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Recovery of RNA and DNA syntheses as an indicator of repair status in p53 deficient Li-Fraumeni syndrome fibroblasts

by

Randy D. C. Barley 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Masters of Science

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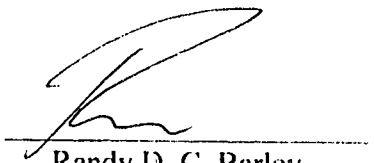

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Recovery of RNA and DNA syntheses as an indicator of repair status in p53 deficient Li-Fraumeni syndrome fibroblasts submitted by Randy D. C. Barley in partial fulfillment of the requirements for the degree of Masters of Science.

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DEDICATION

This thesis is dedicated to the memory of my grandfather, Corwyn Barley, who during the first half of my life provided me with the greatest role model a young man could have.

ABSTRACT

The multifunctional p53 protein is instrumental in maintaining the genomic stability of human cells, due in part to its ability to stimulate the repair of damaged DNA. The aim of this study was to elucidate the role of p53 in regulating nucleotide excision repair (NER), the primary enzymatic mechanism operative on UV-induced photoproducts in cultured human fibroblasts. To this end, recovery of DNA and RNA syntheses was initially measured in UV-treated noncancerous fibroblast strains from patients with Li-Fraumeni cancer family syndrome (LFS) who harbor germline mutations in p53. LFS strains recovered from inhibition of both DNA and RNA syntheses to an extent intermediate between NER-proficient (normal) strains and NER-deficient strains from patients inheriting the UV-hypersensitivity disorders xeroderma pigmentosum and Cockayne syndrome. In follow up studies, a representative p53 deficient strain from an LFS family proved defective in both NER subpathways which act on cyclobutyl pyrimidine dimers, namely, transcription-coupled repair and overall genome repair. It is thus concluded that normal p53 function is vital for proficient operation of the NER machinery in human cells.

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Last, but not least, I would like to thank to the ever-changing members of the tea club for the inspirational discussions (... I certainly will miss them).

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ABBREVIATIONS

A-T	ataxia-telangiectasia
BER	base excision repair
Bq	Becquerel
CDK	cyclin dependent kinase
Ci	Curie
CPD	cyclobutyl pyrimidine dimer
CPM	counts per minute
CS	Cockayne syndrome
dCTP	2'-deoxycytidine 5'-triphosphate
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
dThd	2'-deoxythymidine
EDTA	ethylenediaminetetracetic acid
ERCC	excision repair cross complementing
ESS	enzyme sensitive site
F	female
Gadd	growth arrest and DNA damage
GGR	global genome repair
Gy	gray
HBS	HEPES buffered saline
HCl	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-thanesulfonic
HPV	human papiloma virus
hr	hour
J	Joule
kb	kilobase
KCl	potassium chloride
kDa	kilodalton
l	liter
LFS	Li-Fraumeni syndrome
M	molar
M	male
MWC	molecular weight cutoff

N	normal
NaCl	sodium chloride
NaOH	sodium hydroxide
NER	nucleotide excision repair
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
pRb	retinobiastoma gene product
QMA	quaternary methylamine
RNA	ribonucleic acid
RNase	ribonuclease
RPA	replication protein A
RRS	recovery of RNA synthesis
SDS	sodium dodecyl sulfate
SSC	sodium chloride-sodium citrate
SV40	simian virus 40
TAE	tris-acetate-EDTA
TCA	trichloroacetic acid
TCR	transcription-coupled reapir
TE	tris-EDTA
TEV	T4 endonuclease V
TFIIH	transcription factor IIH
TRIS	tris(hydroxymethyl)aminomethane
TTD	trichothiodystrophy
UDS	unscheduled DNA synthesis
UV	ultraviolet
W	Watt
w/v	weight per unit volume
XP	xeroderma pigmentosum

INTRODUCTION

Historical perspective

Almost three decades ago Cleaver demonstrated that cells derived from patients with the sunlight-sensitive, cancer-prone disorder xeroderma pigmentosum (XP) display a marked intolerance to the cytotoxic effect of ultraviolet light which results from a defect in the repair of UV-induced DNA damage (Cleaver, 1968). This monumental discovery implicated environmental DNA damaging agents as key causative factors in carcinogenesis and, furthermore, suggested that normal enzymatic processing of damaged DNA may afford protection against the development of malignancy. Several hereditary disorders have now been uncovered in which cancer predisposition is associated with abnormal response to a variety of genotoxic agents. In one example, fibroblast strains from affected members of a kindred with the familial cancer syndrome of Li-Fraumeni (LFS) exhibited increased resistance to radiation cytotoxicity (Bech-Hansen, *et al.*, 1981), as opposed to the radiosensitivity typically displayed by cells from patients with the neurovascular disorder ataxia-telangiectasia (A-T) cells. These LFS strains (see pedigree in Figure 1) were subsequently reported to harbor germline mutations in the *p53* tumor suppressor gene, resulting in loss of wild-type p53 function.

The main thrust of the work presented in this thesis was to explore the DNA metabolic properties of fibroblast strains derived from individuals afflicted with LFS, including strains from members of the aforementioned family. Before describing the outcome of this study, the clinical and laboratory features of the UV-sensitive disorders XP and Cockayne syndrome (CS) are reviewed briefly. In addition, the human nucleotide excision repair (NER) pathway is outlined, and recent advances in elucidating the role of the p53 tumor suppressor protein in mediating cellular responses to DNA damage are presented.

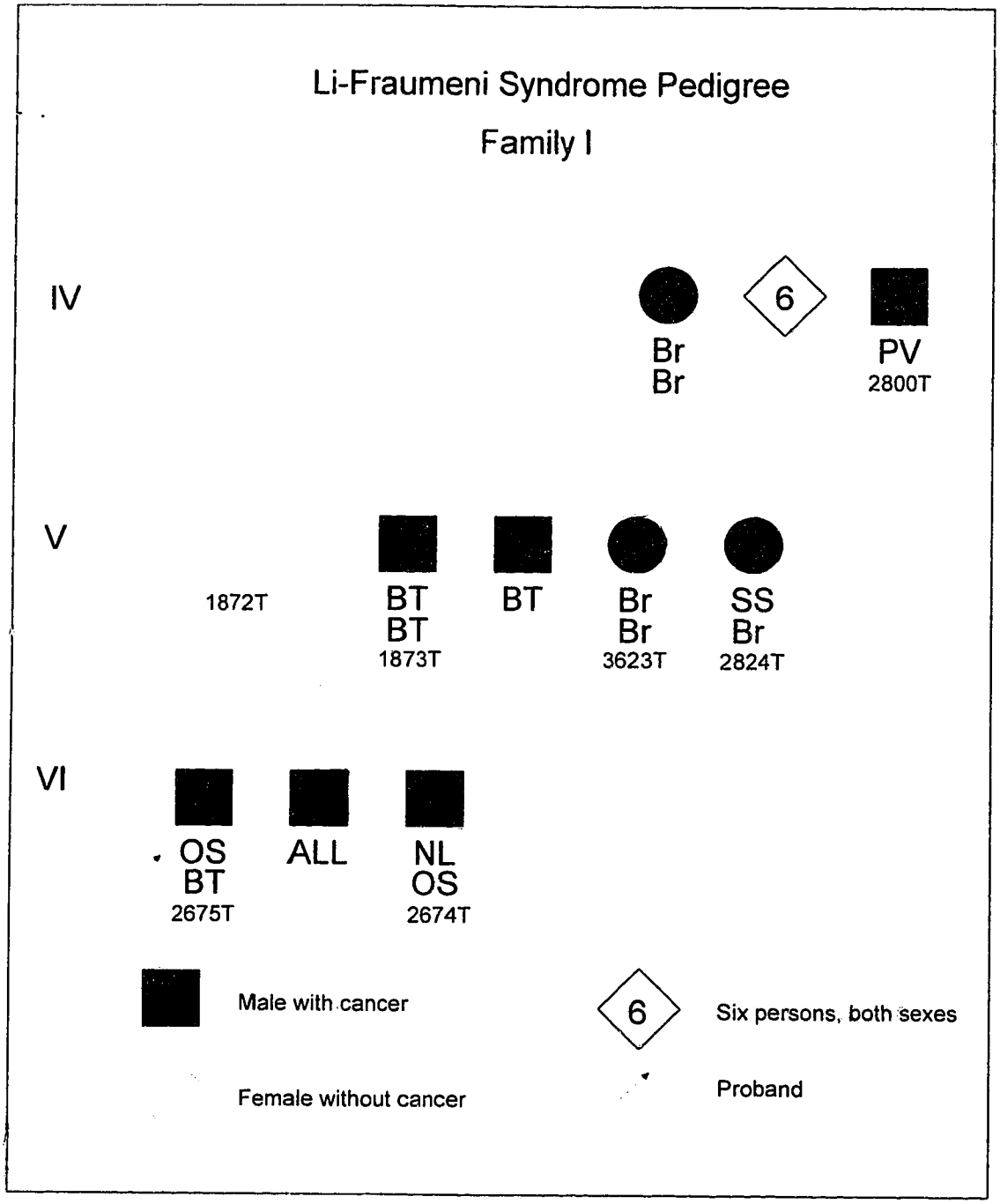

Figure 1. Abridged pedigree of an LFS family with diverse malignancies. The clinical status and cell strain for relevant family members are presented. Abbreviations used: OS, osteosarcoma; ALL, acute lymphoblastic leukemia; NL, neurilemoma; SS, soft tissue sarcoma; BT, brain tumor; Br, breast cancer; PV, polycythemia vera. Br, Br denotes bilateral breast cancer.

NER deficient syndromes

Much has been learned about the molecular basis of XP since the seminal discovery of Cleaver. The consequences of defective DNA repair are evident in the clinical symptoms displayed by the human repair deficiency disorders XP, CS and trichothiodystrophy (TTD). Individuals afflicted with any one of these rare autosomal diseases are extremely sensitive to the UV component of sunlight and frequently exhibit an array of neurologic and developmental abnormalities (reviewed in Bootsma and Hoeijmakers, 1994). Of the three sunlight-sensitive disorders mentioned above, XP is the only one which has an elevated risk for the development of cancer (approximately 2000-fold for squamous and basal cell carcinomas as well as melanomas).

There exists a considerable amount of clinical, genetic and biochemical heterogeneity within each of these repair deficiency syndromes (reviewed in Bootsma and Hoeijmakers, 1994). Cell fusion analyses have shown that XP, the most extensively studied of the three, is composed of seven subgroups designated XPA - XPG, and an eighth known as XP variant. The first seven subgroups are defective in NER, while the XP variant is believed to be deficient in post-replication repair. CS and TTD can each be subdivided into two distinct subgroups: CSA and CSB, and TTD-A and TTD-B, respectively. Interestingly, there are individuals who display clinical and biochemical features common to two or more of the syndromes. For example, CS patients have been reported who share some of the manifestations normally associated with XPB, XPD or XPG. Similarly, cases have been reported wherein TTD patients exhibit characteristics of either XPB or XPD.

i) Xeroderma pigmentosum

XP is an autosomal recessively inherited disease characterized by extreme sun sensitivity, photophobia, keratoses, erythema, ocular abnormalities, hyper- and

hypopigmentation and cancer proneness (reviewed in Kraemer 1994). XP occurs with a frequency of about one per one million live births. Approximately half of all patients with the disease have a history of acute sunburn consequent to minimal sun exposure. Intense freckling usually occurs by the age of two or three, and is restricted solely to sun-exposed regions of the body. A subset of XP patients display, in addition, an array of neurologic abnormalities, which can vary considerably in both severity and age of onset.

The photosensitivity exhibited by XP patients has long been attributed to a deficiency in UV light-induced unscheduled DNA synthesis (UDS), which is a general measure of the proficiency of a cell to repair all types of DNA damage (reviewed in Kraemer, 1994). UDS measures the combined action of all components involved in excision repair, including polymerases mediating the insertion of a repair patch. During the process of repair, the damaged DNA is removed and radiolabelled thymidine is incorporated into the repair patch. The amount of repair is analyzed following exposure to autoradiographic film and densitometry. With the exception of the XP variant, all remaining XP complementation groups are deficient to some degree in UDS following UV exposure. XPA, XPB and XPG show very low levels of UDS, while XPC, XPD, XPE, and XPF typically display UDS levels which range from 15%-50% of the levels seen in normal controls (Paterson, 1982).

In the early 1980's, new techniques were developed which permitted further dissection of the underlying molecular defects in repair. When one such technique, known as the gene specific repair assay (Bohr *et al.*, 1985), was employed in the study of the various XP groups, all but one of the groups were found to be defective in the repair of transcribed genes. The XP group which proved to be proficient in gene specific repair, now known as transcription-coupled repair, was XPC (Venema *et al.*, 1990a). This observation led to the

notion that DNA repair could be divided into two separate components: one which operates on lesions in transcriptionally active genes and the other which deals with damage in the rest of the genome (Table I).

ii) Cockayne syndrome

In 1936 Cockayne first described an autosomal recessive degenerative disease characterized by sun-sensitivity, cachectic dwarfism, deafness, pigmentary retinal degeneration, microcephaly and a milder form of hyperpigmentation than that typically observed in XP patients (reviewed in Kraemer, 1994). Unlike XP patients, individuals afflicted with CS do not exhibit elevated incidences of sun-related malignancies. In the late 1980's, armed with a plethora of new techniques and the knowledge gained from XP, the repair research community began to examine other sunlight-sensitive disorders. Cell strains, derived from patients afflicted with CS, were the next obvious choice, because several years earlier Mayne and colleagues (1982) had shown a strong correlation between survival and recovery of RNA synthesis (RRS) following UV exposure in these cell strains. It was soon discovered that CS cells lacked gene-specific repair (Venema *et al.*, 1990b) while maintaining normal global genome repair, which meant that these mutant cells possessed the exact opposite defect from XPC cells. With this observation, the relationship between UDS, gene-specific repair, global genome repair and recovery of RNA synthesis finally became clear. Cells deficient in gene-specific repair, such as those derived from CS individuals, owe their slower recovery of RNA synthesis compared to that seen in normal cells to the persistence of damage in transcriptionally active genes. Cells derived from XPC patients on the other hand, would be expected to have normal recovery of RNA synthesis, but impaired UDS ability, since their defect lies in the repair of the overall genome, not in the repair of transcribed genes.

