37} was greatly reduced compared to normal in the FA-A and XP strains, but not in the two RS+ strains.

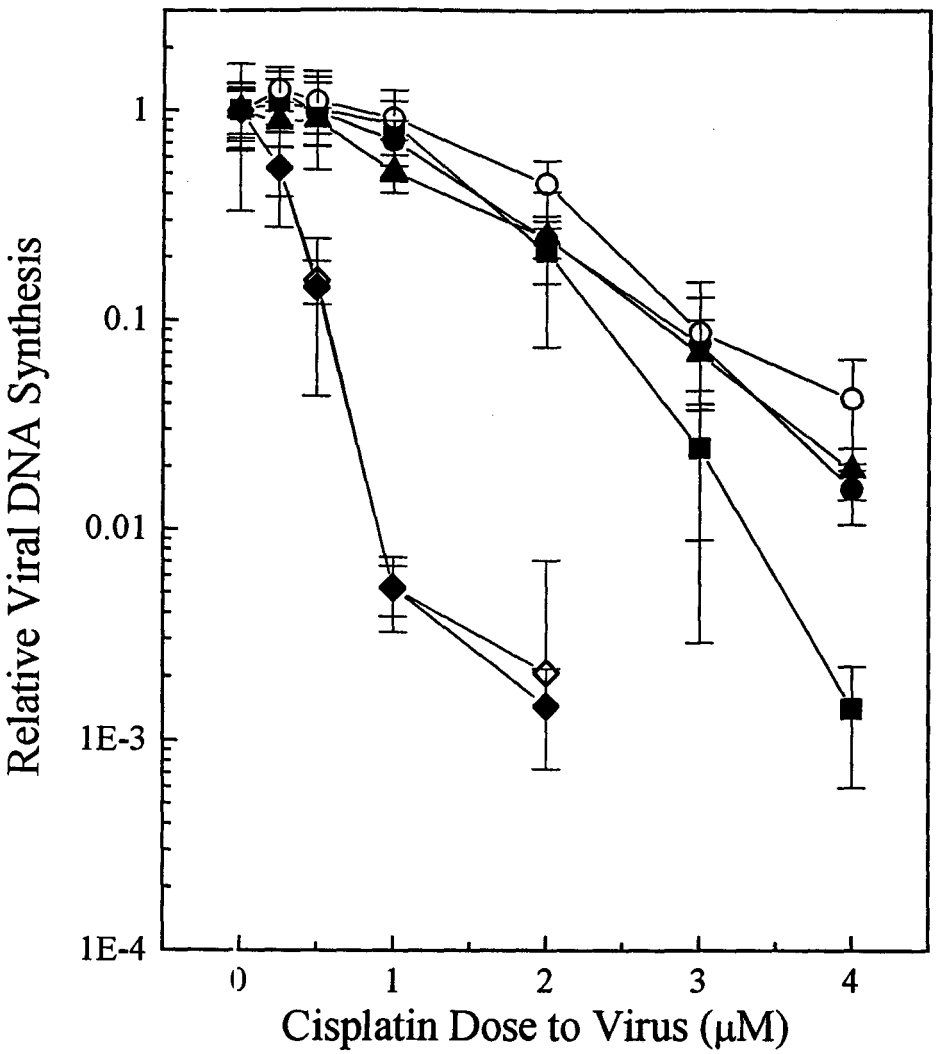

Figure 1. **Host cell reactivation of cisplatin-damaged Ad**. Ad was treated with cisplatin for 24 hrs. at 37°C and used to infect various fibroblast strains. Graph shows typical results for the survival of DNA synthesis for cisplatin-treated Ad. Data points are the mean of duplicate samples shown with standard error. ■GM969 (normal); ●R22, OS6012 (RS+); ▲GM1309B (FA-A); ◆XP1BR (XP-D), ◊XP2BI (XP-G).

Table 1. HCR of cisplatin-treated Ad in various human fibroblast strains.

Cell Strain	Syndrome	Mean Relative D_{37}[a]	Range	Number of Experiments
GM969	Normal	1.01 ± 0.01[b]	1.00-1.07	5
423	Normal	1.04 ± 0.07	0.97-1.12	2
GM3440B	Normal	0.91 ± 0.02	0.88-0.93	2
R22	RS+	1.14 ± 0.17	0.72-1.52	4
S6012	RS+	1.64 ± 0.21	1.22-1.88	3
GM1309B	FA-A	0.99 ± 0.03	0.92-1.06	5
XP12BE	XP-A	0.14 ± 0.01	0.14-0.15	2
XPCS2BA	XP-B	0.18 ± 0.06	0.11-0.24	2
XP1BR	XP-D	0.20 ± 0.01	0.19-0.20	2
XP2BI	XP-G	0.13 ± 0.02	0.10-0.15	2

[a] Relative to the mean D_{37} value for the normal strains used in each experiment
[b] standard error about the mean

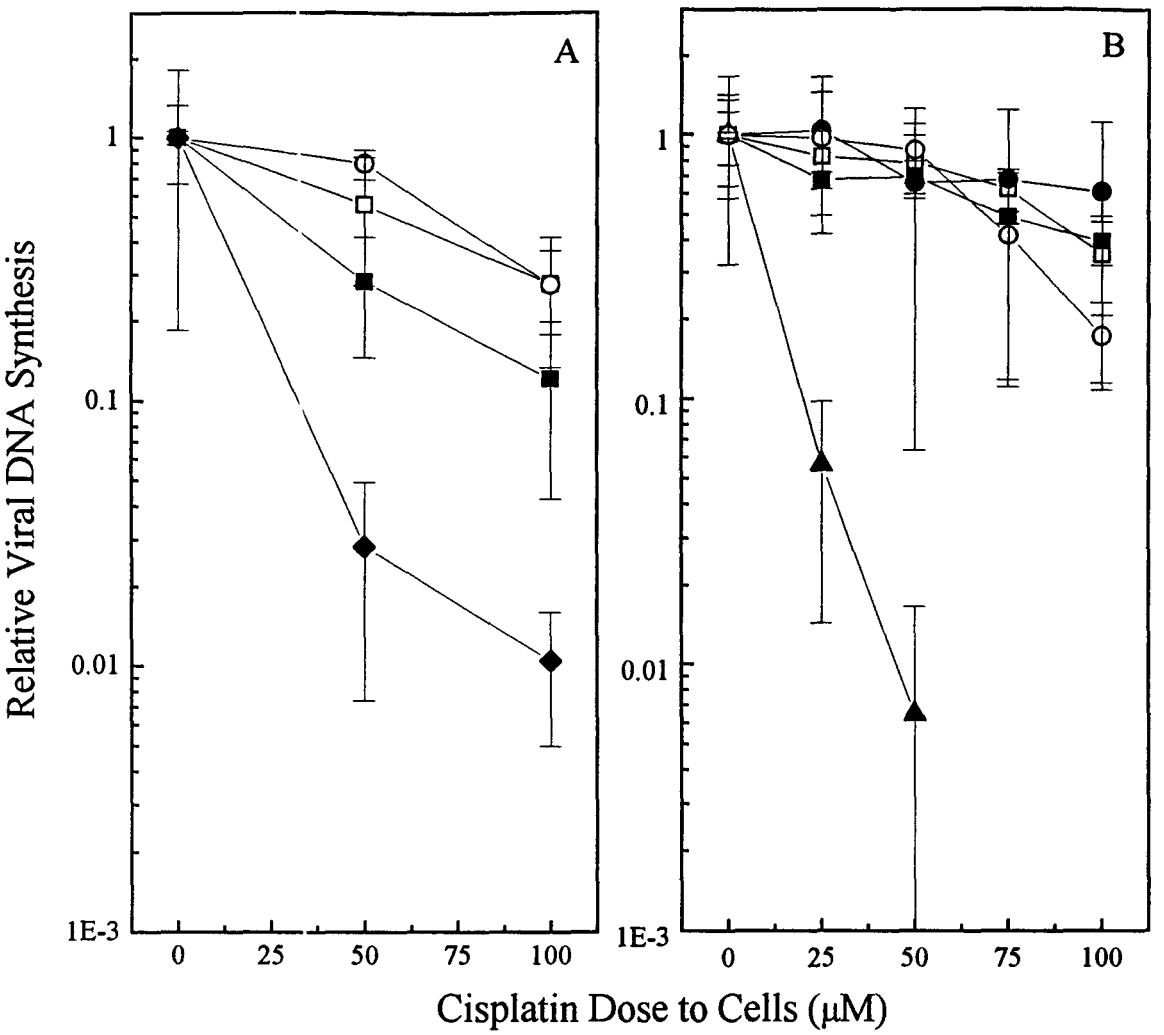

Figure 2. **Capacity of cisplatin-treated fibroblasts to replicate Ad DNA.** Cells were treated with cisplatin for 3 hrs. at 37°C and immediately infected with Ad. Graphs show results of typical experiments. Data points are the mean of duplicate samples shown with standard error. ■GM969, □423 (normal); ●R22, OS6012 (RS+); ▲GM1309B (FA-A); ◆XP1BR (XP-D).

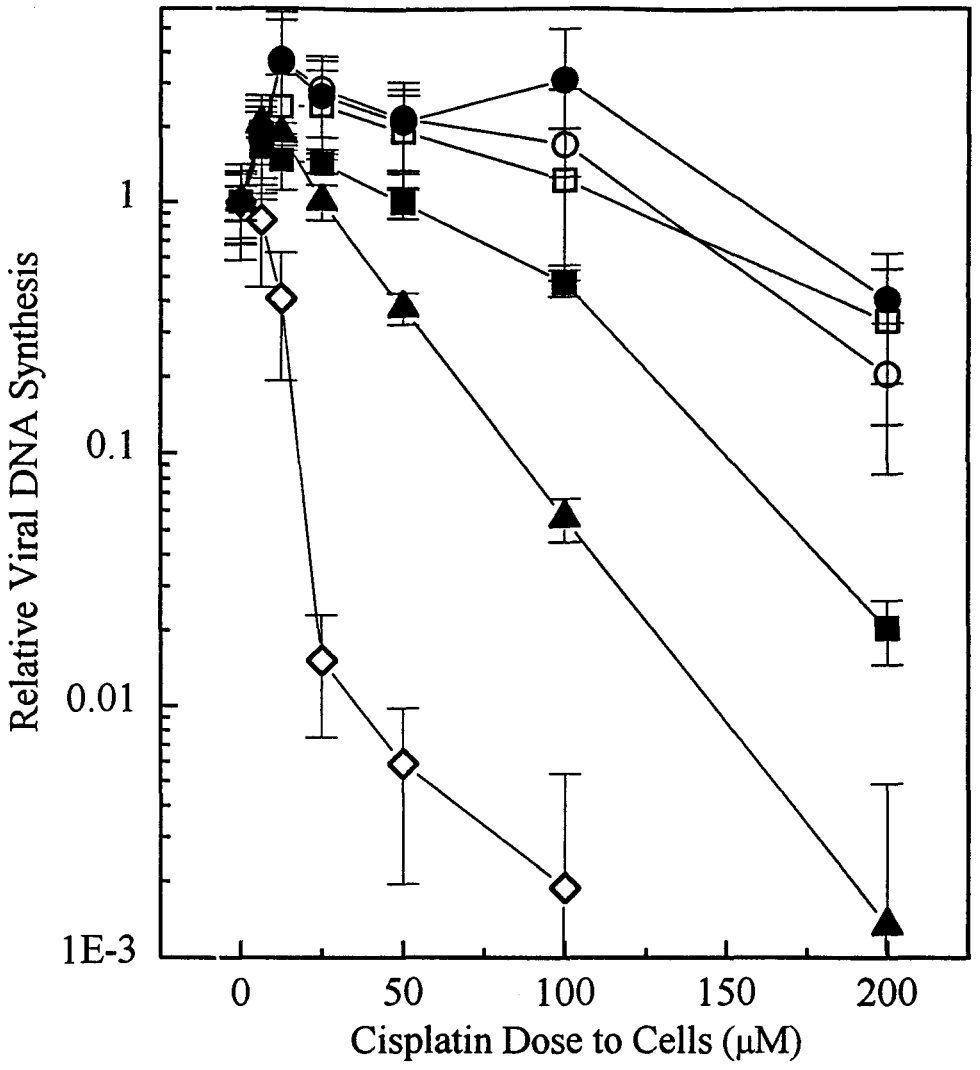

Figure 3. **Delayed capacity of cisplatin-treated fibroblasts to replicate Ad DNA**. Cells were treated with cisplatin for 3 hrs. at 37°C and 24 hrs. later were infected with Ad. Graph shows results of a typical experiment. Data points are the mean of duplicate samples shown with standard error. ■GM969, □423 (normal); ●R22, ○S6012 (RS+); ▲GM1309B (FA-A); ◇XP2BI (XP-G).