Table I. Molecular properties of sunlight-sensitive human disorders.

Strain	Sunlight Sensitivity	Global Genome Repair: Unscheduled DNA Synthesis	Global Genome Repair: 6-4 Photoproduct Removal	Global Genome Repair: Dimer Removal	Transcription-Coupled Repair	RNA Synthesis Recovery
XP-A	++	<5%	defective	defective	defective	defective
XP-B	++	<10%	defective	defective	defective	defective
XP-C	+	15-30%	defective	defective	normal	normal
XP-D	++	15-50%	defective	defective	defective	defective
XP-E	+	>50%	defective	defective	defective	defective
XP-F	+	15-30%	defective	defective	defective	defective
XP-G	++	<10%	defective	defective	defective	defective
XP-V	+	normal	normal	normal	normal	normal
CS-B	+	normal	normal	normal	defective	defective
TTD-A	+	15%	defective	normal	defective	defective

Adapted from Hoeijmakers, 1993
+ sensitive, ++ extremely sensitive
XP-V (variant)

Nucleotide excision repair pathway

Each nucleus of a mammalian cell contains approximately 5 meters of DNA which is continuously subjected to endogenous as well as exogenous DNA damaging agents (Lehmann and Carr, 1994). The UV component of sunlight is one such exogenous agent and promotes dimerization of adjacent pyrimidines, which if unrepaired can lead to mutation, cell death, or neoplasia. Not surprisingly, therefore, cells faced with the daunting task of maintaining their genomic integrity in the face of deleterious agents possess a variety of enzymatic mechanisms to accomplish this, such as mismatch repair, recombinational repair, direct enzymatic reversal, base excision repair (BER) and nucleotide excision repair (NER). Of the various DNA repair mechanisms available to a mammalian cell, NER is perhaps the most extensively studied. Most of the insight gained into the molecular mechanisms of NER in eukaryotic cells has, over the past 30 years, been obtained through the study of repair deficient cell strains. Cells derived from patients with XP, CS, TTD as well as many yeast and rodent DNA repair mutants have greatly facilitated the study of repair. (for review, see Bootsma *et al.,* 1995). NER has recently been shown to involve at least 17 polypeptides in order to successfully complete the multi-step repair process. Seven of the seventeen polypeptides known to be essential to this process constitute proteins whose encoded genes are defective in XPA - XPG (Sancar, 1994).

NER relies on the redundant information in the DNA duplex to remove a lesion, and replace it using the complementary strand as a template. NER is a multi-step process which is capable of recognizing and eliminating a broad spectrum of structurally unrelated DNA lesions such as UV-induced photoproducts, bulky chemical adducts and even certain types of cross-links (Bootsma *et al.,* 1995). The five basic steps of NER are 1) damage recognition; 2) incision of the damaged strand; 3) excision of the damage-containing fragment; 4) resynthesis of the repair-generated gap; and finally, 5) ligation of the newly synthesized patch (Figure 2).

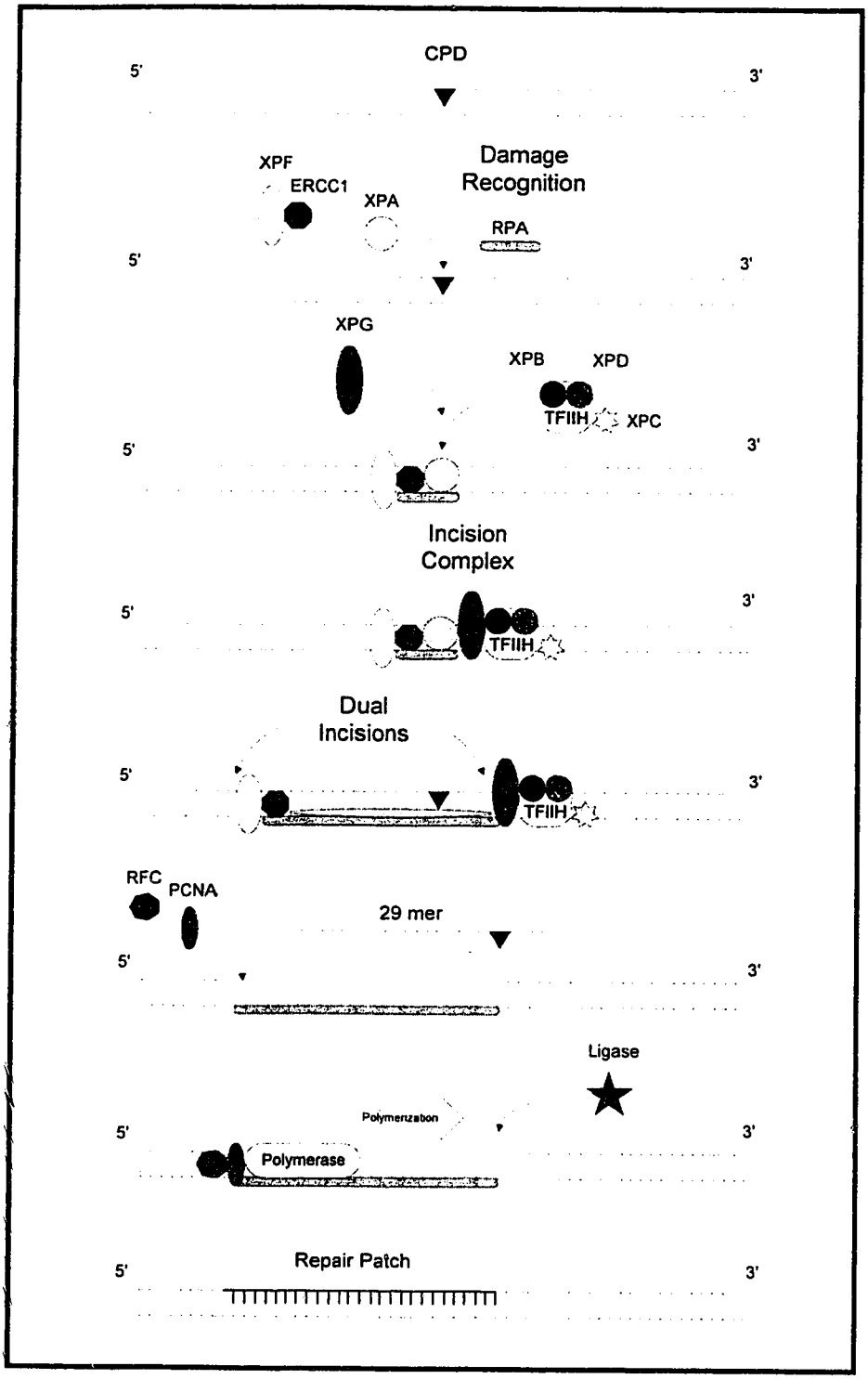

Figure 2. Schematic representation of the mammalian nucleotide excision repair pathway showing the 5 basic steps: damage recognition; incision; excision; resynthesis, and ligation. Adapted from Sancar, 1994 and Wood, 1995.

The NER reaction begins with the recognition of the damaged site which has been shown to be mediated by the XPA protein (Robins *et al.*, 1991) (Figure 2). This protein, in conjunction with RPA (replication protein A which is also involved in DNA replication) binds to the XPF and ERCC1 heterodimer (Park *et al.*, 1995) forming a complex which subsequently binds to the damage-containing site in the DNA. XPB and XPD are 3'=>5' and 5'=>3' helicases, respectively that form an integral part of the multi-subunit transcription factor TFIIH (Guzder *et al.*, 1994) which is recruited to the damaged site by XPA. The next polypeptides to join the preincision complex at the damaged site are the XPC and XPG proteins, which are either loosely associated with, or actively recruited by TFIIH (Mu *et al.*, 1995). With the preincision complex fully formed, TFIIH begins unwinding the helix to provide access for the excinucleases XPG (Habraken *et al.*, 1994) and XPF (Tomkinson *et al.*, 1993) which are responsible for creating the dual incisions. XPG makes the first incision 5 nucleotides 3' to the damaged site (Harrington *et al.*, 1994), while XPF makes the second incision 22 nucleotides 5' to the lesion (Bardwell *et al.*, 1994). Accordingly, the excision fragment is released leaving a gap of approximately 27 - 29 nucleotides, which is then filled by DNA polymerase delta or epsilon. In addition to the polymerase, gap closure requires the presence of PCNA (proliferating cell nuclear antigen) and perhaps the RFC replication factor (Nichols and Sancar, 1992). The repair process is completed when DNA ligase links the newly synthesized patch to the preexisting DNA, thus restoring the damaged site to normal structure and function.

The process of nucleotide excision repair is common to almost all organisms from *Escherichia coli* to elephants, as are the major subpathways of NER. It was discovered in 1985 (Bohr *et al.*) for mammalian cells, and in 1989 (Mellon and Hanawalt) for *E. coli* that transcribed genes are repaired more rapidly than the genome as a whole. This discovery led to the concept of intragenomic heterogeneity of repair. Subsequently, the intrastrand heterogeneity of repair was examined, and it too was found to favor the transcribed strand

over the non transcribed strand within a gene (Venema *et al.*, 1990a). Consequently, nucleotide excision repair can be divided into two subpathways: one which deals with the rapid and efficient removal of lesions that block ongoing transcription, known as transcription coupled repair (TCR), and the other which conducts the slower and less efficient repair of bulk DNA, including the repair of the non-transcribed strand of transcriptionally active genes, known as global genome repair (GGR) (Bootsma and Hoeijmakers, 1994).

Oncogenes vs. tumor suppressor genes

It has been known for many years that faulty regulation of cellular growth and differentiation can result in tumor development (reviewed in Malkin, 1993). Many inappropriately activated growth-potentiating genes, such as oncogenes, have been identified through the study of RNA tumor viruses and the transforming capacity of DNA obtained from malignant tissue specimens. In eukaryotic cells when certain mutations occur in one of the two alleles of a given oncogene, they behave in a dominant manner with respect to the wild-type allele. This is what is commonly referred to as a gain-of-function mutation, and in this case it will perpetually signal the cell to divide. More recently, another family of genes associated with tumorigenesis, known as tumor suppressor genes, have been discovered. Tumor suppressor genes, in contrast to oncogenes, are growth-inhibitory genes that, when mutated, behave in a recessive manner in comparison to the wild-type allele. When both alleles of a tumor suppressor gene are mutated, as is frequently the case in tumor tissue, a loss-of-function mutation results. Of all of the tumor suppressor genes, *p53* is undoubtably the most extensively studied.

i) *p53* tumor suppressor gene

The *p53* tumor suppressor gene is located on the short arm of chromosome 17 and encodes a 53 kDa nuclear phosphoprotein which functions as a negative regulator of cell

proliferation (reviewed in Malkin 1993). The gene is approximately 20 kb in length and consists of 11 exons, the first of which is noncoding. The gene encodes a 2.8 kb mRNA transcript which yields a protein comprised of 393 amino acids. The protein contains five evolutionarily conserved domains, four of which are encompassed by codons 129-146, 171-179, 234-260, 270-287 and the fifth which occurs in the noncoding region of exon 1 (Figure 3). In normal cells, p53 tends to be relatively unstable, with a half-life of 20-35 min. However, under certain circumstances, such as following genotoxic assault, the protein can be stabilized for up to 30 hours (Lu and Lane 1993). Although p53 is not essential for normal development in mice (Donehower et al., 1992), it does play a crucial role in cellular responses to DNA damage. Various workers have demonstrated that p53 is involved in the regulation of the G1-S cell cycle checkpoint (reviewed in Lehmann and Carr, 1994) and the induction of apoptosis in certain cell types (reviewed in Prokocimer and Rotter, 1994). More recently, three groups of workers (Hall *et al.*, 1993, Smith *et al.*, 1994 and Wang *et al.*, 1995) have suggested a direct role for p53 in the processing of DNA damage.

Cellular responses to DNA damage requiring p53 *(G1 arrest, apoptosis and repair)*

Cells harboring DNA damage rapidly increase p53 protein levels by a post-translational protein stabilization mechanism occurring in the C-terminal domain. Induction of p53 results in the immediate transcriptional activation of a set of p53-dependent genes, including *WAF-1* (*p21*) (Xiong *et al.*, 1993), *GADD45* (Kastan *et al.*, 1992), *ERCC3* (Wang *et al.*, 1994), *MDM2* (Chen *et al.*, 1994), *BAX* (Miyashita *et al.*, 1994), and *cyclin G* (Okamoto *et al.*, 1994). This transcriptional activation by p53 is an absolute requirement for both G1 arrest (Pietenpol *et al.*, 1994) and apoptosis (Lowe *et al.*, 1993). When cells are confronted with DNA damage, they may respond by following one of two pathways. They either arrest in G1 or G2, presumably to permit repair of damaged DNA prior to replication or mitosis respectively or they undergo numerous cellular changes as they proceed down an apoptotic pathway presumably to eliminate cells carrying a heavy genotoxic load. Various

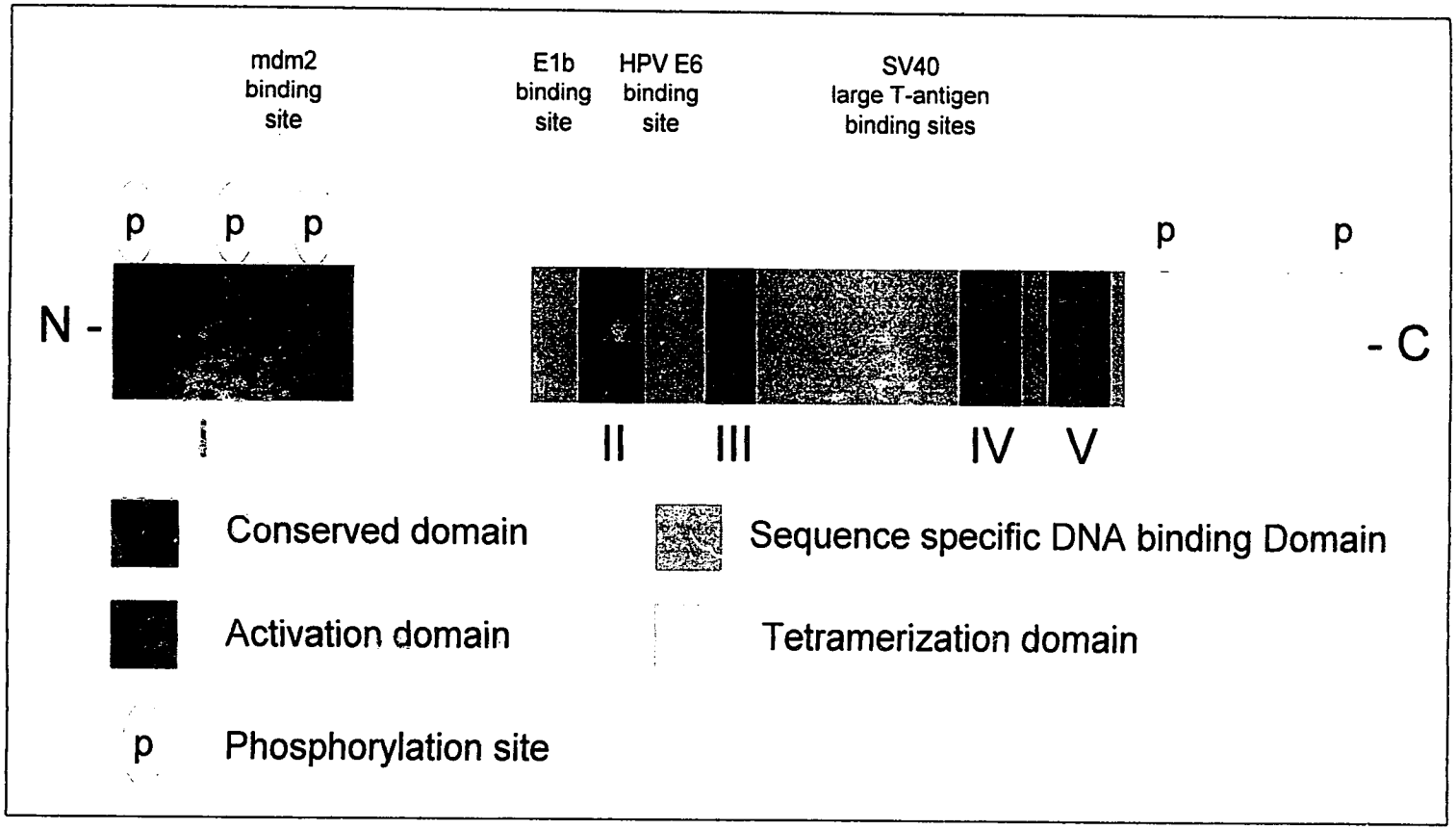

Figure 3: Schematic diagram of the 393 amino acid p53 tumor suppressor protein illustrating: the five highly conserved domains throughout the vertebrate kingdom, the five phosphorylation sites, the C-terminal tetramerization domain, the DNA binding domain which is essential for transcriptional activation, and the protein activation domain. Also included are the SV40 large T-antigen binding sites, the human papillomavirus HPV-E6 binding site, the adenovirus E1b binding site and the mdm2 binding site. Highly conserved domains I toV are encompassed by codons 13-19, 129-146, 171-179, 234-260, and 270-287 respectively. The most commonly mutated amino acids in p53 occur at codons 175, 245 and 248. Adapted from Malkin, 1994.

models have been proposed to explain this cellular decision-making process. The pathway that a cell ultimately selects is dependent on a number of factors such as the level and persistence of DNA damage, cell type and physiological state. Although the actual switch between them may be poorly understood at present, both processes have been extensively studied.

G1 arrest is believed to be mediated by the p53 transcriptional activation of *WAF-1*. The *WAF-1* gene product, p21, has recently been shown to be a universal inhibitor of cyclin kinases (Xiong *et al.*, 1993). This protein exerts its influence on the cell cycle in part by inhibiting the cyclin E-CDK complex, which normally phosphorylates pRb (the retinoblastoma gene product) in G1/S of the cell cycle (Dulic *et al.*, 1994). Hypophosphorylated pRb has been reported to complex tightly with E2F and thereby block its transcriptional activity which is necessary for progression into S phase (Cox and Lane, 1995).

i) G1 arrest following DNA damage

If the objective of G1 arrest is to allow the cell time to repair DNA damage, one might predict that several other cellular processes might be simultaneously coordinated to achieve this end. Recently, several lines of evidence supporting this hypothesis have emerged. In addition to acting as a transcriptional activator for various genes whose products are involved in repair such as *ERCC3* (Wang *et al.*, 1994) and *GADD45*, p53 is also known to directly interact with PCNA, RPA, and TFIIH which also plays a role in repair (Figure 4).

Possible connections between p53 and DNA repair

There currently exist at least three possible mechanisms by which p53 could affect DNA repair in a direct manner. A detailed description of these three mechanisms, which are not mutually exclusive, follows.

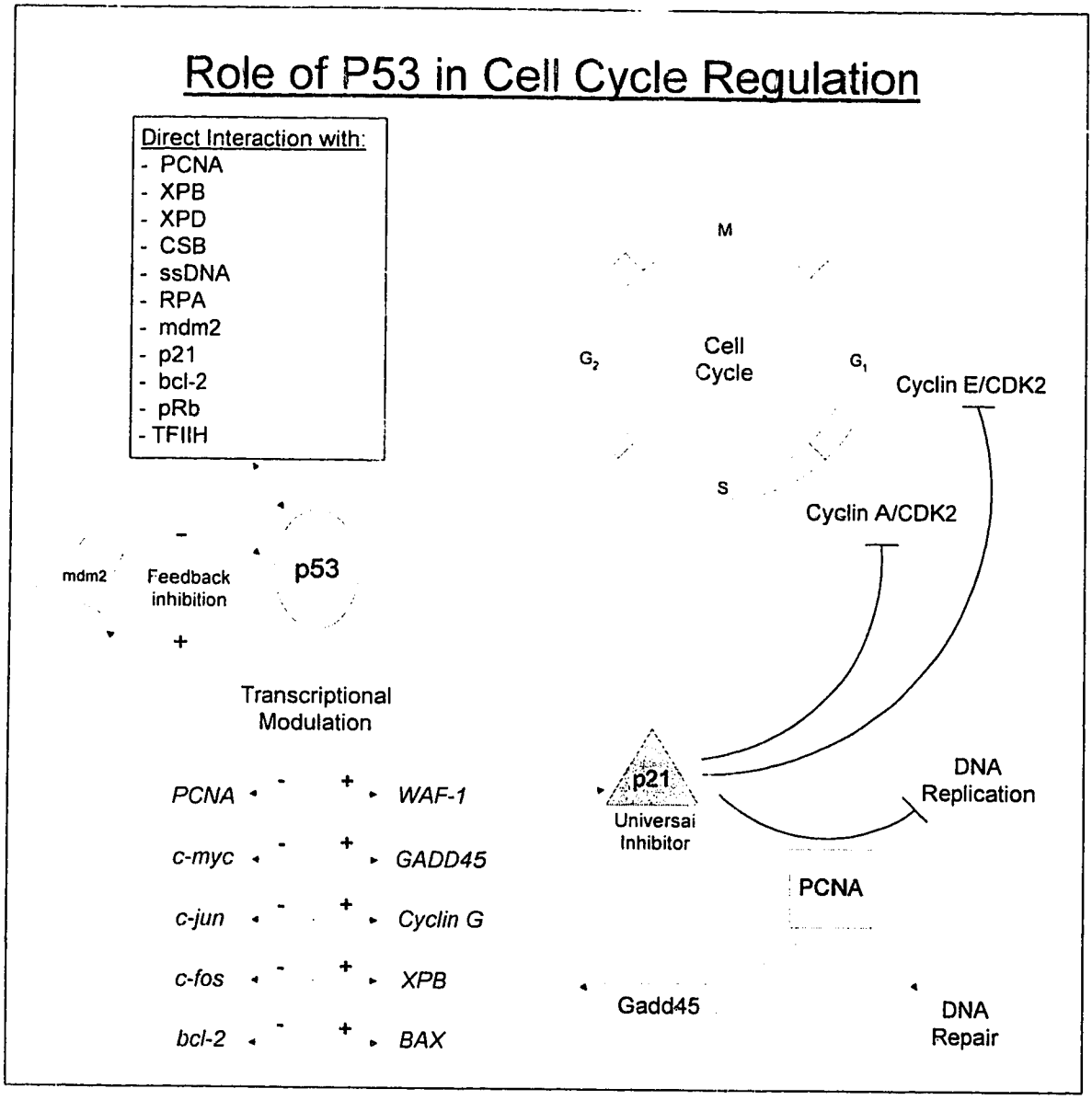

Figure 4. Schematic diagram illustrating the role of p53 in cell cycle arrest, apoptosis and DNA repair. The p53 tumor suppressor protein exerts its effect on these cellular processes by two different mechanisms: transcriptional activation/repression (denoted +/-) and direct protein-protein interaction. The p53 protein has recently been shown to transcriptionally potentiate a variety of genes believed to regulate cell cycle arrest, apoptosis and DNA repair (see body of text for details). The box (upper left) contains a partial list of proteins that p53 has been shown to bind to either *in vitro* or *in vivo*. T denotes inhibition.

i) Mechanism I (PCNA)

PCNA was first identified as a key component of the DNA replication machinery, when it was shown to be required for the *in vitro* replication of SV40 DNA (Prelich *et al.*, 1987). Shortly thereafter, PCNA was discovered to be an auxiliary factor of the mammalian DNA polymerase δ (and later polymerase ε) during semi-conservative replication (Bravo *et al.*, 1987). More recently, Shivji *et al.* (1992) and Nichols *et al.* (1992) have demonstrated that PCNA participates in the gap-filling reaction in NER. Two other recent discoveries are paramount in implicating p53 in the DNA repair process. The first observation further linking p53 and DNA repair was the finding by Hall *et al.* (1993) that PCNA contains a p53 binding site, thus possibly linking the tumor suppressor protein with the repair resynthesis step. Other polypeptides involved in repair synthesis have also been shown to interact with p53 protein [RFC and RPA (Dutta *et al.*, 1993)] (Figure 4). The second observation, by Flores-Rozas *et al.*, (1994), showed that p21 efficiently inhibits the synthesis of long stretches of DNA, while permitting the synthesis of short (repair patch-like) stretches. P21 is thought to mediate the inhibition of DNA synthesis through its interaction with PCNA (Waga *et al.*, 1994). This should not be merely regarded as negative regulation of semi-conservative DNA synthesis following genotoxic assault, but also as positive regulation of repair. Taken together, these findings strongly suggest a direct role for p53 in NER.

ii) Mechanism II (GADD45)

Within the last decade a family of genes, known as GADD (growth arrest and DNA damage inducible), were isolated on the basis of their rapid induction following the occurrence of DNA damage. *GADD45* is of particular interest not simply because its expression has been detected in numerous mammalian cell types, but more specifically because its expression following DNA damage was shown to be p53-dependent (Kastan *et al.*, 1992). The recent observation by Smith *et al.* (1994) and Hall *et al.* (1995) that Gadd45 interacts with PCNA raises the possibility that it too may directly mediate DNA repair

through one of the PCNA-dependent polymerases, δ or ε. An argument could be made that the two mechanisms discussed thus far both relate to the post-excision, gap-filling step, which perhaps has little relevance to the presumed rate-limiting steps in NER, namely, recognition and excision of the damage. This argument is not without its own shortcomings because Cleaver (1987) observed that the inhibition of PCNA-dependent polymerases, in addition to blocking repair synthesis, also adversely affected the excision of various types of bulky lesions from DNA. This clearly ties the resynthesis machinery to some early preincision step of the NER pathway.

iii) Mechanism III (TFIIH)

In the past there has been considerable speculation regarding the involvement of transcription factors in the process of nucleotide excision repair. It was not until recently, however, that Svejstrup *et al.*, (1995) demonstrated the existence of two separate forms of the transcription initiation factor TFIIH: one form which is involved in transcription (holo-TFIIH) and another form which is involved in NER (core-TFIIH). The holo-TFIIH differs from the core form of the complex in possessing four additional peptides, which have been shown to be required for the transcriptional activity. Of the five peptides which comprise the core-TFIIH, two of them (XPB and XPD) were initially discovered as helicases involved in NER. These and similar findings by Drapkin and coworkers (1994) provide concrete evidence for the dual role of TFIIH in both transcription and repair. An exciting observation by Xiao and colleagues (1994) showing an interaction between the activation domain of TFIIH and p53 was the first evidence suggesting that the tumor suppressor protein might be involved in a preincision step of NER. During the period of the present study, another report appeared showing an interaction between p53 and various subunits of the core-TFIIH such as XPB, XPD, and CSB (Wang *et al.*, 1995), This latter observation provides further evidence in support of the notion that p53 might be involved in some preincision step of the NER pathway.

Li-Fraumeni Syndrome

Inactivating mutations of the *p53* gene are the most common genetic alterations observed in human cancers, and have been associated with virtually all sporadically occurring malignancies (Levine *et al.*, 1991). These include soft tissue sarcomas, osteosarcomas, leukemias, brain tumors, and carcinomas of the lung and breast (Malkin, 1993). Together, these tumors account for more than half of the cancers occurring in Li-Fraumeni syndrome (LFS) families (Garber *et al.*, 1991). LFS, first described by Fredrick Li and Joseph Fraumeni, Jr. in 1969, is inherited as a rare autosomal dominant trait and is characterized by the childhood occurrence of a variety of cancers. Due to the spectrum of cancers observed in LFS and what was known about other hereditary cancer syndromes, the *p53* gene was a likely candidate. In 1990, Malkin and coworkers reported that basepair mutations in the *p53* gene were present in the germline of individuals from all five LFS families studied. LFS patients have since been found who harbor no detectable germline mutation in their *p53* gene. They likely do lack some component, either upstream or downstream, in the p53 signal transduction pathway (reviewed in Malkin, 1994). Cells derived from patients afflicted with LFS provide an excellent experimental system in which to study the involvement of p53 in DNA repair, but the mutation spectrum observed in different LFS families must always be taken into consideration. One LFS family, for example, harbors a germline mutation at codon 245, which causes the mutant allele to behave in a dominant negative manner with respect to the wild-type allele.