Table 2. Delayed capacity of cisplatin-treated human fibroblasts to support Ad DNA synthesis.

Cell Strain	Syndrome	Mean Relative D_{37} [a]	Range	Number of Experiments
GM969	Normal	0.79 ± 0.06[b]	0.65-1.00	5
423	Normal	1.26 ± 0.04	1.14-1.34	4
R22	RS+	1.23 ± 0.19	0.74-1.67	5
S6012	RS+	1.25 ± 0.23	0.91-1.68	3
GM1309B	FA-A	0.30 ± 0.16	0.14-0.47	2
XP12BE	XP-A	0.44		1
XP1BR	XP-D	0.13 ± 0.05	0.09-0.18	2
XP2BI	XP-G	0.18 ± 0.08	0.10-0.27	2

[a] Relative to the mean D_{37} value for the normal strains used in each experiment
[b] standard error about the mean

DISCUSSION

Previous reports indicate that RS+ lymphoblastoid cell lines and fibroblast strains show an increased sensitivity to a number of DNA-damaging agents (Allingham-Hawkins and Tomkins, 1991; Burns and Tomkins, 1989; Gentner et al., 1985 and 1986). An increased sensitivity of RS+ compared to normal cells has been reported for mitomycin C (MMC), cisplatin and 8-methoxypsoralen activated by UV light to be 4.5, 4.0 and 2.0 fold, respectively (Gentner et al., 1986) and it has been suggested that the marked sensitivity of RS+ fibroblasts to these agents results from a deficiency in the repair of DNA-interstrand crosslinks. In the present work we have demonstrated that RS+ fibroblasts are not deficient in HCR of cisplatin-damaged Ad, nor in their capacity to replicate Ad DNA following cellular cisplatin treatment. However, HCR of cisplatin-treated Ad was reduced compared to normal in NER-deficient XP cells and the capacity of cisplatin-treated cells for Ad DNA synthesis was reduced compared to normal in both FA-A cells and NER-deficient XP cells. These results indicate that the hypersensitivity of RS+ cells to cisplatin does not result from a deficiency in NER nor from a deficiency in the interstrand-crosslink-repair pathway which is deficient in FA-A cells.

The sensitivity of FA cells to DNA-crosslinking agents has been well established (Ishida and Buchwald, 1982; Hansson et al., 1991; Fujiwara, 1982). As well, FA cells are reduced in their ability to remove cisplatin-induced DNA adducts from the transcriptionally active DHFR gene (Zhen et al., 1993). In particular, the ICL is believed to be the main cytotoxic lesion contributing to FA sensitivity (Kano and Fujiwara, 1981; Gruenert and Cleaver, 1985; Matsumoto et al., 1989). FA cells have been shown to continually accumulate cisplatin-induced DNA ICL during post-treatment incubation, while both normal and XP cells show a decrease in ICL over the same period (Plooy et al., 1985). However, the deficiency of FA-A cells for the repair of ICL did not result in a reduced HCR for cisplatin-treated Ad reported in the present work. Previous experiments have obtained similar findings of normal HCR in FA cells for virus and

plasmid treated with cisplatin and other DNA-interstrand-crosslinking agents. Day et al. (1975) found no difference between FA and normal human fibroblasts in their HCR of psoralen-treated Ad even though ICL were shown to be produced in the Ad DNA, and HCR of cisplatin-treated SV40 DNA was also shown to be normal in FA-A cells (Poll et al., 1984). In a similar type of experiment in which cell extracts were tested for their ability to repair cisplatin-treated plasmid DNA, FA-A cells again showed no reduction in repair compared to normal human cells (Hansson et al., 1991). In other work, a testicular teratoma cell line previously identified as being intrastrand and interstrand crosslink-repair-deficient (Bedford et al., 1988), similar to FA cells, showed normal levels of HCR for cisplatin-treated Ad (Maynard et al., 1989). These results indicate that viral and plasmid assays are unable to detect differences in the repair of DNA-interstrand crosslinks. Several explanations have been suggested for this apparent discrepancy, one being that repair of viral lesions may be somewhat more efficient than the repair of cellular DNA lesions due to the differences in structural complexity and chromosomal organization of the two genomes. Alternatively, it is possible that recombination with or complementation by other viral genomes in the infected cell might account for the apparent repair proficiency of the FA cells. However, it is also possible that interstrand-DNA crosslinks in the Ad genome are not repaired following the infection of normal human cells as suggested by the results of Day et al. (1975)

XP cells are also sensitive to DNA-damaging agents which have been shown to induce DNA crosslinks. Gruenert and Cleaver (1985) concluded that XP cells from complementation group A are deficient in repairing DNA crosslinks caused by 8-MOP. Other reports indicate that XP cells from complementation groups A and F, like FA cells, have a reduced colony forming ability compared to normal human cells following cisplatin treatment (Plooy et al., 1985) and XP group A cells are reduced in their ability to remove cisplatin adducts from the transcriptionally active DHFR gene (Zhen et al., 1993). In contrast, XP cells showed no sensitivity to MMC, while FA cells were similarly sensitive to both MMC and cisplatin (Plooy et al., 1985). Although not reliable for the

detection of DNA-interstrand-crosslink repair, the HCR assay is capable of revealing defects in nucleotide-excision repair (NER) for cisplatin-damaged DNA as evidenced by the reduced reactivation of cisplatin-treated virus and plasmid in XP cells and NER-deficient Chinese hamster ovary (CHO) cells reported in the present work and elsewhere (Poll et al., 1984; Maynard et al., 1989; Sheibani et al., 1989). The normal HCR levels for cisplatin-treated Ad in RS+ cells reported here indicate that the hypersensitivity of RS+ cells to cisplatin does not result from a deficiency in NER.

Physical or chemical treatment of mammalian cells can decrease their capacity to support viral infection (Rainbow, 1981; Defais et al., 1983). The capacity of UV-irradiated cells to support viral infection has been shown to be a cellular, DNA-dependent function (Coohill et al., 1977) which is reduced in several DNA-repair-deficient cell types including several Cockayne syndrome (CS) and XP fibroblasts (Coppey et al., 1979; Coohill, 1981; Ryan and Rainbow, 1986; Rainbow, 1989). The capacity of UV-irradiated cells to support viral infection, as well as the recovery of such capacity, has been employed to study the DNA-repair deficiency in several cell types (Lytle et al., 1976; Rainbow, 1989; Coppey et al., 1979). The capacity of cisplatin-treated cells for viral infection has been shown to reflect clonogenic survival of cells following cisplatin treatment (Clarke and Rainbow, 1994) and Parsons et al. (1986) report that the cellular capacity for Ad infection can be used as an in vitro probe for the drug sensitivity of human tumor cell lines. In the present work we have examined the capacity of cisplatin-treated fibroblasts to support Ad DNA replication. Previous work using the cellular capacity for herpes simplex virus plaque formation has shown that normal cells exhibit a recovery of capacity while repair-deficient XP cells show a further decrease in capacity when there is a delay between UV treatment of cells and viral infection (Lytle et al., 1976). Our results suggest a similar recovery in the capacity of cisplatin-treated RS+ and normal human fibroblasts and a decrease in the capacity of cisplatin-treated XP cells, when infection was delayed.

The replication of Ad DNA requires at least three viral gene products and four host factors. The host factors required for Ad DNA synthesis are nuclear factors 1, 2 and 3 and ORP A (for a review see Horwitz, 1990). DNA damage in these host cell genes and possibly other cellular genes, unless repaired, would be expected to inhibit the expression of the cellular functions necessary for Ad DNA synthesis and thus reduce the capacity of the cisplatin-treated cell to support Ad DNA synthesis. The reduced capacity of cisplatin-treated FA and XP cells for Ad DNA synthesis reported here is thus thought to result from the reduced repair of cisplatin-induced DNA lesions in the transcriptionally active genes of FA and XP cells (Zhen et al., 1993). In the present work we show a normal capacity of cisplatin-treated RS+ cells for Ad DNA synthesis for both immediate and 24 hr-delayed infection. This suggests that the cisplatin hypersensitivity reported for RS+ cells does not result from reduced DNA repair in transcriptionally active cellular genes.

The abnormalities of RS+ fibroblasts include reduced plating efficiency and growth capacity, prolonged mitosis, longer doubling times and abnormal cytokinesis (Tomkins and Sisken, 1984). Other findings indicate that a proportion of RS+ cells is continually being lost in culture due to the failure of some cells to complete mitosis and also to the fact that approximately 11% of daughter cells contain micronuclei as a result of lagging chromosomes and do not further divide (Jabs et al., 1991). These characteristics of RS+ cells complicate and may contribute to the cisplatin hypersensitivity reported for RS+ cells.

Results reported here suggest that RS+ fibroblasts are not deficient in repairing cisplatin-induced DNA lesions that reside within the transcriptionally active euchromatic regions of the chromosomes. This suggests that the cisplatin hypersensitivity of RS+ cells does not result from a deficiency in a DNA-repair pathway of the cell *per se*. The characteristic cellular abnormality that distinguishes RS+ from RS- and normal cells, and that correlates with the hypersensitivity of RS+ cells to several DNA-damaging agents, is the decondensation of the heterochromatic regions of their chromosomes. Due to the

uncondensed, more open structure of these heterochromatic regions, it is possible that these regions of the chromosomes are more susceptible to damage following cisplatin treatment and/or altered accessibility to mechanisms which repair cisplatin-induced damage compared to normal cells. Unrepaired DNA lesions in heterochromatin would be expected to inhibit DNA replication and reduce colony forming ability. The hypersensitivity of RS+ cells to cisplatin and other DNA-damaging agents could thus result directly from the abnormality in the heterochromatic regions of the RS+ cell.

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CHAPTER FOUR

HOST CELL REACTIVATION OF CISPLATIN-TREATED ADENOVIRUS IS REDUCED IN NUCLEOTIDE-EXCISION-REPAIR-DEFICIENT MAMMALIAN CELLS AND SEVERAL HUMAN TUMOUR CELLS

HOST CELL REACTIVATION OF CISPLATIN-TREATED ADENOVIRUS IS REDUCED IN NUCLEOTIDE-EXCISION-REPAIR-DEFICIENT MAMMALIAN CELLS AND SEVERAL HUMAN TUMOUR CELLS

ABSTRACT

Cisplatin is a widely used chemotherapeutic agent in the treatment of a variety of human cancers. Cisplatin exerts its toxic effect by covalently binding to DNA, resulting in monofunctional adducts, intrastrand crosslinks, and interstrand crosslinks. Several recent reports suggest that the cellular capacity for DNA repair, especially nucleotide excision repair (NER), is an important determinant in the sensitivity of cells to cisplatin. We have used a sensitive host cell reactivation (HCR) technique to examine the repair capacity for cisplatin-damaged DNA in several different mammalian cell types. HCR of cisplatin-damaged adenovirus (Ad) was reduced in all UV-sensitive, NER-deficient Chinese Hamster Ovary (CHO) cells examined (complementation groups 1 to 6) compared to NER-proficient CHO cells. HCR of cisplatin-damaged Ad was also reduced in fibroblasts from patients with xeroderma pigmentosum (XP) complementation groups A, B, C, D, F, and G compared to that in normal human fibroblasts. Differences in repair capacities were also detected among human cancer cell lines, indicating a repair deficiency for cisplatin-damaged DNA in some human tumours.

INTRODUCTION

Cisplatin is a chemotherapeutic agent which is prevalent in the treatment of many kinds of cancers (Loehrer and Einhorn, 1984) and is particularly effective in treating tumours of the gonads (Rosenberg, 1985). The cytotoxic effect of cisplatin is widely believed to be as a result of DNA damage (Munchausen, 1974; Harder and Rosenberg, 1970). Cisplatin reacts with DNA through a two-step electrophilic addition to purine bases (Sherman and Lippard, 1987) which results in a spectrum of adducts which cause bending and local unwinding of the DNA (Rice et al., 1988; Malinge et al., 1994) which is sufficient to stall both transcription and replication (Heiger-Bernays et al., 1990; Comess et al., 1992). While cisplatin is an extremely effective drug, the limit to its efficacy is the development of tumours which have acquired resistance (Perez et al.,

1993). The mechanisms by which tumour cells gain resistance are largely unknown although some studies have shown correlation between cisplatin resistance and increased expression of some DNA repair genes (Geleziunas et al., 1991; Dabholkar et al., 1992). Cisplatin-resistant cells have also been shown to have increased capacity to repair cisplatin-damaged DNA (Alaoui-Jamali et al., 1994; Chao, 1994; Calsou, 1993; Parker et al., 1991, Jennerwein et al., 1991) and an increased accumulation of the anti-oncogene, p53 (Brown et al., 1993; Righetti et al., 1996), which has been shown to be involved in DNA repair (Smith et al., 1995; Wang et al., 1995).