As alluded to earlier, not all LFS families share the same germline mutation. However, there do appear to be some *p53* codons which are more frequently mutated than others in LFS. The most commonly mutated codons are 175, 245 and 248 which, as one might speculate, correlate well with hot-spots for mutation in the *p53* gene. In 1994, Tornaletti and coworkers discovered that there is considerable heterogeneity of repair within the transcribed sequence of the *p53* gene. These workers further demonstrated that the

regions of the transcribed strand, corresponding to the mutational hot-spots, were repaired less efficiently than in the remainder of the strand.

In the initial phase of the work presented in this thesis, the DNA metabolic properties of non-cancerous fibroblast strains derived from one or more representative members of three unrelated LFS families are elucidated. These LFS strains, like CS strains, have been found to be deficient in the recovery of both DNA and RNA syntheses following UV exposure (Mirzayans *et al.*, 1996). This observation led to the examination of the rate of repair of cyclobutyl pyrimidine dimers in the genome as a whole as well as within the transcriptionally active *c-myc* gene. In a representative LFS strain, both global and gene-specific repair were reduced in rate to an extent comparable to that observed in certain XP strains. Taken together, these findings support the observations discussed above that the p53 protein functions in GGR as well as TCR.

MATERIALS AND METHODS

Cells and their cultivation

Pertinent characteristics of the fibroblast strains employed in this study are given in Table II. Unless otherwise stated, all cells were cultured at 37°C in Ham's F12 medium supplemented with 10% (v/v) fetal calf serum (Sigma), 1 mM glutamine (Gibco BRL), and 100 units/ml penicillin G sodium/100 ug/ml streptomycin sulfate (Gibco BRL) in a humidified atmosphere of 5% CO_2 in air.

Ultraviolet irradiation

Cells were exposed to a bank of two 15 watt (low-pressure mercury vapor) germicidal lamps (model GE 15T8, General Electric, Toronto, ON) which emitted 97% of their radiant energy at a wavelength of 254 nm. The fluence rate of the UV source for the duration of this period of experimentation was 1.1 W/m^2. The UV fluences administered to cells ranged between 5 and 20 J/m^2 as indicated.

Preparation of T4 endonuclease V for dimer detection

i) Bacterial culture and cell lysis

E. coli containing the expression vector pTac-denV was a gift from Dr. J. K. de Riel of Temple University. A 24-hour culture was used to inoculate 2 x 1-liter flasks of LB broth containing 50 ug/ml ampicillin (Sigma). Cultures were grown in a shaking incubator at 37°C until a cell density of 1 x 10^8 cells/ml was attained, whereupon IPTG (Gibco-BRL) was added to a final concentration of 1 mM. The cultures were incubated for an additional 8 hours to allow sufficient induction of T4 endonuclease V (TEV). Cells were harvested by centrifugation in a Sorvall GS3 rotor driven by a Sorvall centrifuge model RC-5C at 5000 rpm for 15 minutes at 4°C. From this point on, all procedures were carried out in a cold room and all instruments and solutions were prechilled to 4°C. The supernatant was

discarded and the pellet was washed in sucrose-free lysis buffer (50 mM Tris pH 8.0-1 mM EDTA-100 mM NaCl-10 mM β-mercaptoethanol-10% sucrose) followed by resuspension of the pellet in 30 mls of lysis buffer. Lysozyme (Sigma) was added to a concentration of 0.5 mg/ml, and the mixture was incubated in an ice bath for 30 min. Lysis was achieved by sonication of the cell suspension on ice with a Branson Sonifier 200 for 15 minutes at a microtip setting of #5 with 10% pulsed action. Cellular debris was removed by centrifugation in a Sorvall GSA rotor for 20 minutes at 10 000 rpm. The supernatant was collected and proteins were precipitated by the addition of 60% (final volume) ice-cold acetone (Anachemia). Acetone was added in a stepwise manner with constant stirring. The protein mixture was centrifuged in a GSA rotor for 35 minutes at 10 000 rpm. The supernatant was discarded and the pellet was resuspended in 30 ml buffer A (50 mM Tris, pH 7.4-2 mM EDTA-300 mM NaCl-2 mM β-mercaptoethanol-1% glycerol). Unless otherwise stated, all solutions were prepared with reagents purchased from Sigma Chemical Company.

ii) Chromatography

All chromatography was performed using the Pharmacia-LKB GradiFrac protein station. The protein suspension was gravity fed through a 60 ml QMA (Waters-Millipore) column that had been pre-equilibrated with buffer A. The column was subsequently washed with an additional 30 mls of buffer A and all the flow-through was collected. This flow-through was then applied to a 30-ml Blue Sepharose (Pharmacia) column at a rate of 0.5 ml/min, which had also been equilibrated with buffer A. The column was washed with an additional 30 mls of buffer A to ensure that all unbound proteins had been removed. Proteins were eluted from the Blue Sepharose column by developing a linear gradient from 0.3-3.0 M NaCl in buffer A. The elution peaks were monitored using an optical unit (set at 280 nm) and a chart recorder attached to the GradiFrac, while the protein activity was measured using the pBR322 gel-based assay described below. The fractions corresponding to the TEV peak

Table II. Characteristics of human fibroblast strains studied.

Fibroblast Strain	Clinical Description	Donor			*in vitro* Age	p53 Gene	
		Age	Sex	Family		Mutant Codon	Exon
GM38	NORMAL	9	F	-	16-22	-	-
GM43	NORMAL	32	F	-	15-23	-	-
CRL1170	XPC	27	F	-	9-18	-	-
GM1856	CSA	13	M	-	9-22	-	-
GM739	CSB	3	F	-	10-20	-	-
2675T	LFS	16	M	I	17-22	245	7
2800T	LFS	71	M	I	15-23	234*	7
3546T	LFS	n/a	n/a	I	10-16	245	7
3649T	LFS	n/a	F	II	11-17	unknown	unknown
1860T	LFS	n/a	M	III	10-16	unknown	unknown

* Non-germline mutation

NCI family designation (Family I, #0165; Family II, #0101; Family III, #0221)

were collected and pooled. These fractions were then concentrated using 10 K Centricons (Amicon) according to the manufacturer's directions. The concentrated samples were dialyzed in 12 000 - 14 000 MWC dialysis bags (Spectra/Por) against buffer B (50 mM potassium phosphate buffer, pH 6.5-1mM β-mercaptoethanol-2% glycerol) for 8 hours with buffer changes every hour. The dialyzed samples were then applied at a rate of 0.5 ml/min to a 40-ml CM sephadex column (Waters-Millipore) that had been pre-equilibrated with buffer B. The column was then washed with 40 mls of buffer B and the fractions containing TEV were eluted by developing a gradient from 0 to 0.5 M KCl. Once again Centricon 10 centrifugal concentrators were used to concentrate the fractions corresponding to the predominant peak. To remove a minor contaminating nuclease from the enzyme preparation, the sample was run through a 100 ml Sephacryl 100 HR (Pharmacia) column at a rate not exceeding 0.3 ml/min. Before injection onto the column, the sample was dialyzed against buffer C (100 mM potassium phosphate, pH 6.5-300 mM KCl-5 mM EDTA-5 mM β-mercaptoethanol-5% glycerol) which was also used to pre-equilibrate the column. Finally, the sample was concentrated to a final volume of less than two mls.

iii) Verification of enzyme purity and determination of specific activity

An aliquot of the TEV preparation was electrophoresed beside both a low-range molecular weight (MW) prestained SDS-PAGE ladder and a broad-range MW non-prestained ladder (Bio-Rad) on a denaturing 15% SDS PAGE gel in a Bio-Rad mini-gel apparatus for 2 hours at 90 volts. Gels were silver stained to enhance detection of any contaminating bands. Once the purity had been assessed, both the specific and non-specific activities of the preparation were measured. To this end, a gel-based assay was developed which monitored the change in conformation of UV-irradiated plasmid DNA from the supercoiled to the nicked circular state. Supercoiled pBR322 (Pharmacia) DNA at a concentration of 15 ug/ml was exposed in 12-ul droplets to a total fluence of 20 J/m^2. This fluence was selected because it introduced, on average, one cyclobutane pyrimidine dimer

per plasmid molecule. Irradiated and non-irradiated aliquots of DNA were then incubated with 1 ul of the TEV preparation in 1x TEV reaction buffer for 15 min at 37°C. The negative control consisted of an aliquot of DNA to which TEV buffer C and 1x reaction buffer were added. After adding loading buffer, the samples were electrophoresed in a mid-sized BRL submerged gel electrophoresis apparatus for 2 hours at 70 volts on a 0.7 % agarose TAE gel. Agarose gels were stained with Cyber Green (Molecular Probes) for 40 minutes and the DNA was visualized by using a transilluminator emitting primarily 254 nm light. The bands were photographed using an MP-4 camera system and polaroid 567 print/negative film. The negative from this photograph was then used for densitometric analysis. A ratio of supercoiled-to-nicked circular plasmid of 1:2.6 indicated that the reaction had gone to completion. One unit of TEV was sufficient to cleave all dimer-containing sites in 10 ug of UV-irradiated (10 J/m^2) genomic DNA when incubated for 30 min at 37°C. One unit of TEV contained 2.5 ng of protein based on the Bradford protein assay.

Assay of DNA synthesis recovery

Cellular DNA was labelled by incubating exponentially growing cultures for 24 hrs in medium containing 2 x 10^9 Bq per mmol [methyl-^{14}C] dThd at 3.7x 10^2 Bq per ml (New England Canada, Lachine, Quebec) (Figure 5). Prelabelled cultures were seeded in 60-mm dishes at a density of about 1 x 10^5 cells/plate and incubated at 37°C for a minimum of 16 hours in dThd-free medium. After removal of the medium and rinsing with PBS, the cultures were irradiated with 254 nm UV light. Both UV-treated and sham-treated (control) cultures were incubated in non-radioactive dThd-free medium for various times (eg. 5 hrs), and then pulse-labelled for 40 mins in medium containing 3 x 10^{12} Bq per mmol ^{3}H-dThd at 3.7 x 10^5 Bq/ml. Cells were lysed on the plates using 0.02 M NaOH, and the trichloroacetic acid-insoluble radioactivity in the lysates was determined according to standard procedures (Sambrook *et al.*, 1989).

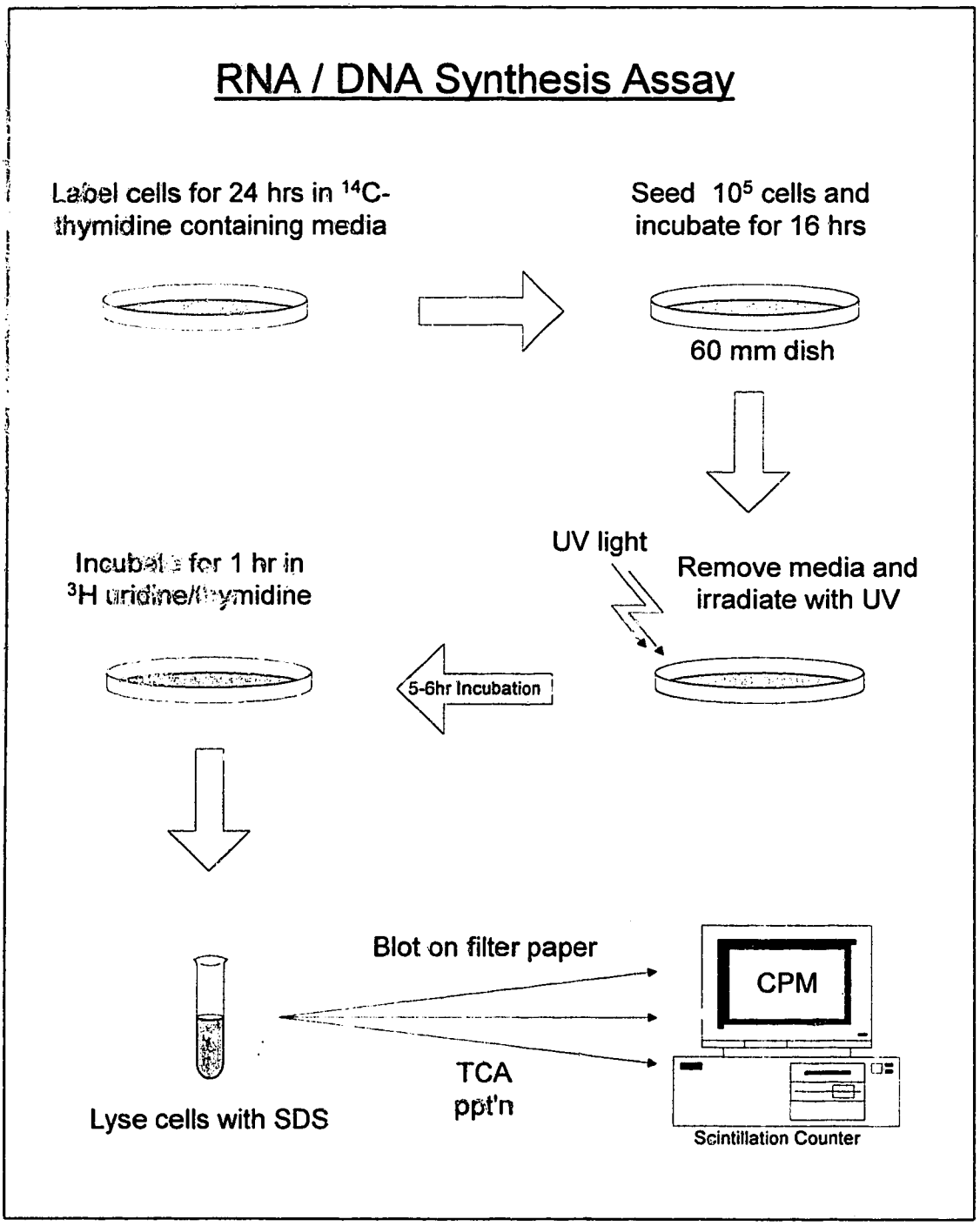

Figure 5. Illustration of the technique employed for measuring the recovery of nucleic acid synthesis post-UV irradiation in normal and LFS fibroblast strains. See text for details.