Nucleotide excision repair (NER) has been shown to be an important pathway in the repair of cisplatin-damaged DNA (Hansson et al., 1990; Sheibani et al., 1989). NER has been studied extensively through the use of repair-deficient mammalian cells. Particularly useful have been a number of of Chinese hamster ovary (CHO) cell lines isolated based on their sensitivity to UV light (Busch et al., 1989). These CHO mutants have been valuable in the isolation of human DNA repair genes by complementation analysis (as reviewed by Hoeijmakers, 1993). Defects in some of these genes have been discovered to cause the UV-sensitive, human syndromes xeroderma pigmentosum (XP), Cockayne syndrome (CS) or trichothiodystrophy (TTD). Many of these repair-deficient mammalian cells show an increased sensitivity to cisplatin (Plooy et al., 1985).

DNA repair capacity has been effectively assessed in the past using host cell reactivation (HCR) of DNA-damaged virus which can be quantitated in several ways such as: viral antigen production (Rainbow, 1980; Maynard et al., 1989; Eady et al., 1992), plaque forming ability (Aaronson and Lytle, 1970; Day, 1974), reactivation of a reporter gene which has been inserted into a recombinant virus (Francis and Rainbow, 1995; Valerie and Singhal, 1995), and amount of viral DNA synthesis (Arnold and Rainbow, 1996).

We report here a convenient HCR assay which measures the synthesis of viral DNA in cells that have been infected with cisplatin-damaged adenovirus type 5 (Ad5). We show that both human and CHO cells with known defects in nucleotide excision repair (NER) are reduced in their capacity to reactivate cisplatin-treated Ad. DNA repair has been shown to be important in the acquired resistance of some tumour cells to cisplatin (Zeng-Rong et al., 1995; Zhen et al., 1992; Chao, 1994; Yen et al., 1995; Lai

et al., 1995). In this work we show that HCR of cisplatin-treated Ad is capable of revealing differences in repair among several human tumour cell lines.

MATERIALS AND METHODS

Cell Strains

Parental CHO-AA8 cells and UV-sensitive UV20, UV5, UV24, UV41, UV135, and UV61 mutant CHO cell lines (from complementation groups 1 to 6 respectively) were provided by Dr. Larry Thompson, Lawrence Livermore National Laboratory, Livermore, CA, with the help of Dr. Gordon Whitmore, Physics Division, Ontario Cancer Institute, Toronto, Ontario. The UV-sensitive mutant CHO cell line UV86, from complementation group 6, was obtained from Dr. David Busch, Department of Environmental and Toxicological Pathology, Armed Forces Institute of Pathology, Washington, D.C., USA.

The normal human fibroblast strain GM969 and xeroderma pigmentosum cell strains XP12BE (GM5509B), XPCS2BA (GM13026), XP1MI (GM2096), XP2BE (GM677), XP1BR (GM3615), XP2YO (GM4313), XP2BI (GM3021A) (from complementation groups A, B, C, C, D, F, and G respectively) were obtained from the NIGMS Human Genetic Cell Repository, Coriell Institute for Medical Research, Camden, N.J., USA.

The human cervical carcinoma, HeLa cell line was obtained from the American Type Culture Collection, Rockville, MD, USA. Medical Research, Camden, N.J., USA. The human colon carcinoma cell line HT29 and the ovarian carcinoma cell line 2008 were obtained from Dr. G. Singh, Hamilton Regional Cancer Center, Hamilton, Ontario, Canada. The ovarian carcinoma cell line SKOV-3 was obtained from Dr. S. Bacchetti, Department of Pathology, McMaster University, Hamilton, Ontario, Canada. The squamous cell carcinoma cell line SCC-25 was obtained from Dr. J.S. Lazo, University of Pittsburgh School of Medicien, Pittsburgh, PA, USA and human 293 cells were obtained from Dr. F. Graham, Departments of Biology and Pathology, McMaster University, Hamilton, Ontario, Canada.

All cells were grown in monolayer in alpha-minimal essential medium (α-MEM) (GIBCO-BRL Canada) with 1% antibiotic-antimycotic supplemented with 10% newborn calf serum (NCS) for CHO cells or 10% fetal bovine serum (FBS) for cells of human origin.

Virus

The virus used was Ad5(lacZ), a recombinant virus containing the β-galactosidase gene inserted into the deleted non-essential E3 region. This construct was kindly provided by Dr. F. L. Graham, Departments of Biology and Pathology, McMaster University, Ontario, Canada. Viral stocks were grown as described previously (Hitt et al., 1995) on monolayers of 293 cells (Graham et al., 1977). Stocks were titred by plaque assay on human 293 cells and suspended in PBS to a titre of about 10^9 plaque forming units (pfu)/mL.

Cisplatin Treatment of Virus

Ad5(lacZ) was treated with cisplatin in a suspension in which the chloride ion concentration was adjusted to 50 mM using an appropriate ratio of low chloride PBS and α−MEM containg 1% antibiotic-antimycotic solution. Cisplatin solution was then added to the viral suspensions to give the appropriate final drug concentration. This suspension was left at 37°C for 24 hrs.

Quantification of Viral DNA Synthesis

CHO cells and human tumour cells were seeded, either in Corning 96-well plates at $3.8x10^4$ cells/well, or in Nunc 24-well plates at $2x10^5$ cells/well, whereas human fibroblasts were seeded at $1.9x10^4$ cells/well in 96-well plates or $1x10^5$ cells/well in the 24-well. Cell monolayers just reached confluence at about 24 hrs. after seeding and were infected with either untreated or cisplatin-treated Ad5(lacZ) at a multiplicity of infection (MOI) of 40 pfu/cell in a volume of 40 μL/well for 96-well plates or 200 μL/well for 24-well plates. Following viral adsorption for 90 mins. at 37°C, the infected cells were overlayed with 160 μL for 96-well plates or 1 mL for 24-well plates, of warm α−MEM supplemented with 10% NCS or FBS. 2-4 hrs. after infection, virus which had not adsorbed to the cells was removed by aspiration and replaced with warm α-MEM containing 10% serum and 1% antibiotic solution.

At an appropriate time after infection, infected cells were lysed and DNA was transferred to a nylon membrane following one of two protocols. Cells which were seeded to 24-well plates were lysed by addition of 200 μL of pronase solution (4 mg/mL pronase, 40 mM Tris pH 8.0, 40 mM EDTA pH 8.0, 2.4% SDS) and incubated for 1.5-3

hrs. The cell lysates were then stored at -20°C until phenol:chloroform extractions were performed and DNA was precipitated with two volumes of cold absolute ethanol with 0.2 M NaCl. DNA was then pelleted by centrifugation at 13 000 rpm for 20-30 minutes at 4°C after which pellets were resuspended in 40 μL of TE buffer (10 mM Tris, 1 mM EDTA) and slot-blotted onto Gene Screen Plus (Dupont Cat.#NEF-976) according to the manufacturer's specifications.

Infected cells from 96-well plates were lysed by first aspirating the medium and adding 40 μL of proteinase K solution per well. After 1.5 - 2 hrs. at 37°C, DNA was denatured by addition of 80 μL/well of 1.0 N NaOH with 50 mM EDTA. After a further 2 hrs. at 37°C, samples were transferred directly onto Gene Screen Plus according to the manufacturer's specifications.

Viral DNA in each sample was probed with ^{32}P-labelled Ad2 DNA and the relative viral DNA content in each sample was determined from the radioactivity of each slot-blot using a phosphorimaging system.

RESULTS

Host Cell Reactivation of Cisplatin-treated Virus in NER-deficient Cells

The survival of viral DNA synthesis for cisplatin-treated Ad was examined following the infection of both excision-repair proficient and excision-repair deficient mammalian cells. The amount of viral DNA synthesized by each cell line following infection with cisplatin-treated virus was expressed as a fraction of that synthesized in the same cell line following infection with non-treated virus. Figure 1 shows typical results for the survival of Ad DNA synthesis following cisplatin treatment for infection of the nucleotide excision repair (NER)-proficient CHO-AA8 cells and the NER-deficient CHO cell mutants representing complementation groups 1 to 6 (Thompson et al., 1980; Busch et al., 1980). It can be seen that survival of cisplatin-treated virus was reduced in all the excision repair-deficient CHO cell lines tested compared to that in CHO-AA8 cells.

Survival curves were fitted to a linear quadratic equation, (ln relative amount of viral DNA) = $-(\alpha x+\beta x^2+\gamma)$) and the parameters α, β and γ were determined by least squares analysis in order to obtain a D_{37} value for each survival curve. For each experiment, the D_{37} value obtained for each CHO cell mutant was expressed relative to the D_{37} value obtained in the CHO-AA8 parental line. The relative HCR values so

obtained were taken as a measure of the DNA repair capacity of the cell and pooled results for a number of experiments are shown in Table 1. It can be seen that a large reduction in HCR for cisplatin-treated Ad was detected following infection of all the NER-deficient CHO cell lines tested representing complementation groups 1 to 6, compared to that of the NER-proficient CHO-AA8 parental cells with no overlap of error bars.

Typical results for the survival of Ad DNA synthesis for cisplatin-treated Ad in human fibroblasts are shown in Figure 2. It can be seen that survival of cisplatin-treated virus was reduced in all the NER-deficent XP strains tested compared to that in the NER-proficient normal human fibroblast strain. Relative HCR values for the various XP fibroblast strains obtained from a number of experiments are shown in Table 1. It can be seen that a large reduction in HCR for cisplatin-treated Ad was detected following infection of the XP fibroblast strains representing complementation groups A, B, C, D, F and G, compared to that in the normal fibroblasts with no overlap of error bars.

Host Cell Reactivation of Cisplatin-treated Virus in Human Tumour Cells

Figure 3 shows typical results for the survival of cisplatin-treated Ad in various human tumour cell lines. For comparison purposes, each experiment included the HeLa cell line. Relative HCR values for the different tumour cell lines are shown in Table 1. It can be seen that HCR of cisplatin-treated Ad varied by about two fold for the five different tumour cell lines tested and was reduced in all the tumour cell lines tested compared to that in the normal human fibroblast strain, indicating some deficiency in the repair of cisplatin-damaged DNA for these tumour cells. The HCR was greatly reduced in HeLa, 2008 and SCC-25 cells compared to that in the normal human fibroblast strain.

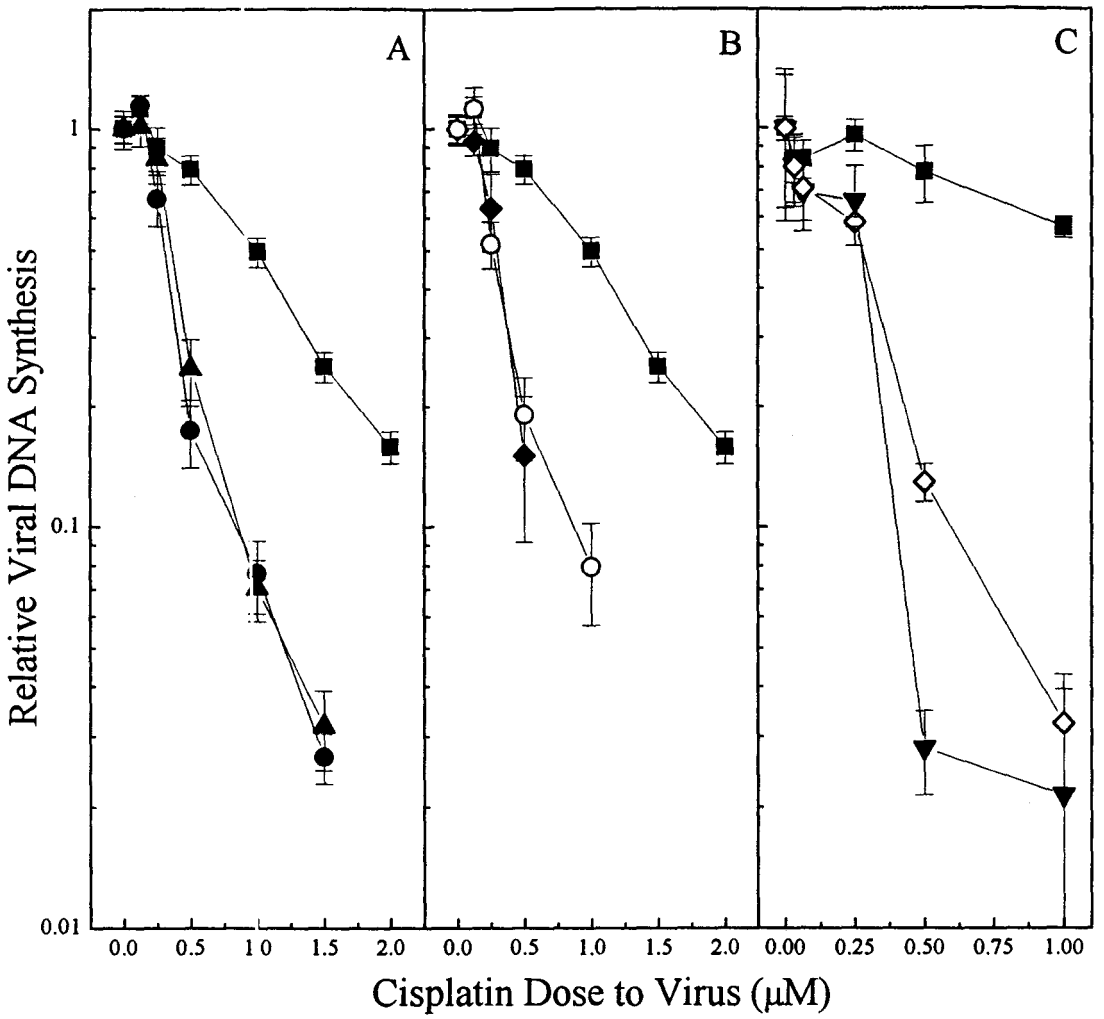

Figure 1. **HCR of cisplatin-damaged Ad in CHO cells**. Ad was treated with cisplatin for 24 hrs. at 37°C to damage the viral DNA. Data points are the mean of at least triplicate samples shown with standard error. ■AA8 (repair-proficient parental line). (A) ●UV20 (group 1), ▲UV5 (group 2). (B) ◆UV24 (group 3), ○UV135 (group 5). (C) ▼UV41 (group 4), ◊UV86 (group 6).

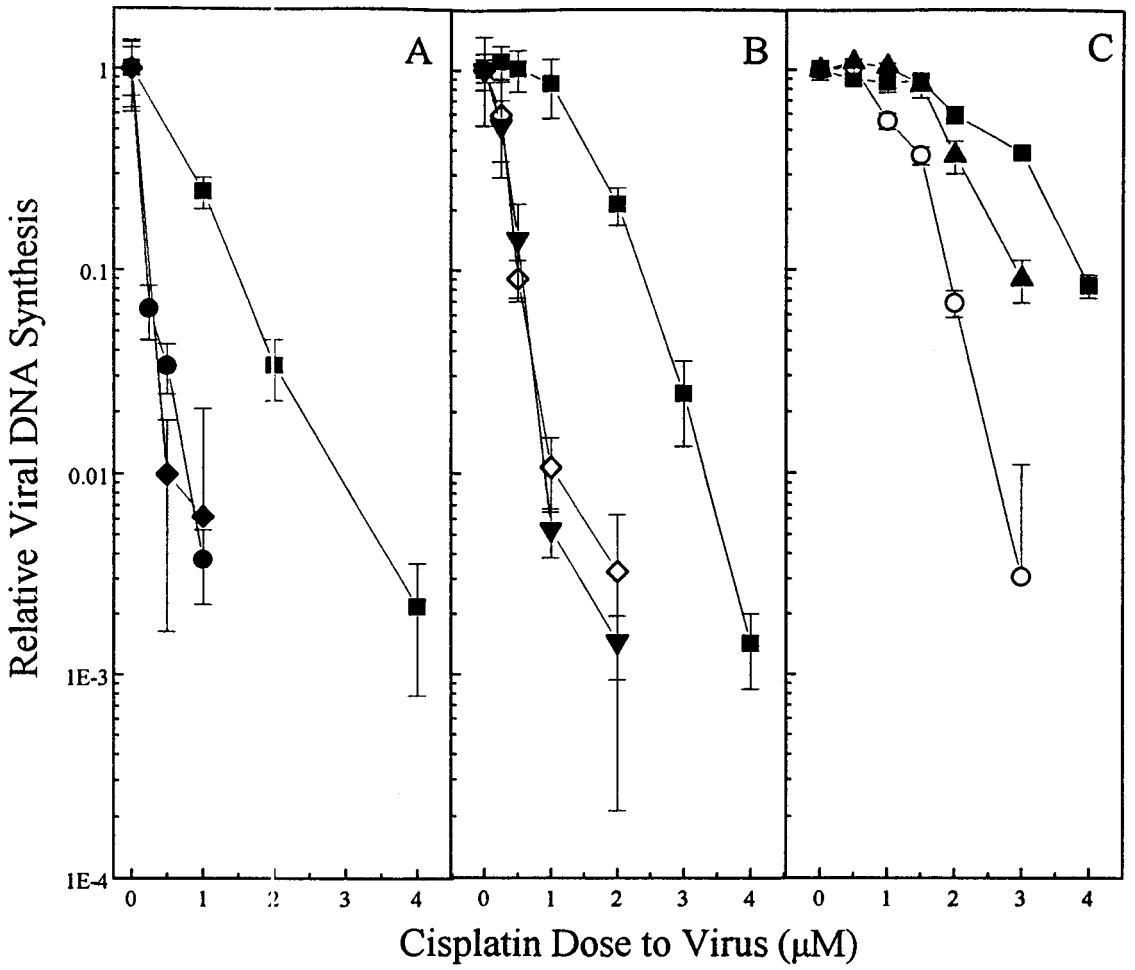

Figure 2. **HCR of cisplatin-damaged Ad in XP fibroblasts**. Ad was treated with cisplatin for 24 hrs. at 37°C to damage the viral DNA. Data points are the mean of at least duplicate samples shown with standard error. ■GM969 (normal). (A) ●XP12BE (XP-A), ◆XP2BI (XP-G). (B) ◊XPCS2BA (XP-B), ▼XP1BR (XP-D). (C) ○XP1MI (XP-C), ▲XP2YO (XP-F).

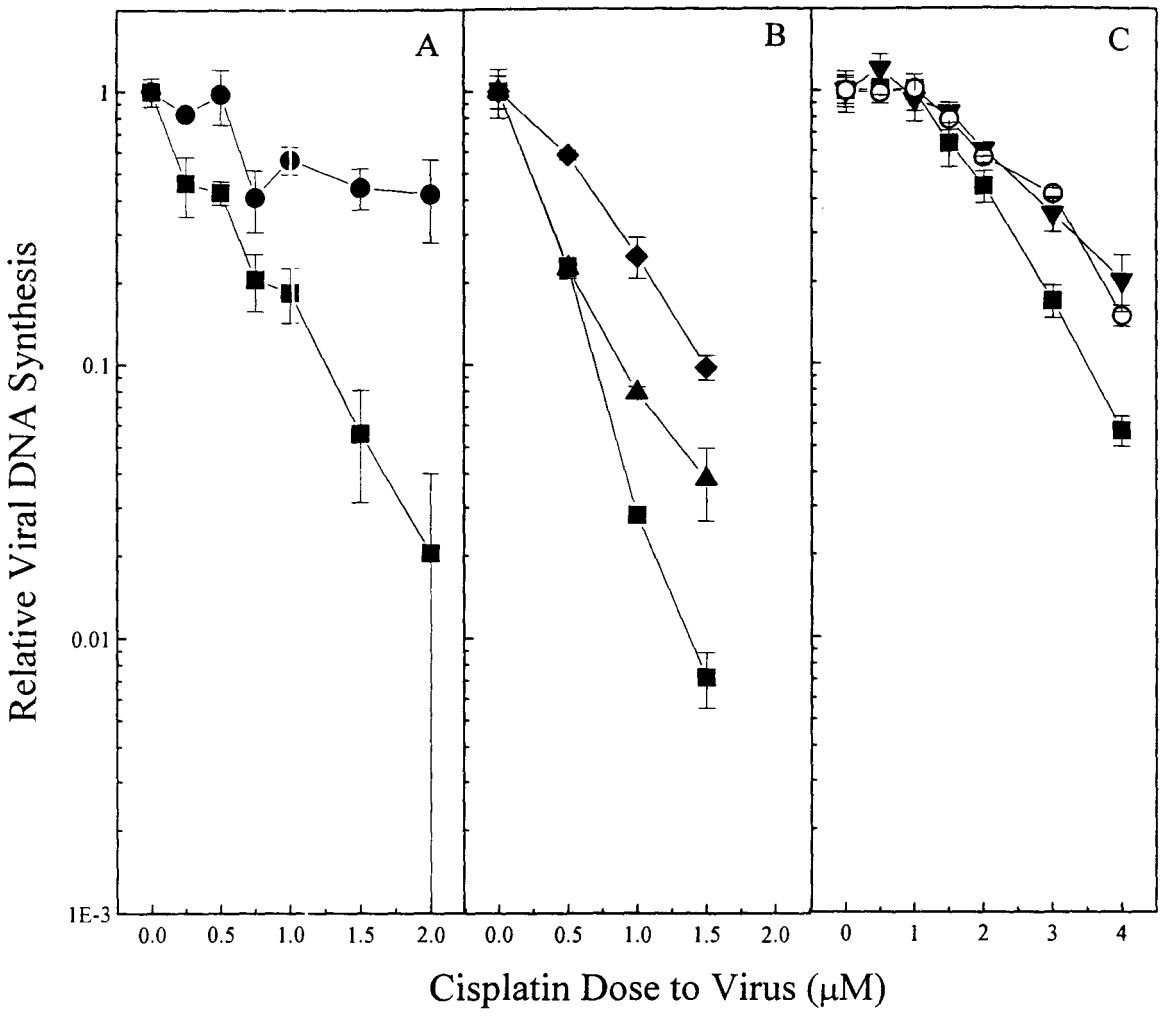

Figure 3. **HCR of cisplatin-damaged Ad in human tumour cells.** Ad was treated with cisplatin for 24 hrs. at 37°C to damage the viral DNA. Data points are the mean of at least triplicate samples shown with standard error. ■HeLa (cervical carcinoma) are included in each plot for comparison. (A) ●GM969 (normal fibroblasts). (B) ◆HT29 (colon carcinoma), ▲SSC-25 (squamous cell carcinoma). (C) ○2008 (ovarian carcinoma), ▼SKOV-3 (ovarian carcinoma).

Table 1. HCR of cisplatin-treated Ad.

Cell Strain/Line		Mean Relative D_{37}[a]	Number of Experiments
CHO Cells:	AA8(parental)	1	8
	UV20 (1[b])	0.17 ± 0.03[c]	8
	UV5 (2)	0.22 ± 0.05	5
	UV24 (3)	0.30 ± 0.05	7
	UV41 (4)	0.21 ± 0.06	4
	UV135 (5)	0.25 ± 0.08	4
	UV85 (6)	0.24 ± 0.05	3
XP Cells:	GM969(normal)	1	7
	XP12BE (XP-A)	0.20 ± 0.06	3
	XPCS2BA (XP-B)	0.27 ± 0.11	3
	XP1MI (XP-C)	0.53	1
	XP2BE (XP-C)	0.48	1
	XP1BR (XP-D)	0.27 ± 0.12	3
	XP2YO (XP-F)	0.70	1
	XP2BI (XP-G)	0.18 ± 0.04	3
Tumour Cells:	HeLa	1	6
	2008	1.81 ± 0.22	6
	SKOV-3	2.37 ± 0.75	4
	HT29	1.97 ± 0.48	3
	SCC-25	0.89	1
	GM969	2.93 ± 0.80	2

[a]values made relative to the first cell line given in each group
[b]NER-deficient complementation group
[c]standard error about the mean

DISCUSSION

Cisplatin causes cell death by reacting with DNA, forming a spectrum of adducts (Sherman and Lippard, 1987). While it is unclear at this time which cisplatin DNA adduct is the primary cause of cytotoxicity of the drug, comparison of cisplatin with its less toxic analogue, carboplatin, shows that cytotoxicity of the drug correlates best with the number of intrastrand adducts induced between purine bases (Fichtinger-Schepman et al., 1995). This is consistent with observations that NER-deficient mutants show extreme sensitivity to cisplatin treatment (Sheibani et al., 1989; Plooy et al., 1985) since, presumably, NER is involved in the repair of intrastrand adducts.