The amount of DNA synthesis, expressed as a percentage of that occurring in the untreated controls, was calculated using the following formula:

$$[(\text{cpm } ^3\text{H/cpm } ^{14}\text{C})_{\text{treated}} - (\text{cpm } ^3\text{H/cpm } ^{14}\text{C})_{\text{control}}] \times 100.$$

Assay of RNA synthesis recovery

Cellular DNA was labelled by incubating exponentially growing cultures for 24 hrs in medium containing 2 x 10^9 Bq per mmol [methyl-^{14}C] dThd at 3.7x 10^2Bq per ml (New England Canada, Lachine, Quebec) (Figure 5). Prelabelled cultures were seeded in 60-mm dishes at a density of about 1 x 10^5 cells /plate and incubated at 37°C for a minimum of 16 hrs in dThd-free medium. After removal of the medium and rinsing with PBS, the cultures were irradiated with either 5 or 10 J/m^2 UV light. The UV-treated and sham-treated (control) cultures were incubated in non radioactive dThd-free medium for various times (ie. 6 hrs), and then pulse-labelled for 60 minutes in medium containing 1.37 x 10^{12} Bq per mmol ^{3}H-Urd at 1.8 x 10^5 Bq/ml. SDS was used for lysis while radiometric analysis was performed as outlined above for the measurement of recovery of DNA synthesis.

Analysis of global DNA repair by alkaline sucrose sedimentation

For this assay cellular DNA was labelled by incubating exponentially growing cultures for 24 hrs in medium containing 2.4 x 10^{11} Bq (i.e. 6.7 uCi) per mmol [methyl-^{3}H] dThd at 1.8 x 10^4Bq (i.e. 0.5 uCi) per ml (New England Canada, Lachine, Quebec). Prelabelled cultures were seeded in 60-mm dishes at a density of about 1 x 10^5 cells /plate and incubated at 37°C for a minimum of 16 hours in dThd-free medium. Cells were then rinsed twice with prewarmed (37°C) phosphate-buffered saline (PBS), irradiated with 254 nm ultraviolet light and incubated in complete medium for various intervals to permit DNA repair (eg. 6 hours). Following incubation, cells were again washed twice with PBS and subjected to a brief saponin treatment (0.25 % saponin in HBS, pH 7.4) in order to achieve

cell permeabilization (Mirzayans et al., 1994). The resulting permeabilized cells on each plate were then washed repeatedly with 1x TEV reaction buffer. Cells were harvested by gentle scraping with a rubber policeman, and collected in 2 ml Eppendorf microfuge tubes; corresponding ^{3}H-labelled (treatment) and ^{14}C-labelled (control) cells were collected in the same tube. Excess TEV was added and the tubes were incubated for 30 min at 37°C. Following this incubation, 150-ul aliquots of the reaction mixture were combined with 100 ul of lysis solution (1 M NaOH 0.1 M EDTA) and carefully layered on top of 6-ml gradients. The gradients were 5-20 % (w/v) sucrose in 2 M sodium chloride-0.3 M sodium hydroxide-10 mM EDTA (pH 12.5). After holding for 1 hour (to allow denaturation of native DNA), the gradients were placed in a Sorvall TST-60.4 rotor and centrifuged at 30 000 rpm for 3 hours in a Sorvall RC70 ultracentrifuge. Once centrifugation was complete, gradients were fractionated into about 22 seven-drop fractions using a P-1 peristaltic pump (Pharmacia) and the TCA-insoluble radioactivity in each fraction was determined according to the methods in Sambrook et al (1989). These values were then used to generate gradient profiles that yielded insight into the relative sizes of DNA molecules produced under different treatment conditions (Figure 6).

Analysis of DNA damage and repair in defined genes

i) DNA isolation and restriction enzyme treatment

For the measurement of gene-specific repair, non-radiolabelled fibroblasts were seeded into 150-mm petri plates at a density of ~ 1 x 10^6 cells/dish and incubated at 37° for 48 hours. Cells were then rinsed twice with prewarmed PBS, irradiated at a dose of 5 J/m^2 and either incubated in dThd-free media containing hydroxyurea for 6 hours to allow repair or harvested immediately to serve as controls. Hydroxyurea (HU), an inhibitor of ribonucleotide reductase (Timson, 1975), was added to a concentration of 2 mM to inhibit replicative DNA synthesis. Cells were harvested by the addition of 1 ml of lysis solution (10 mM Tris-0.5% SDS-1 mM EDTA) per plate. Proteinase K (Sigma) was added to the lysate at

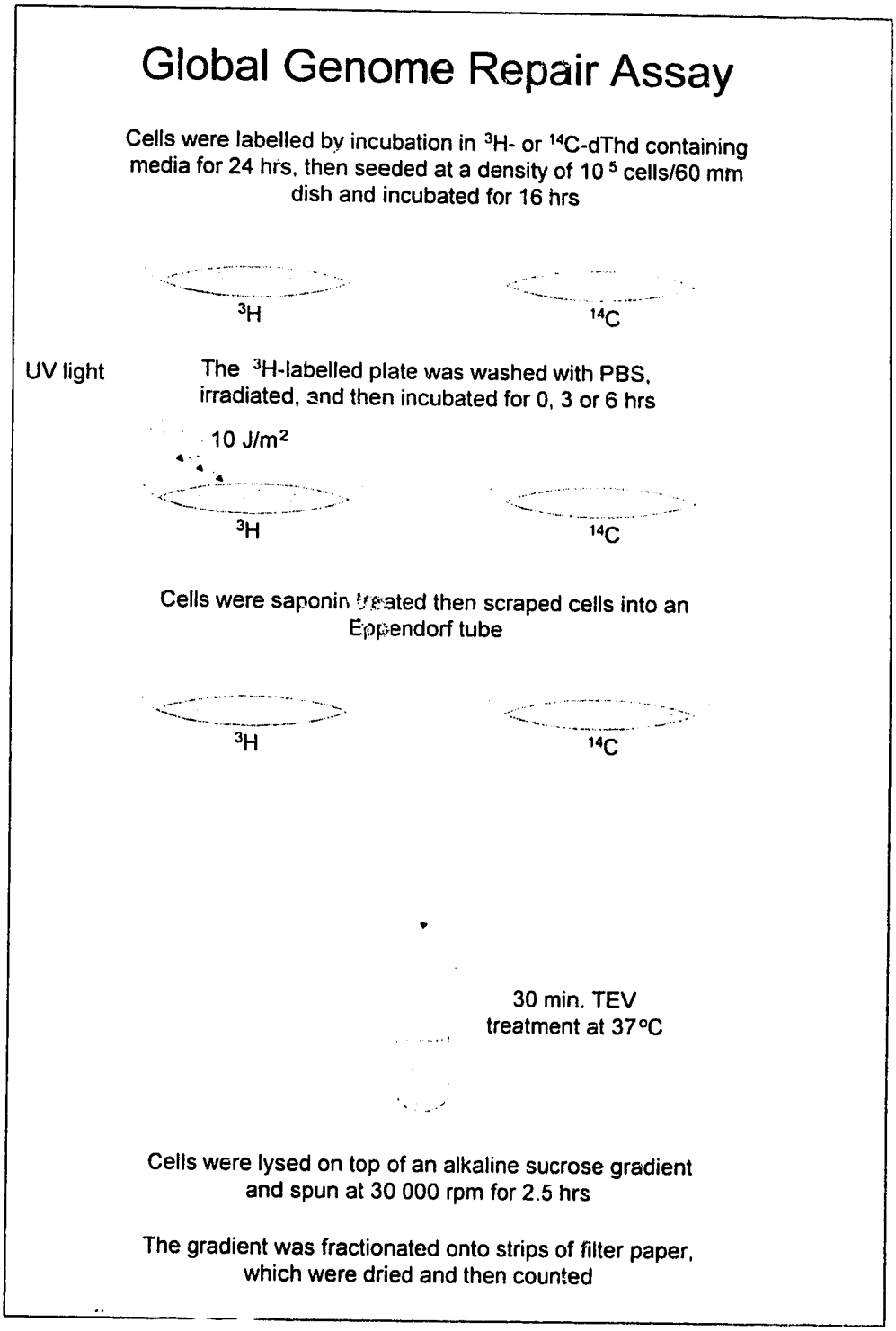

Figure 6. Illustration of the technique employed for the study of global genome repair in UV-irradiated normal and LFS fibroblasts (see text for details).

a concentration of 0.1 mg/ml and incubated for 12-16 hours at 37°C. High molecular weight DNA was then extracted by using sequentially Tris-buffered phenol pH 8.0, phenol-chloroform (1:1), and finally chloroform-isoamyl alcohol (24:1), all in a 1:1 ratio with the lysate solution. Nucleic acids were precipitated by the addition of 1/10 volume of 2.5 M sodium acetate (pH 5.2) and 2 volumes of ice-cold ethanol (100%). Once precipitated, the pellet was washed with 70% ethanol and resuspended in TE pH 8.0. RNase A (Sigma) was added to a concentration of 50 ug/ml and the mixture was incubated for 3 hours at 37°C (adapted from Bohr *et al.*, 1987). After precipitation, resuspension and quantitation of the DNA, it was incubated with 3 unit/ug of *BamHI* (Pharmacia) at 37°C for 2 hours. For optimum results, BamHI was removed by phenol-chloroform treatment, and the pure DNA was reprecipitated and resuspended in 200-500 ul of TE pH 8.0. The final DNA concentration was about 0.5 ug/ul.

ii) T4 endonuclease V treatment and electrophoresis

To measure dimer-containing sites in DNA, 10-ug of *BamHI* digested DNA was incubated with 2 units of TEV at 37°C for 30 min in 1x TEV reaction buffer (20 mM Tris pH7.5-1 mM EDTA-100 mM NaCl-1 mM β-mercaptoethanol). Quadruplicate enzyme reactions for each treatment were prepared. Once the reactions were complete, all samples were quantitatively loaded onto a 0.7 % denaturing agarose gel, which was prepared in 1x alkaline running buffer (30 mM NaOH-1 mM EDTA). The samples were electrophoresed in a mid-sized BRL submerged gel electrophoresis apparatus for 20 hours at 33 volts. Following electrophoresis, the gel was neutralized in 100 mM Tris pH 8.0 with 3 changes. Finally, the gel was stained in a 0.5 ug/ml solution of ethidium bromide and photographed.

iii) Southern transfer, hybridization, autoradiography and densitometry

Prior to Southern transfer of the DNA, the gel was depurinated in 0.25 M HCl for precisely 20 minutes, and then rinsed briefly in distilled water. Southern transfer, with the

exception of the transfer buffer (0.4 M NaOH-0.6 M NaCl), was conducted according to procedures outlined in Sambrook *et al.* (1989). The positively charged Hybond N^+ membrane (Amersham) containing the DNA was prehybridized according to the manufacturer's recommendations, and hybridized with 10 ug/ml of ^{32}P-labelled DNA corresponding to exon 3 of the *c-myc* gene. Hybridization was done overnight (16 hours) in a hybridization oven and the membrane was washed five times in buffer containing 0.1x SSC-0.1% SDS at 60^{o}C.

Membranes were placed in plastic bags which were taped to Kodak X-Omat autoradiographic films. The films were then placed inside autoradiographic cassettes containing reflective screens and stored at -70^{o}C for periods ranging from 8-16 hours. Densitometric analysis was performed on the autoradiographs using Gel Scan XL (LKB-Bromma), which was employed for both scanning the intensity of the full length 20-kb *c-myc* containing fragment, and interpretation of the data (Figure 7).

iv) Hybridization probes

Plasmids containing the 1.2-kb *EcoRI/Cla* I insert corresponding to exon 3 of the *c-myc* gene were purified from bacteria by alkaline lysis (Sambrook et al., 1989). The insert was excised from the plasmid using the two aforementioned restriction endonucleases (Pharmacia), and gel purified. The insert was recovered from the gel by electroelution followed by ethanol precipitation. The purified insert was resuspended in TE pH 8.0. The DNA concentration was determined by absorbance measurements taken at a wavelength of 260 nm. The DNA was stored in 50 ng aliquots in 0.5 ml microfuge tubes at -20^{o}C. Radiolabelled probes were generated by using one such tube in conjunction with a random priming kit (Pharmacia). Unincorporated ^{32}P-labelled dCTP was removed by loading the random priming reaction mixture onto a Bio-Rad P-30 spin column. The specific activity of the resultant probes was typically 1 x 10^9 cpm per ug of DNA.