The HCR values for DNA synthesis of cisplatin-treated Ad in the UV-sensitive, NER-deficient CHO cell mutants from complementation groups 1-6 were found to be reduced 3 to 5 fold compared with that in parental NER-proficient CHO-AA8 cells (Figure 1 and Table 1). Similar HCR values have been reported for DNA synthesis of UV-irradiated Ad in CHO cell mutants from these complementation groups 1-6 (Arnold and Rainbow, 1996) The relative amount of HCR for UV-irradiated Ad in these and other NER-deficient cells correlates well with the UV-sensitivity of the cells themselves as measured by colony forming ability, suggesting that HCR of UV-irradiated Ad reflects the cellular DNA-repair capacity important for clonagenic survival (Rainbow, 1981; Defais et al., 1983; Arnold and Rainbow, 1996). However, the CHO cell mutants from complementation groups 1 and 4 are substantially more sensitive to cisplatin and other DNA-interstrand-crosslinking agents compared to CHO cell mutants from other complementation groups (Sheibani et al., 1989; Hoeijmakers, 1993; Jones, 1994; Collins, 1993), yet HCR values for cisplatin-treated Ad were similar for the CHO cell mutants from complementation groups 1-6 tested. Similarly reduced HCR values for cisplatin-damaged plasmid have been reported for the NER-deficient CHO cell lines UV20, UV5 and UV41 from complementation groups 1,2 and 4 respectively (Sheibani et al., 1989). Taken together, these results might suggest that viral and plasmid reactivation assays are unable to detect differences in the cellular capacity for DNA interstrand crosslinks. This is supported by the results of Poll et al. (1984) which show normal HCR levels for reactivation of cisplatin-treated SV40 DNA in Fanconi anemia (FA) cells, which are known to be deficient in cisplatin-induced interstrand DNA crosslinks. Normal HCR levels in FA cells have also been reported for reactivation of Ad treated with psoralen

plus near UV light (Day et al., 1975). These results suggest that interstrand DNA crosslinks in adenovirus produced by psoralen plus near UV light are not repaired during reactivation of adenovirus in normal human cells.

Our results indicate a substantial decrease in the capacity of NER-deficient CHO cell lines to repair cisplatin-damaged DNA. We also show a reduced capacity for human fibroblasts from XP patients from complementation groups A, B, C, D, F, and G to reactivate cisplatin-damaged Ad (Figure 2). This is consistent with the finding that repair synthesis of a cisplatin-treated plasmid is reduced in cell extracts from XP groups A, C, D, and G (Hansson et al., 1990) and that HCR of cisplatin-damaged Ad is reduced in XP-A fibroblasts relative to that in a normal human cell line or several ovarian and testicular cell lines (Maynard et al., 1989; Bubley et al., 1991). XP-A and XP-F fibroblasts have also been shown to have a reduced colony forming ability following cisplatin exposure compared to normal fibroblasts (Plooy et al., 1985) and XP-A fibroblasts are reduced in their ability to remove cisplatin adducts from the active DHFR gene (Zhen et al., 1993). Collectively, these results demonstrate the central importance of NER in the cellular response to cisplatin treatment and show an involvement of the following genes in the repair of cisplatin-damaged DNA: ERCC1, ERCC2/XPD, ERCC3/XPB, ERCC4/XPF, ERCC5/XPG, ERCC6/CSB, XPA and XPC.

Stable transfection of the UV20 CHO cell mutant from complementation group 1 with the ERCC1 gene results in an increased cellular resistance to cisplatin (Bramson and Panasci, 1993; Lee et al., 1993; Larminat and Bohr, 1994) providing further evidence for the involvement of the ERCC1 gene in the repair of cisplatin-damaged DNA. The reduced HCR of cisplatin-treated Ad for the UV24 CHO cell mutant from complementation group 3 and the XP-B cells presented here indicates an involvement of the ERCC3 gene in the repair of cisplatin-damaged DNA. In contrast, stable transfection of the CHO 27-1 cell mutant from complementation group 3 with the ERCC3 gene does not result in an increased resistance to cisplatin, suggesting that the ERCC3 gene may not be a determinant of cisplatin resistance (Lee et al., 1993). However, there is evidence that the CHO 27-1 cell is a double mutant having a defect in the repair of both DNA alkylation damage and UV-induced lesions (Kaina et al., 1990). It is thus possible that both mutations in CHO 27-1 cells affect the repair of cisplatin-damaged DNA, such that

stable transfection with the ERCC3 gene alone does not lead to an increased cisplatin resistance of the cells.

The mechanism by which tumours acquire resistance to cisplatin has been the subject of intense research (as reviewed by Chu, 1994; and Eastman, 1991). The primary mechanisms proposed to account for this resistance are decreased intracellular drug accumulation (Bungo et al., 1990), cytosolic drug inactivation by increased expression of the thiols, glutathione and metallothionein (Meijer et al., 1990), increased replicative bypass of platinated DNA (Mamenta et al., 1994) and increased capacity to repair cisplatin-DNA adducts (Masuda et al., 1988). While each of these mechanisms has been described in cisplatin-resistant human tumour cell lines, increased DNA repair capacity has been shown to correlate best with cellular resistance in many tumour cell lines. Studies of the murine leukemia cell line L1210 and its cisplatin-resistant derivatives showed a marginal decrease in drug accumulation, while there was approximately a four-fold increase in the removal of intrastrand platinum adducts (Eastman and Schulte, 1988). HCR of a plasmid bearing the CAT reporter gene has also been used to demonstrate increased DNA repair in L1210 sublines with acquired resistance to cisplatin as well as in a human breast carcinoma cell line, MCF7-MLNr, selected for its resistance to alkylating agents (Sheibani et al., 1989; Yen et al., 1995).

We show here a reduced HCR of cisplatin-damaged Ad in several human tumour cells (Figure 3) suggesting a reduced capacity for repair of cisplatin-damaged DNA in these cells. Many other investigators have reported various types of DNA-repair defects in human tumour cell lines (Day et al., 1980; Parshad et al., 1984; Rainbow, 1989; Evans et al., 1996), suggesting that various deficiencies in DNA repair may be an important aspect of their malignant phenotype.

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CHAPTER FIVE

REDUCED CAPACITY FOR ADENOVIRAL DNA SYNTHESIS IN CISPLATIN-TREATED LI-FRAUMENI SYNDROME FIBROBLASTS

REDUCED CAPACITY FOR ADENOVIRAL DNA SYNTHESIS IN CISPLATIN-TREATED LI-FRAUMENI SYNDROME FIBROBLASTS

ABSTRACT

Li-Fraumeni syndrome (LFS) is a rare, autosomal dominant disorder that is characterized by a high incidence and early onset of multiple cancers. Patients are predisposed to tumour formation as a result of an inherited mutant allele of the p53 tumour suppressor gene. Normal functioning of the p53 protein includes modulating cellular response to DNA damage, and more recent results suggest a direct role for p53 in the repair of UV-induced DNA damage. We have investigated the ability of Li-Fraumeni fibroblasts to repair cisplatin-damaged DNA in host cell reactivation (HCR) and cellular capacity assays using adenovirus (Ad) as a probe. The HCR of cisplatin-damaged Ad in p53-heterozygous LFS fibroblasts was similar to that in normal fibroblasts, while the repair-deficient xeroderma pigmentosum (XP) fibroblasts showed a reduced HCR of the virus. Studies with the spontaneously immortalized p53-hemizygous LFS cells also show a normal HCR of cisplatin-damaged Ad. In contrast, the capacity of cisplatin-damaged Li-Fraumeni fibroblasts to support untreated Ad DNA replication was greatly reduced compared to normal cells. The reduced capacity of the LFS cells was similar to the level of reduction seen in the repair-deficient XP cells. These findings suggest that the repair of cisplatin-induced DNA lesions in actively transcribed cellular genes necessary for viral replication is deficient in the LFS fibroblasts.

INTRODUCTION

The Li-Fraumeni syndrome (LFS) is a rare, autosomal dominant disease that is associated with a high incidence and early onset of several forms of cancer, namely soft tissue sarcomas, breast carcinomas, acute leukemias, osteosarcomas, brain tumours and adrenocortical carcinomas (Li and Fraumeni, 1969). The predisposing factor in LFS patients is an inherited germline mutation in one p53 allele (Malkin et al., 1990). Loss

of the wild type (wt) allele leads to tumour formation in LFS patients (Srivastava et al., 1992) and to immortalization of LFS fibroblasts in culture (Yin et al., 1992).

The p53 tumour suppressor gene appears to function in several ways. It is involved in regulation of the cell cycle (Kuerbitz et al., 1992; Yin et al., 1992), in mediating cellular response to DNA damage (Kastan et al., 1991), and in controlling p53-dependent apoptosis (Lowe et al., 1993a and 1993b). Studies have shown that γ-irradiation of normal human fibroblast cells results in activation of wild type p53 levels which induce a G_1 cell cycle checkpoint (Kastan et al., 1992). However, it has also been shown that loss of this G_1 checkpoint does not influence cell survival following ionizing radiation (Slichenmeyer et al., 1993). In mouse thymocytes, γ-irradiation leads to p53-induced apoptosis (Lowe et al., 1993a) suggesting perhaps a differential p53 response based on cell type. It is believed that the induction of p53 arrests damaged cells in G_1 of the cell cycle in order to allow sufficient removal of DNA damage before cells begin to synthesize DNA for replication. It is postulated that if the cell is damaged beyond repair, then programmed cell death, or apoptosis, results. Therefore, loss of normal p53 function may facilitate the onset of cancer by permitting the replication and/or survival of genetically altered cells.

The accumulation of p53 protein, leading to either G_1 arrest or apoptosis, has been shown to occur following cellular DNA damage. Agents such as γ-rays, etoposide, mitomycin C (MMC) and cisplatin induce an increase in nuclear p53 levels (Fritsche et al., 1991) as do 4NQO (Maltzman and Czyzyk, 1984), x-rays, actinomycin D (Kastan et al., 1992), and UV-irradiation (Maltzman and Czyzyk, 1984; Fritsche et al., 1993; Yamaizumi and Sugano, 1994). The role of p53 in DNA repair is becoming evident; however, it is not clear whether p53's involvement in repair is a direct or indirect one. One study, using DNA-repair-deficient fibroblasts, has linked the nuclear accumulation of p53 to DNA-repair capacity suggesting that the signal for p53 induction is the presence of unrepaired DNA lesions in actively transcribed genes (Yamaizumi and Sugano, 1994). Nucleotide excision repair (NER)-deficient xeroderma pigmentosum (XP) cells from

complementation groups A, F and G were shown to have an eightfold lower minimum required dose for induction of p53 accumulation than normal cells did. Furthermore, Cockayne syndrome (CS) fibroblasts, deficient only in preferential NER of actively transcribed genes, had a similar low minimum required dose whereas, XP-C cells, which have normal preferential NER but are deficient in overall genomic repair, exhibited a minimum required dose similar to that of normal fibroblasts (Yamaizumi and Sugano, 1994). These results suggest that nuclear accumulation of the p53 protein is induced by damage located in the actively transcribed regions of the genome.

Two immortalized Li-Fraumeni cell lines, hemizygous for p53 mutations in codons 184 and 248, show an enhanced cellular UV resistance in comparison to normal and p53-heterozygous LFS cells. This resistance is thought to result from a reduction in UV-induced apoptosis in these LFS cells (Ford and Hanawalt, 1995). The complete absence of wt p53 in these cells was shown to decrease levels of global repair as well as repair of the nontranscribed strand of active genes while transcription-coupled repair of the transcribed strand was normal (Ford and Hanawalt, 1995). In constrast, Wang et al. (1995) showed that LFS fibroblasts, heterozygous for a mutation at codon 245, were significantly reduced in their gene-specific repair of UV-induced lesions. Reduced repair of UV damage in actively transcribed genes was also observed in human pampilloma virus (HPV)-infected cervical carcinoma cells which have reduced p53 activity due to the abrogation of wt p53 by the HPV-E6 protein (Evans et al., 1996). Mutant p53 and HPV-E6 protein expression were reported to sensitize wt p53-expressing tumour cells to UV light and to reduce the NER capability of these cells when assayed by HCR of a reporter gene and by *in vitro* radionucleotide incorporation (Smith et al., 1995). Cellular sensitivity of p53-heterozygous LFS fibroblasts to UV and other DNA-damaging agents, however, has been reported to be normal. Normal clonogenic survival was observed following treatment with UV light, x-rays, MNNG and the DNA-crosslinking agent mitomycin C (MMC) (Little et al., 1987).

The induction of p53-mediated apoptosis is an important mechanism of chemotherapeutic cell death following treatment with antitumour agents such as cisplatin (Lowe et al., 1993b). Studies with ovarian tumour cell lines have shown a correlation between p53 expression and sensitivity to cisplatin. Resistance of these cells to cisplatin was accompanied by an elevation in the constitutive levels of p53 protein (Brown et al., 1993). Therefore, the altered p53 activity in LFS cells would be expected to affect their response to cisplatin damage. This is an important point to consider when treating human tumours, especially since p53 mutations are the most common genetic alterations in human cancer (Hollstein et al., 1991).

Adenovirus (Ad) is a DNA tumour virus that depends on host cell mechanisms in order to replicate its genome. Ad encodes several early gene products, some of which function to activate host cell replicative processes. The early region 1A and 1B genes (E1A and E1B) act by binding to specific cellular proteins that normally function in regulating cell growth. E1A targets the retinoblastoma gene product pRb (Whyte et al., 1988), while E1B binds to wt p53 (Sarnow et al., 1982). It has also been shown that E1A increases levels of p53 protein by post-translational stabilization (Lowe and Ruley, 1993) and increases cellular susceptibility to anticancer agents (Lowe et al., 1993b). It has been suggested that the complexes formed between p53 and viral proteins may function to prevent p53 from affecting cellular genes that are essential to viral replication and/or transformation. This idea is supported by the observation that wt p53 can inhibit the replication of SV40 viral DNA (Braithwaite et al., 1987).

We have used Ad as a probe to examine the repair of cisplatin-induced DNA damage in p53-heterozygous LFS fibroblast strains and their immortalized hemizygous counterparts expressing only mutant p53. Host cell reactivation for viral DNA synthesis of cisplatin-treated Ad and the capacity of cisplatin-treated cells for Ad DNA synthesis were examined. It was found that both p53-heterozygous and -hemizygous LFS cells showed a similar HCR of cisplatin-damaged virus compared to normal cells, whereas the HCR of nucleotide excision repair (NER)-deficient XP cells was greatly reduced. We

have also carried out cellular capacity experiments in order to investigate the ability of cisplatin-damaged LFS fibroblasts to support Ad DNA synthesis. It was shown that the capacity of the p53-heterozygous LFS fibroblasts to replicate Ad DNA was greatly reduced compared to normal. As well, the levels of Ad DNA synthesized during infection of untreated normal and untreated LFS host cells was compared. The amount of Ad DNA detected in the p53-heterozygous LFS fibroblasts was much less than that detected in the normal cells.

MATERIALS AND METHODS

Cell Strains

Normal cells: Normal fibroblast strains GM969 and GM9503 were obtained from the NIGMS Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ). The normal 423 strain was kindly provided by Dr. Patricia Chang, McMaster University, Hamilton, Ont. *Xeroderma pigmentosum cells*: The xeroderma pigmentosum strains XP12BE (GM5509B), XPCS2BA (GM13026), XP1BR (GM3615) and XP2BI (GM3021) were all obtained from the NIGMS Human Genetic Mutant Cell Repository. These strains belong to complementation groups A,B,D and G, respectively. *Li-Fraumeni cells*: The 041 and 087 LFS fibroblasts are heterozygous for p53 mutations at codons 184 and 248 respectively. Their spontaneously immortalized counterparts (041mut and 087mut) are hemizygous, expressing only mutant p53 (Yin et al., 1992). All LFS cells were kindly provided by Dr. M.A. Tainsky, M.D., Anderson Cancer Center, Houston, Texas.

All cells were grown in 75 cm^2 tissue culture flasks (Corning Incorporated, Corning, NY) in α-minimal essential medium (α-MEM) supplemented with 10% (v/v) fetal bovine serum (Sigma Chemical Co., St. Louis, MO) and 1% (v/v) antibiotic/antimycotic containing 10 000 U/mL penicillin G sodium, 10 000 ug/mL streptomycin sulfate and 25 ug/mL amphotericin B as Fungizone® in 0.85% saline (Gibco BRL, Life Technologies Inc., Grand Island, NY). Cells were incubated in a 5% CO_2,

100% humidified, water-jacketed incubator (Forma Scientific Inc., Marietta, OH) at 37°C. At confluency, cells were passaged by aspirating spent growth medium, rinsing in 1x phoshpate buffered saline (PBS) and by the addition of 2x trypsin-EDTA containing 0.5% trypsin and 5.3 mM EDTA•4Na (Gibco BRL, Life Technologies Inc., Grand Island, NY). Once the cells rounded up, they were resuspended in medium and subcultured at appropriate dilutions.

Cisplatin

Cisplatin (*cis*-diamminedichloroplatinum(II)) was obtained from Sigma Chemical Company (St. Louis, MO). Stock solutions of 1 mM were made in 1x PBS (1.4 M chloride ion) for the treatment of cells or in 1x low chloride PBS (6.6 mM chloride ion) for the treatment of virus. Stock solutions were made fresh and filtered through a 0.45 μm disposable filter (Nalge Company, Rochester, NY) just prior to use.

Virus

The human recombinant Ad5(lacZ) virus was used for infection of cells. The Ad5(lacZ) virus contains the *Escherichia coli* β-galactosidase (lacZ) gene inserted into the deleted, non-essential E3 region of the Ad viral genome and was generously provided by Dr. Frank Graham, Departments of Biology and Pathology, McMaster University, Hamilton, Ont. Viral stocks were grown on monolayers of human 293 cells and were titred by plaque assay as described previously (Hitt et al., 1995). Stock virus was suspended in PBS to a titre ranging from 10^8-10^{10} plaque forming units (pfu)/mL.

Host Cell Reactivation

Cells were seeded at a density of $1.9x10^4$ cells/well in 96-well Microtest III tissue culture plates (Falcon, Becton Dickinson & Company, Lincoln Park, NJ) and were incubated for 24 hrs. prior to infection. Ad5(lacZ) was treated with varying doses of cisplatin in serum-free medium containing 1% antibiotic/antimycotic for 24 hrs. at 37°C to damage the viral DNA. Because cisplatin molecules contain two chloride atoms as

leaving groups, the chloride ion concentration of the treatment solution can affect the amount of damage induced in the viral DNA during treatment. For this reason, the chloride ion concentration was set to 50 mM for all HCR experiments in order to maintain consistency from experiment to experiment. The medium was aspirated from the wells and cells were infected with 40 μL of viral suspension at a multiplicity of infection (MOI) of 20 pfu/cell. After a 90 min. incubation at 37°C, the infection was terminated by aspirating the virus and adding 200 μL of fresh growth medium to the wells. Plates were then incubated for 72 hrs. at 37°C to allow for repair and replication of viral DNA.

Cellular Capacity of Ad DNA Synthesis

Cells were seeded at a density of $1.9x10^4$ cells/well in 96-well plates. After a 24 hr. incubation, the growth medium was aspirated and cells were treated with varying doses of cisplatin in growth medium for 3 hrs. at 37°C to damage cellular DNA. In this assay, chloride ion concentration was not set to a specified value as the environment inside the cells provides an appropriate chloride ion concentration for cisplatin activity. The cisplatin was then aspirated and cells were rinsed with 1x PBS to remove any residual drug. Delayed infection of cell monolayers was done 24 hrs. following cisplatin treatment. Cells were infected with 40 μL of Ad5(lacZ) viral suspension at an MOI of 100 pfu/cell, and were incubated for 90 mins. at 37°C. Infection was terminated as above. Plates were incubated for 48 hrs. at 37°C to allow for repair of cellular DNA and subsequent replication of virus.

Quantification of Viral DNA Synthesized

Following the appropriate incubation time, the growth medium was aspirated from the wells and 50 μL of proteinase K solution (1 mg/mL proteinase K, 1% SDS, 0.2x SSC) was added to the wells for 1.5 hrs. at 37°C in order to lyse the cells. To denature the viral DNA for blotting, 50 μL of NaOH solution (1 M NaOH, 1 mM EDTA), was

added to the wells and plates were incubated another 1.5-2 hrs. before slot-blotting to nylon membrane (GeneScreen Plus®, DuPont, NEN Research Products, Boston, MA) using the Minifold II Slot-Blot system (Schleicher and Schuell Inc., Keene, NH). The membrane was then air dried before probing with ^{32}P-labelled Ad2 DNA. To quantitate the amount of radioactivity, blots were exposed to a phosphor screen and were analyzed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Host Cell Reactivation of Cisplatin-damaged Ad in Li-Fraumeni Cells

The survival of viral DNA synthesis for cisplatin-treated Ad was examined following the infection of normal, Li-Fraumeni, as well as NER-deficient XP fibroblasts. The amount of viral DNA synthesized by each cell strain at 72 hrs. after infection with cisplatin-treated virus was expressed as a fraction of that synthesized in the same cell strain following infection with untreated virus. Results of the HCR experiments are shown in Figure 1. It can be seen that both the normal and the p53-heterozygous Li-Fraumeni strains show a similar host cell reactivation of cisplatin-damaged Ad, whereas the XP strains show a greatly reduced HCR. These results suggest normal levels of repair of cisplatin lesions in viral DNA for both the normal and the Li-Fraumeni cells, but show that repair of cisplatin-damaged virus is deficient in the XP cells. Similar reduced HCR of cisplatin-treated virus in XP cells has been reported previously (Poll et al., 1984; Bulmer et al., 1996).

A single experiment of the HCR of cisplatin-damaged virus in the spontaneously immortalized, p53-hemizygous LFS cells was conducted and is shown in Figure 2. As for the p53-heterozygous LFS fibroblasts, the p53-hemizygous LFS cells do not appear to have reduced reactivation of the virus compared to the normal controls, whereas the NER-deficient XP cell strain showed reduced HCR of cisplatin-damaged Ad.

Capacity of Cisplatin-damaged Li-Fraumeni Fibroblasts to Support Ad DNA Synthesis

It has been demonstrated previously that the cellular capacity assay can be used to examine the recovery and/or repair of mammalian cells following exposure to DNA-damaging agents (Lytle et al., 1976; Coppey et al., 1979; Rainbow, 1989). When the infection of cells was delayed following UV exposure, it was found that the capacity of the repair-deficient XP cells for herpes simplex viral infection was not restored, whereas the normal cells exhibited a restoration of capacity (Lytle et al., 1976; Coppey et al., 1979). Initial cellular capacity experiments were conducted by infecting the cells immediately following cisplatin treatment (data not shown). Results from these experiments indicated a reduced capacity of the LFS cells to support Ad DNA replication. Further experiments were performed using a delayed protocol in which the cells were infected with virus 24 hrs. after cisplatin treatment. Figure 3 shows representative capacity results for delayed infection of cisplatin-treated cells. It can be seen that the capacity of damaged cells to support Ad replication is reduced in both the p53-heterozygous Li-Fraumeni and the XP fibroblasts compared to the normal fibroblasts strains. The Li-Fraumeni and XP cells do not appear to differ from each other, but are significantly reduced compared to normal with no overlap of error bars. Cellular capacity survival curves for viral DNA synthesis were fitted to a linear quadratic equation (ln relative amount viral DNA= $-(\alpha x+\beta x^2+\gamma)$) and the parameters α, β, γ were obtained by least squares analysis in order to calculate D_{37} values for each survival curve. For each experiment, the D_{37} value obtained for each cell strain was expressed relative to the average D_{37} value obtained from all the normal strains used in that experiment. The mean relative D_{37} values for delayed capacities are shown in Table 1. It can be seen that the mean relative D_{37} was greatly reduced in the p53-heterozygous Li-Fraumeni and the NER-deficient XP fibroblasts compared to that of the normal strains.

Relative Ad DNA Synthesis in p53-Heterozygous and -Hemizygous LFS Cells

The Ad E1B protein has been shown to bind to wt p53 during infection of cells (Braithwaite et al., 1991) and it has been suggested that this interaction is required for efficient Ad DNA synthesis. It was therefore considered of interest to examine the level of Ad DNA synthesis in LFS cells that contain wt p53 and those that do not. For each LFS cell strain or cell line used in capacity experiments, the amount of viral DNA synthesized in the untreated sample was expressed relative to the mean amount of viral DNA synthesized in the untreated samples of normal fibroblasts. Table 2 shows the mean relative values of viral DNA synthesis from a number of experiments. It can be seen that Ad infection of p53-heterozygous LFS fibroblasts resulted in a reduced amount of viral DNA detected at 72 hrs. compared to infection of normal cells, whereas greater than normal amounts of Ad DNA were detected following infection of the p53-hemizygous LFS cells.