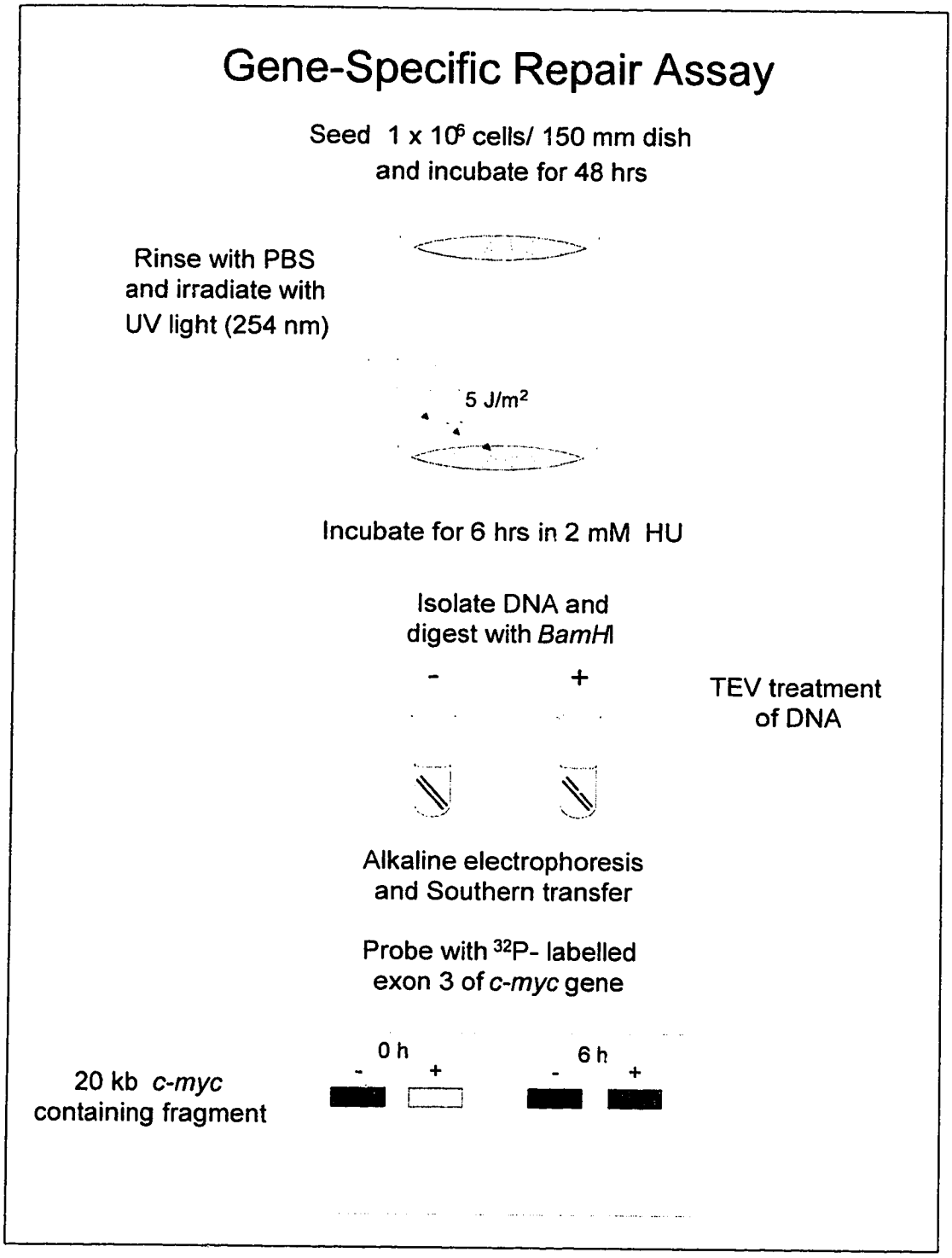

Figure 7. Illustration of the technique employed for the study of gene specific repair in UV-irradiated normal and LFS fibroblasts (see text for details).

Post-UV survival assay

Exponentially growing cultures of GM38 (normal) and 2800T (LFS) were seeded in 100 mm petri plates at 2 different densities (1 x 10^5 cells/plate and 5 x 10^4 cells/plate). The cells were given 30 hrs in which to attach prior to the commencement of the experiment. Following this incubation, the media was removed and the cells were rinsed once with sterile PBS, at which point they were exposed to UV of varying fluences (0-20 J/m^2). The cells were then incubated in fresh Ham's F12 medium for up to seven days. On either the 6th or 7th day (depending upon initial seeding density) the cells were washed with PBS, trypsinized and counted in a Coulter counter.

Modified alkaline sucrose sedimentation assay

For this assay exponentially growing GM38 fibroblasts were seeded in 60-mm dishes at a density of about 1 x 10^5 cells/plate and incubated at 37°C for a minimum of 24 hours in complete medium. At this point, cells were either exposed to 100 Gy of γ-radiation or sham-irradiated, then washed twice with PBS. Cells were harvested by gentle scraping with a rubber policeman, combined with lysis solution (1 M sodium hydroxide-0.1 M EDTA) and carefully layered on top of a 6-ml gradient. The gradients were 5-20 % (w/v) sucrose in 2 M sodium chloride-0.3 M sodium hydroxide-10 mM EDTA (pH 12.5). After holding for 1 hour (to allow denaturation of native DNA), the gradients were placed in a Sorvall TST-60.4 rotor and centrifuged at 30 000 rpm for 2 hours in a Sorvall RC70 ultracentrifuge at 20°C. Once centrifugation was complete, gradients were fractionated sequentially into 23 wells in a BioRad dot-blot apparatus. The vacuum was then applied and the DNA was transferred to a nylon membrane (Hybond N^+) where it was fixed by brief alkali treatment (0.4 M NaOH). The membrane was then prehybridized for 2 hrs at 60°C according to manufacturer recommendations. A ^{32}P-labelled probe was prepared using 25 ng of genomic DNA isolated from GM38 cells. The DNA was radiolabelled with a random-priming kit from Pharmacia and ^{32}P-dCTP from Amersham according to manufacturer's recommendations. Following a

5-hr hybridization, the membrane was washed 4 times with 1x SSC/0.1% SDS, and exposed to an autoradiographic film for ~90 sec. An image analyzer was then used to determine the intensity of each dot on the autoradiogram. These values were used to generate gradient profiles that yielded insight into the relative sizes of DNA molecules produced under different treatment conditions.

RESULTS

Recovery of nucleic acid synthesis in UV-irradiated fibroblasts

In normal cells, exposure to UV radiation results in a transient depression in the rate of DNA synthesis which is manifested as a decrease in 3H-dThd incorporation into genomic DNA at early times after irradiation (Rude and Friedberg, 1977). This transient depression is followed by a time-dependent recovery of DNA synthesis. At about six hours following 5 J/m^2 of UV radiation, the level of DNA synthesis in irradiated cultures recovers to levels observed in sham-irradiated control cultures. There is a direct correlation between the ability of human cells to recover from the initial depression of DNA synthesis after UV and the ability to conduct repair of UV-induced DNA damage (Lehmann et al., 1979). XP strains, which are defective in NER, consequently show impaired recovery of DNA synthesis in response to UV exposure. Exposure of normal cells to UV radiation similarly results in the inhibition and subsequent recovery of RNA synthesis, which can be monitored by measuring the rate of incorporation of ^{3}H-Urd into RNA. The post-UV recovery of RNA synthesis in CS cells is defective (Mayne et al., 1982) due to a defect in transcription-coupled repair. In contrast, XP group C cells exhibit normal TCR and hence appear normal for the recovery of RNA synthesis after UV irradiation.

The objective of this study was to gain insight into the ability of LFS strains to conduct NER. To this end, five strains from LFS family members (each heterozygous for a mutation in the *p53* gene) and five control strains (two normal, one XP and two CS) were screened for their ability to recover from UV-induced inhibition of both DNA and RNA syntheses (see Table II for a description of the strains). One of the LFS strains (2800T) was subsequently compared to a normal control strain for the ability to remove CPDs from global DNA as well as from within the transcriptionally active *c-myc* gene.

i) Recovery of DNA synthesis in UV-irradiated fibroblasts

In order to determine the optimal duration for post-UV incubation, a control strain (GM38) was UV-irradiated and incubated in fresh Ham's F12 medium for various time periods from 0-7 hours. Cultures were then pulse-labeled with ^{3}H-dThd for 10 min and the amount of radioactivity incorporated into genomic DNA was determined. A summary of the results are presented in Figure 8. As expected, there was a characteristic inhibition followed by a time-dependent recovery of DNA synthesis. The recovery of DNA synthesis in the normal control (GM38) strain after 5 J/m^2 UV light reached levels similar to that which was observed in sham-irradiated cultures (~97%), while cultures that received 10 J/m^2 did not exceed 60 % of the level of DNA synthesis observed in non-irradiated cultures. Based on these results, 5 J/m^2 was chosen as the optimal fluence with which to conduct all subsequent recovery of DNA synthesis experiments.

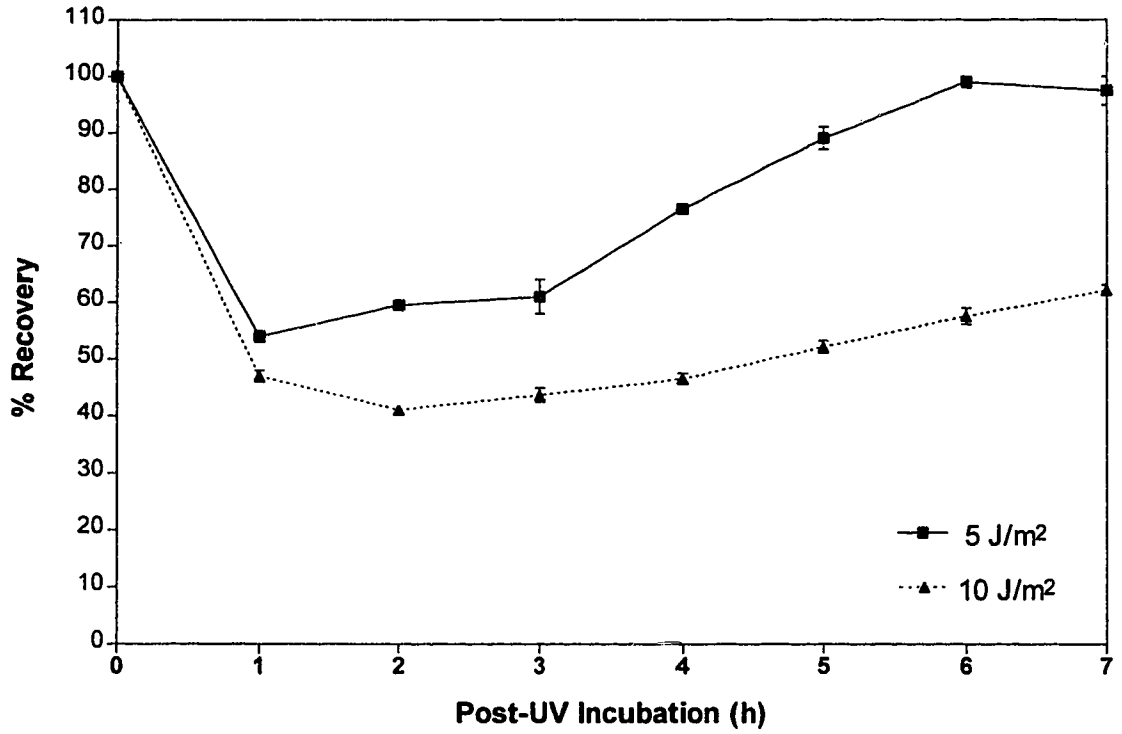

Figure 8. Recovery of DNA synthesis after exposure to either 5 or 10 J/m^2 of UV light in GM38 fibroblasts. The means of triplicate samples and their associated standard errors are shown. Values are normalized to those of sham-irradiated controls.

In a subsequent experiment, five LFS and five control strains were irradiated with 5 J/m^2 UV light, and then incubated for 5 hours whereupon the cells were pulsed for 40 min, and the radioactivity incorporated was determined. As shown in Figure 9, the recovery of DNA synthesis in normal fibroblast strains (GM38 and GM43) reached about 90% of non-irradiated control levels by 5 hours post-UV irradiation, while the repair deficient XP (CRL1170) and CS (GM1856 and GM739) fibroblast strains did not exceed 46% of the levels observed in non-irradiated controls (Figure 9). With the exception of strain 3649T (90%) belonging to Family II, LFS fibroblasts displayed a level of DNA synthesis recovery following UV exposure which was clearly intermediate when compared to the levels seen in normal and CS fibroblasts. The recovery of DNA synthesis in the remaining LFS strains ranged from 51.2% (2800T) to 70.0% (3546T) (Figure 9). These results suggest that, although the majority of LFS strains are defective for the recovery of DNA synthesis, there is considerable heterogeneity in response to UV exposure, not only between LFS families, but also within each family as illustrated by the differences in recovery of DNA synthesis between 2800T and 3546T (both members of LFS Family I). If the recovery of DNA synthesis is an indicator of GGR capacity, then based on these results, all LFS strains with the exception of 3649T should be defective to some extent at GGR as determined by alkaline sucrose gradient sedimentation.

ii) Recovery of RNA synthesis in UV-irradiated fibroblasts

Although initially several post-UV recovery periods were considered, the 6-hour time point yielded the greatest difference in the recovery of RNA synthesis between normal and CS control strains. Two fluences, 5 J/m^2 and 10 J/m^2, were used to follow the recovery of RNA synthesis in LFS and control strains. As expected for the lower fluence, the two normal controls (GM38 and GM43), following the 6 hour post UV incubation period, attained levels comparable to their non-irradiated counterparts, i.e. 99 % and 100 %, respectively (Figure 10). Since XPC fibroblasts are proficient at gene-specific repair, these

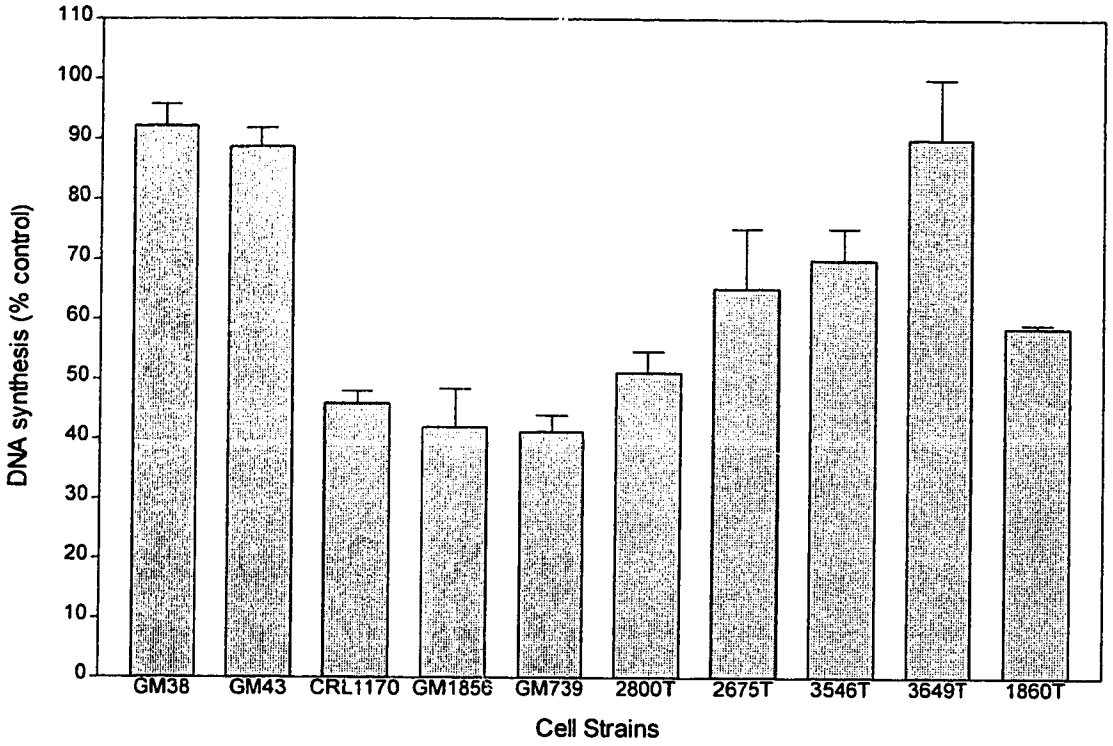

Figure 9. Recovery of DNA synthesis in control and LFS strains following exposure to 5 J/m^2 of 254 nm UV light. Levels of DNA synthesis were measured 5 hours after UV exposure. These values represent the means of replicates and their standard errors. Percent values are calculated based on the recovery of DNA synthesis in corresponding sham-irradiated controls. The number of replicates for each point is not less than four.

cells were also expected to exhibit a "normal" level of recovery of RNA synthesis. The XPC strain CRL1170 responded normally, reaching 95.5 % of the level seen in non-irradiated cultures. Conversely, the two CS strains GM1856 and GM739, which have a defect in TCR, yielded a range in recovery of RNA synthesis between 54.4 % and 69.1 % of the level observed in non-irradiated cultures (Figure 10). At a UV fluence of 5 J/m^2 only two of the LFS strains, (2800T and 2675T) belonging to Family I, showed some deficiency in the recovery of RNA synthesis. Strains 2800T and 2675T, with values of 79.5 % and 81.0 %, respectively, are clearly intermediate in the level of recovery of RNA synthesis when compared to normal and CS control strains. The remaining three LFS strains exhibited near normal levels of recovery of RNA synthesis following 5 J/m^2 of UV (Figure 10).

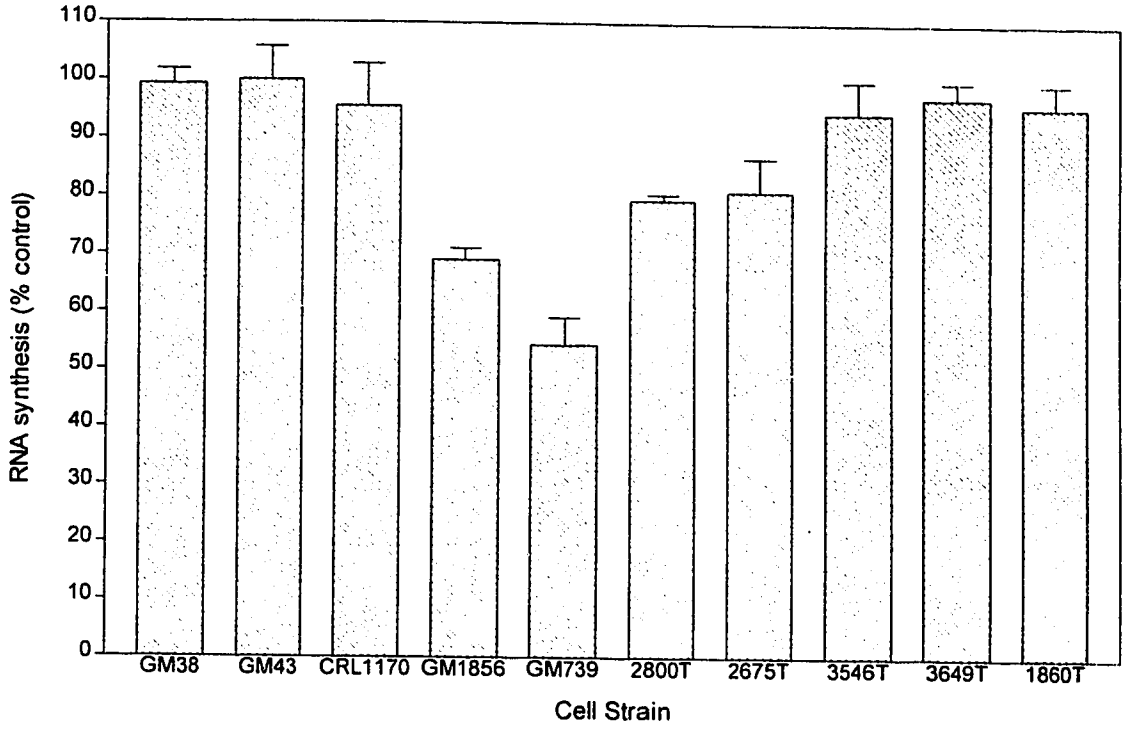

Figure 10. Recovery of RNA synthesis in LFS and control strains following exposure to 5 J/m^2 of UV light. Levels of RNA synthesis were measured 6 hours after UV exposure. Values represent the means of replicates and their standard errors. (14>n > 4)

When cells were irradiated at the higher fluence (10 J/m^2) there was no change in the qualitative response of any of the control strains; however, there was a marked change in the response of LFS strains, 3546T and 1860T (Figure 11). These two strains, which appeared to behave normally at 5 J/m^2, achieved significantly lower levels of recovery of RNA synthesis at the higher dose (10 J/m^2) when compared to normal controls. Even at the higher fluence, LFS strain 3649T retained its "normal" level of recovery of RNA synthesis at 6 hours post-UV exposure. A summary of the results from the 5 and 10 J/m^2 recovery of RNA synthesis experiments is presented in Table III.

Table III. Recovery of RNA synthesis in LFS and control strains 6 hours following 5 J/m^2 and 10 J/m^2 UV light. Values are expressed as a percent of sham-irradiated controls. Each point represents the mean of replicate samples and its appropriate standard error.

Strain	5 J/m^2	10 J/m^2
GM38	99.2 +/- 2.6	69.2 +/- 2.1
GM43	100.0 +/- 5.7	64.2 +/- 3.1
CRL1170	95.5 +/- 7.3	63.4 +/- 3.7
GM1856	69.1 +/- 2.0	46.8 +/- 4.0
GM739	54.4 +/- 4.8	31.9 +/- 3.6
2800T	79.5 +/- 1.0	39.5 +/- 1.1
2675T	81.0 +/- 5.8	40.6 +/- 2.7
3546T	94.6 +/- 5.5	48.5 +/- 2.5
3649T	97.3 +/- 2.8	60.8 +/- 3.5
1860T	95.8 +/- 4.0	54.0 +/- 1.7

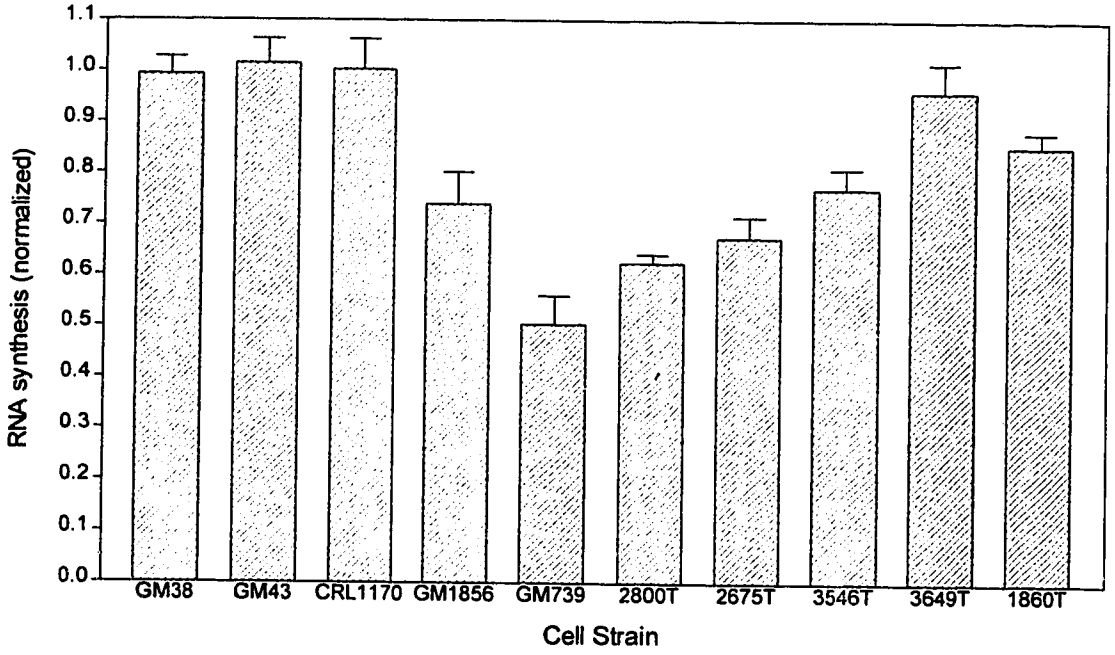

Figure 11. Recovery of RNA synthesis in LFS and control strains following exposure to 10 J/m^2 of UV light. Levels of RNA synthesis were measured 6 hours after UV exposure. Values represent normalized means of replicates and their standard errors. Values for the levels of recovery of RNA synthesis were normalized against the mean for the two normal controls. The levels of recovery of RNA synthesis in the controls did not exceed 70%. (14>n >4)

Monitoring repair of dimers in UV-irradiated fibroblasts

The defect observed in the recovery of DNA synthesis at 6 hours post-UV exposure in LFS strains suggests that DNA damage throughout the genome may persist longer in those strains than in normal controls. This delay in the recovery of DNA synthesis therefore may indicate a defect in the repair of the genome overall. When an indirect assay which relies on polymerase inhibitors to reveal damage containing sites was used, two LFS strains from two members of Family I (2800T and 2675T) were clearly shown to be defective in overall genome repair (Mirzayans *et al.*, 1996).

i) Monitoring repair in the overall genome

In this study the ability of LFS cells to remove dimers from the genome overall was assessed using TEV, a dimer specific endonuclease which specifically introduces a nick in DNA at dimer containing sites. Alkaline sucrose sedimentation was employed to separate the TEV-treated DNA samples and controls according to their molecular weight. Based on the data presented in Table IV, there appears to have been no repair by 3 hours post-UV exposure in either GM38 or 2800T cells. By 6 hours post-UV, there had been significantly more repair in the GM38 fibroblasts than in the 2800T fibroblasts. As predicted by the defect in the recovery of DNA synthesis, 2800T cells were markedly defective in global repair of pyrimidine dimers at 6 hours following 10 J/m^2 of 254 nm UV light. By the end of the 6 hour post-UV incubation period, repair in the normal control strain (GM38) exceeded 57 percent, while the representative *p53* mutant strain (2800T) only attained 36 percent repair of TEV sensitive sites (see Table IV and Figure 12).

ii) Monitoring repair in the *c-myc* gene of UV-irradiated fibroblasts

As mentioned earlier, nucleotide excision repair is comprised of two major subpathways, transcription-coupled repair which operates predominantly on the lesions

Table IV. Mean number of dimer-containing sites remaining in the DNA per 10^8 daltons as revealed by TEV following the indicated repair period in a representative *p53* mutant and control strain. Numbers in brackets represent percentages of sites remaining in the DNA .

	Repair Times		
	0 hrs	3 hrs	6 hrs
GM38	14.3 (100%)	15.8 (110%)	6.2 (43%)
2800T	27.8 (100%)	30.0 (106%)	18.2 (64%)

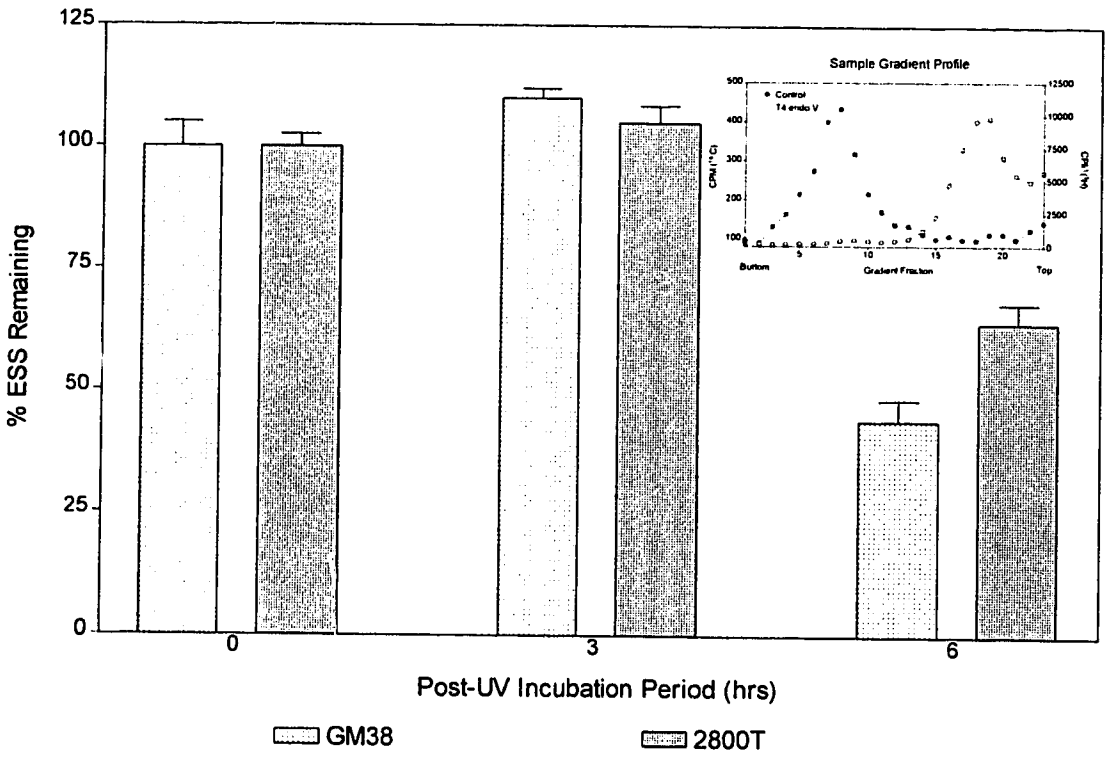

Figure 12. Percentage of TEV sensitive sites (ESS) remaining in the genomic DNA of GM38 (normal) and 2800T (LFS) cells, at indicated incubation periods, following exposure to 10 J/m^2 UV light. Intact cells were permeablized with saponin, then subjected to TEV treatment. Data points represent means and their associated standard deviations. The insert shows alkaline sucrose gradient profiles of DNA from UV-irradiated cultures (open squares) and from non-irradiated cultures (solid squares) which were subjected to TEV treatment.

occurring in the transcribed strand of active genes, and global repair which accounts for repair in the non-transcribed strand of active genes as well as in the remainder of the genome. The basic machinery is thought to be identical in these two subpathways, the only difference being the association of the repair machinery with a chromatin accessibility factor for global repair and a transcription coupling factor for TCR. Just as the rate of recovery of DNA synthesis can be used as a predictive tool for the status of global repair in a cell, so too should the rate of recovery of RNA synthesis be predictive for TCR status. Using inhibitors of long-patch repair polymerases, we have recently demonstrated that an LFS fibroblast strain (2800T) is markedly defective in the removal of UV photoproducts from the transcriptionally active *c-myc* gene (Mirzayans et al., 1996). In this study, this observation was followed up using a more direct assay, which measures the persistence of dimers as opposed to the appearance of repair-resynthesis-associated gaps.