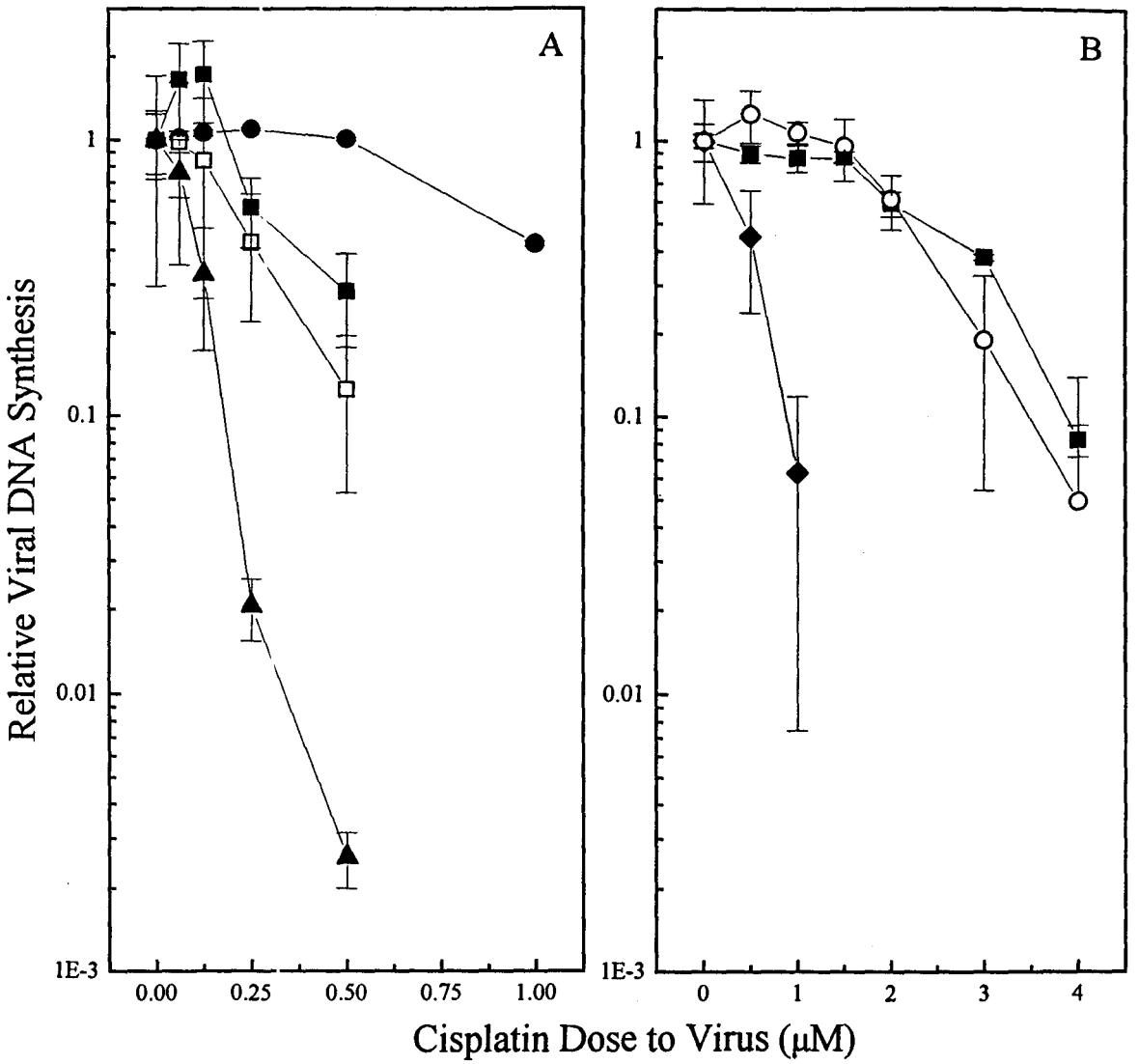

Figure 1. **HCR of cisplatin-damaged Ad in p53-heterozygous LFS fibroblasts**. Ad was treated with cisplatin for 24 hrs. at 37°C to damage viral DNA. (A) Data points are the mean of duplicate samples shown with standard error. (B) Data points are the mean of triplicate samples shown with standard error. ■GM969, □423 (normal); ●041, ○087 (LFS); ▲XPCS2BA (XP-B), ◆XP2BI (XP-G).

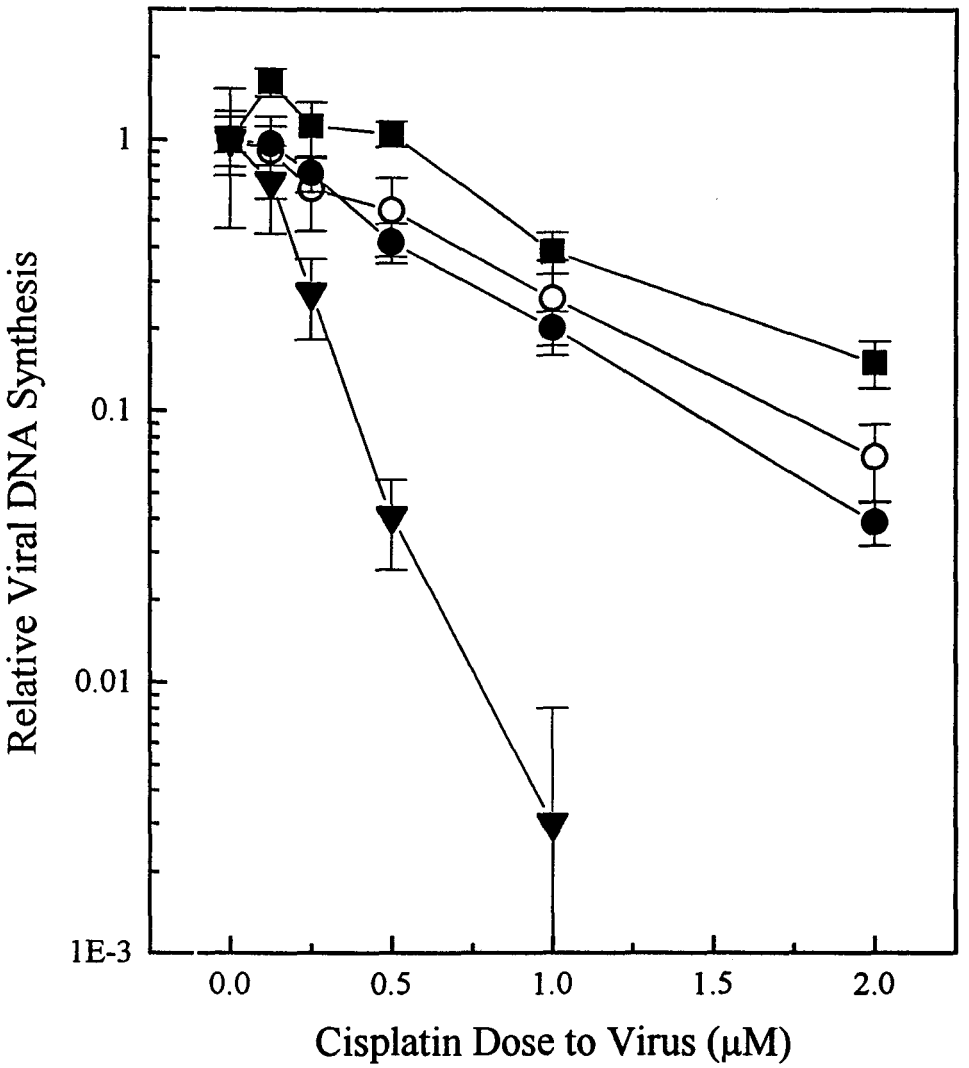

Figure 2. **HCR of cisplatin-damaged Ad in p53-hemizygous LFS cells.** Ad was treated with cisplatin for 24 hrs. at 37°C to damage viral DNA. Data points are the mean of triplicate samples shown with standard error. ■GM969 (normal); ●041, ○087 (LFS); ▼XP12BE (XP-A).

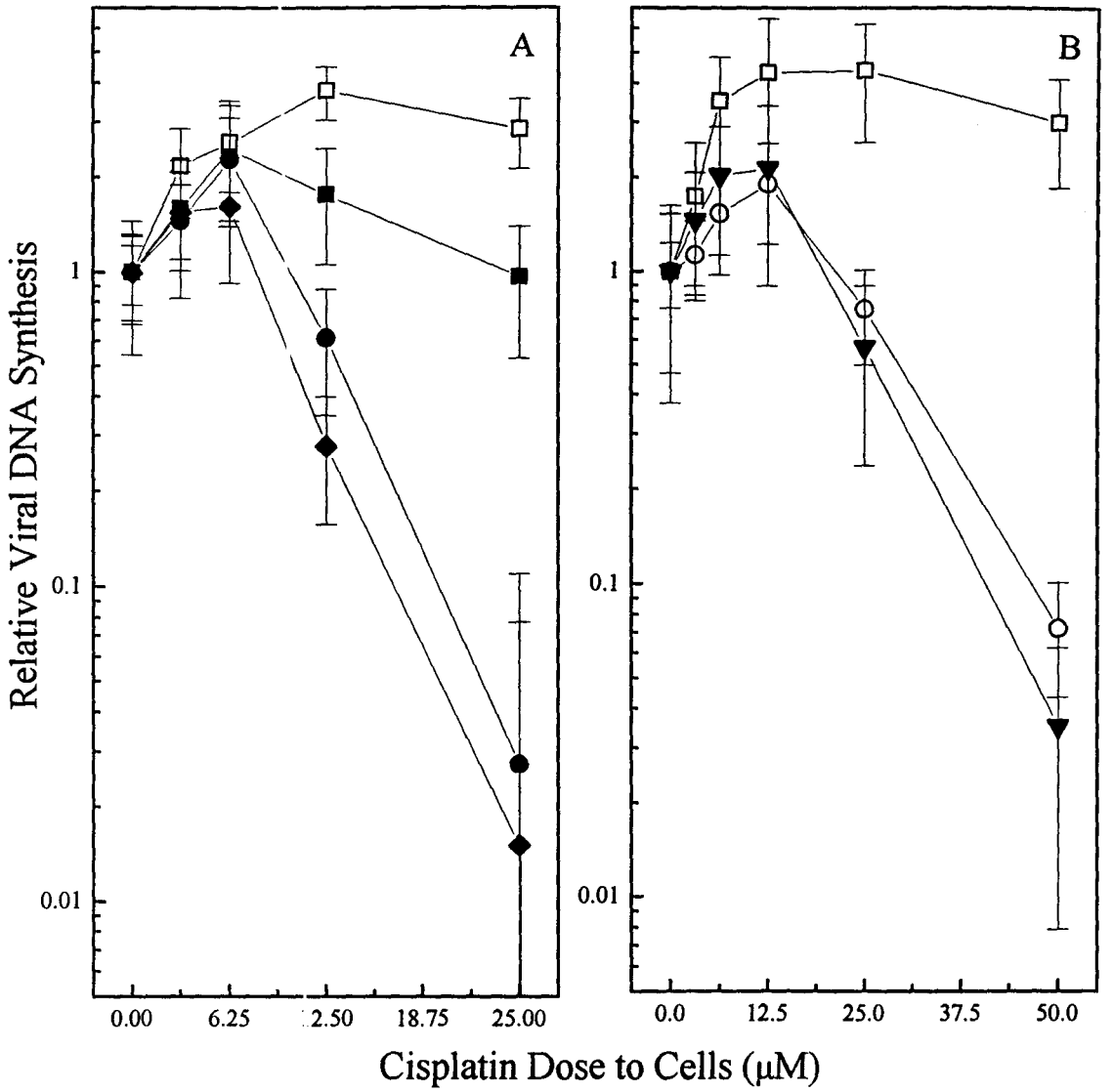

Figure 3. **Capacity of cisplatin-damaged LFS fibroblasts to replicate Ad DNA.** Cells were treated with cisplatin for 3 hrs. at 37°C to damage cellular DNA. Cells were then infected with untreated Ad 24 hrs. after cisplatin treatment. (A) Data points are the mean of quadruplicate samples shown with standard error. (B) Data points are the mean of triplicate samples shown with standard error. ■GM969, □423 (normal); ●041, ○087 (LFS); ◆XP2BI (XP-G), ▾XP12BE (XP-A).

Table 1. Capacity of cisplatin-damaged human fibroblasts to support Ad replication.

Cell Strain	Syndrome	Mean Relative D_{37}[a]	Number of Experiments
GM969	Normal	0.87 ± 0.07[b]	6
423	Normal	1.26 ± 0.10	3
GM9503	Normal	1.0	1
041	Li-Fraumeni	0.56 ± 0.15	3
087	Li-Fraumeni	0.60 ± 0.08	4
XP12BE	XP-A	0.59 ± 0.14	4
XP2BI	XP-G	0.23	1

[a] Relative to the mean D_{37} value for the normal strains used in each experiment
[b] Standard error about the mean

Table 2. Relative amount of Ad DNA synthesis in untreated LFS cells

Cell Strain	Syndrome	Mean Relative Amount of Viral DNA Synthesized[a]	Number of Experiments
GM969	Normal	1.1 ± 0.06[b]	6
GM9503	Normal	0.85	1
041 wt/mut	LFS	0.41 ± 0.22	3
087 wt/mut	LFS	0.26 ± 0.07	4
041 mut	LFS	4.6 ± 1.4	4
087 mut	LFS	5.7 ± 1.7	4

[a] Relative to the mean amount of viral DNA synthesized by the normal strains in each experiment

[b] Standard error about the mean

DISCUSSION

There have been few studies regarding the sensitivity of Li-Fraumeni cells to DNA-damaging agents. In one study, the p53-heterozygous 041 and 087 LFS fibroblasts were shown to exhibit normal cytotoxic responses to x-rays, UV light, MNNG and MMC when assayed by colony forming ability (Little et al., 1987). There was no hypersensitivity or increased resistance seen in the p53-heterozygous LFS cells. More recently, these same two cell strains were again reported to have normal colony forming ability following UV treatment (Ford and Hanawalt, 1995). In contrast, disruption of normal p53 function in colon carcinoma cells by transfection with either HPV-E6 gene or a mutant p53 transgene results in reduced clonogenic survival following UV damage (Smith et al., 1995); however, these cells would be expected to have a different level of functioning wt p53 than that present in the p53-heterozygous LFS fibroblasts. The HPV-E6 protein is known to promote proteolytic degradation of p53 (Scheffner et al., 1990) while mutant p53 expressed from the transgene would abrogate normal p53 by binding to it. The same sequestering of wt p53 protein would take place in the p53-heterozygous LFS cells; however, these cells have only one wt copy of p53 while the colon carcinoma cells contain two wt p53 alleles resulting in greater levels of wt p53 protein. Results from our lab (data not shown) indicate that the immortalized, p53-hemizygous 041 and 087 LFS cells containing no wt p53 have an increased clonogenic survival following cisplatin treatment (Bulmer, personal communication). This correlates with the increased UV survival observed for these same cells by Ford and Hanawalt (1995) which was attributed to a reduction in UV-induced apoptosis. Evidence for a similar mechanism in the case of cisplatin comes from studies with cisplatin-resistant human tumour cells. Induction of wt p53 expression in human glioblastoma cells has been observed in association with the cisplatin-induced apoptosis of these tumour cells (Kondo et al., 1995). Further support for this idea comes from studies of ovarian carcinoma cell lines in which cellular resistance to cisplatin is paralleled by a reduced susceptibility to cisplatin-induced

apoptosis. p53 analysis of these cells revealed mutations within the DNA-binding domain of the p53 protein. The induction of p53 accumulation following ionizing radiation or cisplatin treatment was absent in the resistant cells harbouring p53 mutations but was normal in the cisplatin-sensitive cells (Perego et al., 1996). Therefore, it appears that the absence of normal p53 function can result in a cellular resistance to DNA-damaging agents via increased cell survival directly resulting from reduced apoptosis, and that p53 may be a determinant of the chemosensitivity of cancer cells.

Studies of the repair of DNA damage in Li-Fraumeni cells has focused so far on UV-induced DNA lesions. The 041 and 087 p53-heterozygous strains were shown to have reduced levels of global UV repair 6 hrs. after irradiation; however, their repair capacity reached normal levels at 24 hrs. following irradiation (Ford and Hanawalt, 1995). Cell extracts of the colon carcinoma cells transfected with either the HPV-E6 viral oncogene or a mutant p53 transgene both exhibit a reduced level of NER when measured by HCR of a UV-damaged reporter gene and *in vitro* radionucleotide incorporation (Smith et al., 1995). Studies have found that the most effective inducers of p53 accumulation in cells are the cytotoxic antitumour drugs such as cisplatin (Fritsche et al., 1991). In this study, we examined the HCR of cisplatin-damaged Ad in LFS cells and found that both the p53-heterozygous and -hemizygous cells were not reduced in their reactivation of the virus compared to normal whereas the NER-deficient XP cells did show a greatly reduced HCR of the damaged virus (Figs. 1 and 2). NER has been shown to be an important pathway in the repair of cisplatin damage (Hansson et al., 1990; Sheibani et al., 1989) and previous studies have reported a reduced HCR for cisplatin-damaged DNA in XP cells (Poll et al., 1984; Hansson et al., 1991; Maynard et al., 1989; Bulmer et al., 1996). The normal HCR of cisplatin-damaged Ad for LFS cells reported here suggests that untreated LFS cells have normal excision repair of cisplatin lesions, but does not preclude the possibility that HCR of cisplatin-treated Ad may be deficient in cisplatin-treated cells (enhanced reactivation), which would indicate a deficiency in an induced repair pathway. The reduced HCR of UV-damaged plasmid in the colon carcinoma cells may arise from

the different p53 environment in these cells (two wt p53 alleles) compared to the LFS cells (one wt p53 allele).

Investigations of gene-specific repair and studies with cells from DNA-repair-deficient syndromes have suggested that p53's role in the repair of UV-induced DNA damage may be specifically linked to the preferential repair of actively transcribed regions of the genome. Repair of DHFR fragments in p53-heterogygous, UV-treated LFS fibroblasts, with a mutation at codon 245, was shown to be significantly reduced at 4, 8 and 24 hrs. after UV irradiation (Wang et al., 1995). Experiments conducted with XP and Cockayne syndrome (CS) cells have demonstrated that the nuclear accumulation of p53 is related to the repair of actively transcribed genes (Yamaizumi and Sugano, 1994). The minimum required dose (MRD) of UV irradiation necessary to induce p53 accumulation in XP-A cells is eightfold lower than that required for normal cells. For CS cells, which are deficient only in the preferential repair of active genes, the MRD is similarly low as for XP-A cells. In XP-C cells, which are deficient in global repair but have normal levels of preferential repair, the MRD is the same as for normal cells (Yamaizumi and Sugano, 1994). These results provide evidence that p53 accumulation is associated with DNA damage and may be particularly involved in eliciting a response to damage within active genes. The presence of mutant p53 in the heterozygous LFS cells can have a dominant negative effect on wt p53 and, therefore, may not permit a normal response to the UV damage leading to deficient DNA repair. Ford and Hanawalt (1995) have demonstrated that complete absence of wt p53 in 041 and 087 immortalized LFS cells decreased the repair of the nontranscribed strand of an active gene while the transcription-coupled repair (TCR) of the transcribed strand was normal. Cells harbouring mutant or no p53 genes have been shown to continue through G1 into S phase following DNA damage with no G1 arrest (Kastan et al., 1991). The extra repair time during cell cycle arrest for cells with wt p53 is not available to p53-mutant cells, therefore making repair of the transcribed strand of active genes a priority would be beneficial to cell survival.

We also investigated the capacity of cisplatin-treated cells to support Ad DNA synthesis. It is known that physical or chemical treatment of cells can decrease their ability to support viral infection (Rainbow, 1981; Defais et al., 1983). Unrepaired DNA damage in the host cell genes that are required for Ad replication would be expected to reduce the capacity of the cells to synthesize viral DNA. The capacity of UV-irradiated cells to support viral infection is reduced in several DNA-repair-deficient syndromes including CS and XP (Coppey et al., 1979; Coohill, 1981; Ryan and Rainbow, 1986; Rainbow, 1989). We have found that the two p53-heterozygous LFS fibroblast strains showed a reduced capacity to support Ad DNA replication that was similar to the level of reduced capacity seen in the repair-deficient XP strains (Figure 3). This is thought to result from the reduced repair of cisplatin-induced lesions in the transcriptionally active host genes of the Li-Fraumeni and XP cells. Therefore, our results suggest that a deficiency of normal p53 function, as present in the LFS fibroblasts, can affect the repair of cellular genes to a similar extent as that found in the NER-deficient XP fibroblasts. Our results also support the hypothesis that p53 is involved in the repair response of actively transcribed regions of the genome, often referred to as transcription-coupled repair (TCR).

Recent work in our lab using UV has led to similar observations for HCR and capacity experiments with LFS cells as those seen with cisplatin. The capacity of UV-damaged, p53-heterozygous LFS fibroblasts to support Ad DNA replication was reduced compared to normal UV-damaged fibroblasts. The p53-hemizygous LFS cells also had a reduced cellular capacity following UV-treatment (McKay, personal communication). These results suggest a deficiency in the repair of UV-induced lesions in the host cellular genes required for Ad DNA synthesis, as was observed for cisplatin-induced lesions. The HCR data for UV-treated Ad also lends support to the idea that p53 is involved in TCR. HCR of a β-galactosidase (lacZ) reporter gene expressed from a non-replicating adenoviral construct was enhanced by pretreatment of cells with either UV or heat shock in normal and XP-C fibroblasts but was absent in CS cells (McKay and Rainbow, 1996). XP-C cells have normal preferential repair of active genes while CS cells are deficient in

preferential repair, therefore, enhanced reactivation of reporter genes is dependent on repair of an active gene and is detected only in TCR-competent cells. It has been shown that UV-enhanced HCR of a UV-damaged β-gal reporter gene is reduced or absent in p53-deficient human tumour cells and that coinfection of these cells with a recombinant Ad vector expressing wt p53 results in enhanced HCR (Rainbow et al., 1995). This suggests that the inducible repair component of the TCR pathway involves p53. McKay et al. (1996) have reported that HCR of a UV-damaged β-gal reporter gene is normal in both p53-heterozygous and p53-hemizygous LFS cells; however, pretreating the cells with either UV or heat shock results in enhanced HCR only in the normal controls. The absence of the UV- and heat shock-enhanced HCR in the LFS cells suggests that they are deficient in an inducible DNA repair response which repairs the reporter gene. This supports a role for p53 in the transcription-coupled repair of active genes.

The use of adenovirus 5 in the HCR and capacity assays carried out here merits mention of the interactions between adenoviral early region E1A and E1B proteins and p53. E1A is known to induce the stabilization of the wt p53 protein, increasing its half-life by 5-10 fold in untransformed cells expressing the Ad5 E1A oncoprotein (Lowe and Ruley, 1993). It also increases cellular sensitivity to DNA-damaging agents like cisplatin, doxorubicin and γ-radiation (Sanchez-Prieto et al., 1995; Lowe et al., 1993b), and has been shown to promote cell death by apoptosis (Lowe and Ruley, 1993). Infection of cells with our Ad5(lacZ) viral construct introduces both E1A and E1B genes into the cells. E1B protein binds to p53 in transformed cells (Sarnow et al., 1982) as well as in Ad-infected cells (Braithwaite et al., 1991). It was shown to have no additional effect on p53 levels or half-life in cells expressing both E1A and E1B compared to E1A alone (Lowe and Ruley, 1993). The presence of the E1B protein in cells was also observed to inhibit the increase in E1A-associated apoptosis (Lowe and Ruley, 1993). Therefore, it is possible that the E1A protein introduced into the wt p53-expressing fibroblasts by Ad viral infection may be stabilizing the p53 protein in those cells but may also be sensitizing them to the cytotoxic effects of the cisplatin treatment. For the LFS cells with

altered p53 expression, the presence of E1A and E1B could produce different responses to cisplatin damage than those induced in cells with normal p53 status. Our results suggest that p53 status does affect the amount of Ad DNA that can be detected 72 hrs. following infection of the host cells (Table 2). The higher level of Ad DNA detected in the p53-hemizygous LFS cells may be the result of a more efficient viral infection of these cells due to the absence of wt p53. Since the presence of wt p53 can inhibit replication of SV40 DNA (Braithwaite et al., 1987), it follows that the lack of wt p53 could allow for ease of viral DNA replication in the Ad-infected LFS cells.

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SUMMARY

This study of the repair of cisplatin-damaged DNA in human cells has led to several observations. Preliminary work with RS+ cells showed that lymphoblastoid cell lines are suitable for use in HCR experiments by analysis of either viral DNA synthesis or expression of a β-gal reporter gene. This could prove a useful method of quickly assessing the drug sensitivity of human cells which could be used to better predict the tumour response of cancer patients to chemotherapy.

The previous observed cellular hypersensitivity of RS+ cells to DNA-damaging agents, particularly DNA-crosslinkers, had been suggested to possibly result from a DNA repair deficiency in these cells. Studies of the repair of cisplatin-damaged DNA in RS+ fibroblasts by use of HCR and capacity assays showed no deficiency in the ability of these cells to repair DNA damage. By comparison with NER-deficient XP and interstrand crosslink repair-deficient FA cells, it was determined that RS+ cells are not deficient in NER, nor in the repair of DNA interstrand crosslinks induced by cisplatin. These results suggest that abnormal DNA repair is not the cause of the RS+ mutagen hypersensitivity. However, our assays reflect repair only within the euchromatic regions of chromosomes, therefore, future work on the assessment of repair within the abnormal, decondensed heterochromatic regions of the RS+ chromosomes would be necessary to rule out a deficiency of DNA repair within those regions of the genome.

Work with XP fibroblasts showed a reduced HCR of cisplatin-damaged virus in these NER-deficient cells. Analysis of cells from complementation groups A, B, C, D, F and G revealed a deficiency in the ability to reactivate DNA-damaged virus within all these groups. These results implicate NER as an important pathway in the repair of cisplatin-damaged DNA. The cellular capacity of cisplatin-treated XP fibroblasts to support Ad replication was also significantly reduced compared to normal cells, suggesting a deficiency in the ability of the XP cells to repair transcriptionally active cellular genes.

Use of the HCR assay to assess repair in Li-Fraumeni syndrome cells revealed normal reactivation of cisplatin-damaged virus in both the p53-heterozygous and p53-hemizygous LFS cells studied. This suggests that bulk NER in these cells is not affected by p53 status. The p53-heterozygous LFS fibroblasts showed a reduced capacity to support Ad replication following cellular cisplatin damage, suggesting a role for p53 in the repair of actively transcribed cellular genes. These results support the mounting evidence that p53 is specifically involved in transcription-coupled DNA repair, and further reveal how mutations in the p53 tumour suppressor gene can lead to cancer.

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