Table V. Presence of cyclobutyl pyrimidine dimers in a 20-kb restriction fragment containing the *c-myc* gene in GM38 and 2800T fibroblasts at 0 and 6 hrs after exposure to 5 J/m^2 UV light. TEV was use as a probe for CPD-containing sites.

		0 Hours Repair		6 Hours Repair		Overall
	TEV	Absorbance	ESS / 20 kb	Absorbance	ESS / 20 kb	Repair (%)
GM38	-	0.230 +/- 0.040	1.6	0.265 +/- 0.020	0.25	84
	+	0.046 +/- 0.002		0.207 +/- 0.020		
2800T	-	0.11 +/- 0.010	1.2	0.140 +/- 0.010	1.17	3
	+	0.033 +/- 0.003		0.043 +/- 0.006		

In the control fibroblast strain (GM38) 84% of the TEV sensitive sites were removed from the *c-myc* gene by 6 hrs after exposure to 5 J/m^2 of UV_{254} light. In contrast, the LFS strain 2800T only removed 3 percent of the sites during the same 6-hr period (see Table V and Figure 13). The number of ESS is calculated according to the formula ESS = -ln b/a, wh : "b" represents the intensity of the band corresponding to the TEV treated sample and "a" represents the intensity of the band corresponding to the non-TEV treated sample.

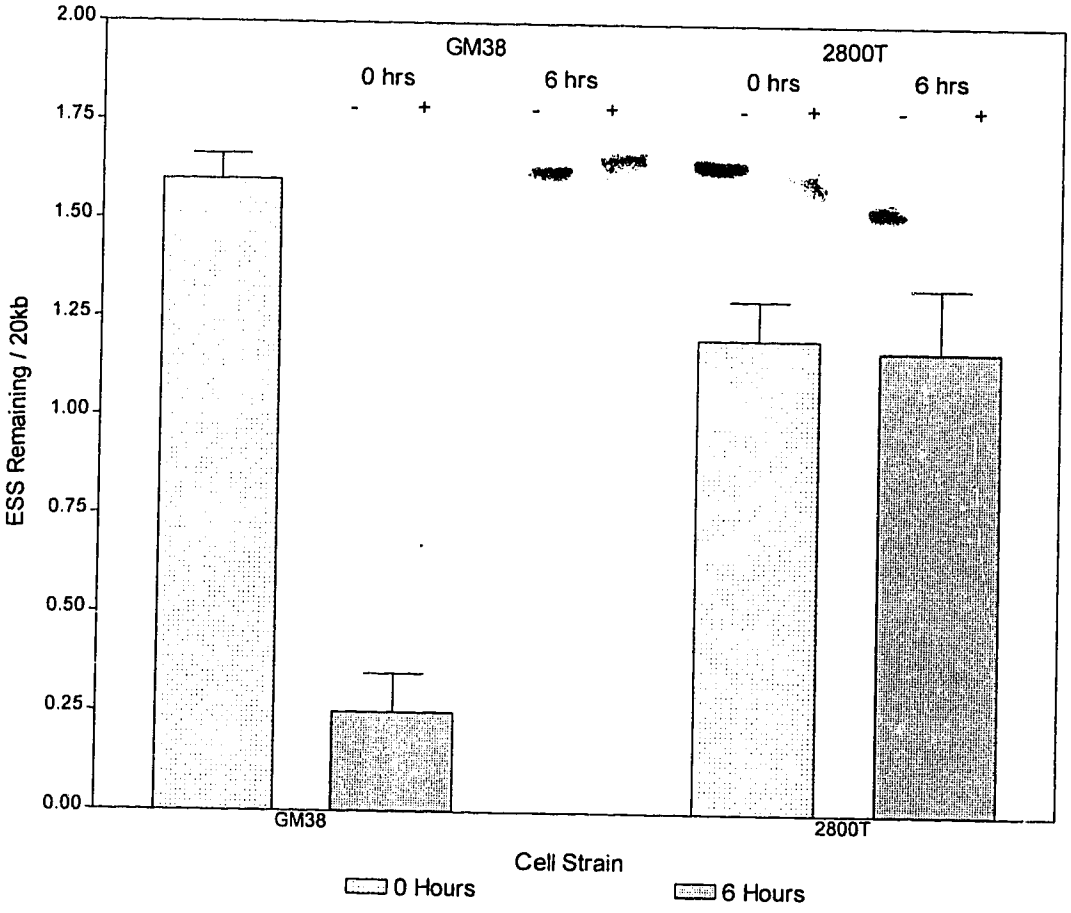

Figure 13. Removal of enzyme sensitive sites (ESS) from the *c-myc* gene in GM38 and 2800T fibroblasts following irradiation with 5 J/m^2 UV_{254} light and a 6 hour post-UV incubation. Each datum point represents the mean (+/- standard deviation) of six samples from two independent experiments. The insert shows autoradiographic bands corresponding to the intact 20 kb *c-myc* containing fragment (+ and - denote the presence or absence of enzyme treatment, respectively

Post-UV survival in normal and LFS fibroblasts

Post-UV survival was measured at various fluences in both normal and LFS fibroblast strains to determine the biological effects, if any, of reduced global and transcription-coupled repair capacity. To monitor survival, exponentially growing cultures of GM38 and 2800T were seeded in 100 mm petri dishes at 2 different cell densities. Cells were then irradiated at varying fluences, and incubated for 6 days (Figure 14) or 7 days (data not shown) prior to cell counting. Employing this assay, XP cells have been shown to be markedly sensitive to UV radiation (Mirzayans and Waters, 1985). In contrast, 2800T cells proved to be resistant to UV-induced inhibition of cell growth. For example, a dose of 5 J/m2 reduced the extent of cell growth to 70 % in GM38 cells without having any noticeable effect in 2800T cells. Fluences of UV required to induce 50 % inhibition of cell growth (IC_{50}) were 1.5 fold greater for 2800T than for GM38 cells (12 J/m^2 vs. 8 J/m^2 respectively). This experiment was also repeated at an initial cell density of 5×10^4 cells per dish with a 7 day post-UV incubation period and the graphs are virtually superimposeable.

Modified alkaline sucrose sedimentation assay

Given the number of overlapping cellular processes affected by the p53 tumor suppressor protein, it may be difficult to measure any particular one in isolation without biasing the outcome. The study of the effects of p53 status on DNA repair exemplifies this difficulty. Traditionally, many of the assays used to measure DNA repair have been dependent on the prelabelling of genomic DNA with radioactive nucleotide precursors. Since even low levels of prelabelling have been shown to upregulate *p53* expression (Dover *et al.*, 1994), it was necessary to develop an assay which circumvents the need for prelabelling. Performing only slight modifications to the traditional alkaline sucrose sedimentation assay would not only meet this objective, but would also retain retro-compatibility with previously generated data, thus facilitating meaningful comparisons. In short, the modifications involve the post-labelling rather than prelabelling of genomic DNA for detection purposes.

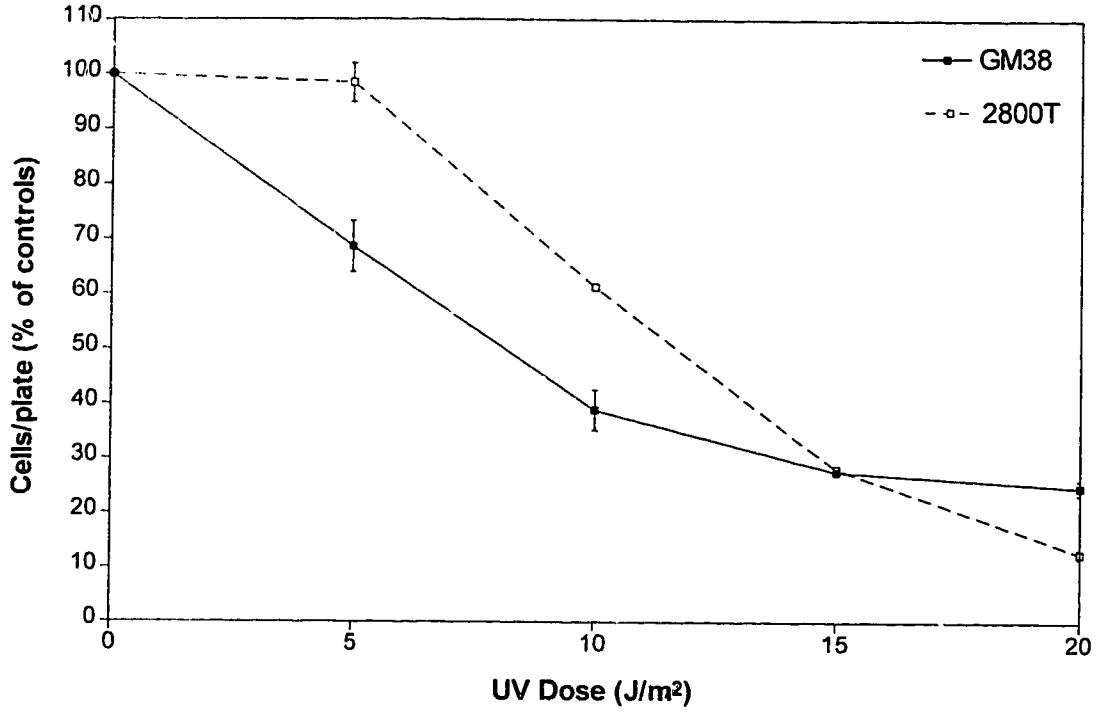

Figure 14. Cell survival 6 days post-UV in a normal (GM38) and an LFS (2800T) strain. Values are expressed as a percentage of the number of cells in non-irradiated cultures. The initial seeding density was 1 x 10^5 cells/100mm plate. The maximum number of fibroblasts per dish at 6 days post-UV did not exceed 5.1 x 10^5.

To simulate the results obtained in this type of experiment, one dish of fibroblasts was exposed to 100 Gy of γ-irradiation (heavily fragmented DNA), and a second dish was sham-irradiated (high molecular weight DNA). The cells on each dish were lysed on top of separate alkaline sucrose gradients and the gradients were centrifuged for 2 hours. Each gradient was then sequentially fractionated into 23 different wells of a dot-blotting apparatus. After transfer and alkali fixation of the DNA to a nylon membrane, the membrane was probed with a ^{32}P-labelled total genomic DNA probe. Following hybridization, an autoradiographic film was exposed to the membrane for about a minute and developed (Figure 15). A laser densitometer was used to measure the intensity for each of the spots. These readings permit a quantitative comparison of the relative amounts of DNA in each of the fractions (Figure 16). Gradient fractions 1-12 go from left to right while gradient

fractions 13-23 are immediately below gradient fractions 1-12 and run from right to left. From simple inspection, it is clear that the bulk of the DNA is in fractions 1-6 for series A and 13-17 for series B which in itself suggests a dramatic shift in molecular weight. The mean molecular weight for the non-irradiated control sample was 474 x 10^8 Daltons, while the molecular weight of the γ-irradiated sample was only 3.7 x 10^8 Daltons.

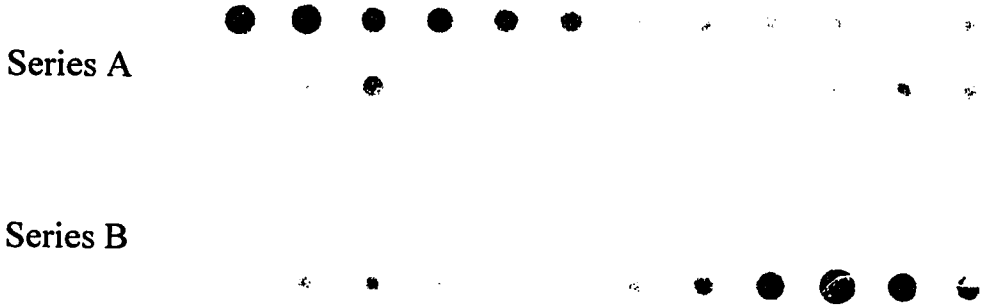

Figure 15. Illustration of an autographic exposure of the type generated using a modified alkaline sucrose sedimentation assay. Series A was used to generate a profile of the gradient from non-irradiated cells. Series B was used to generate the gradient profile for irradiated cells.

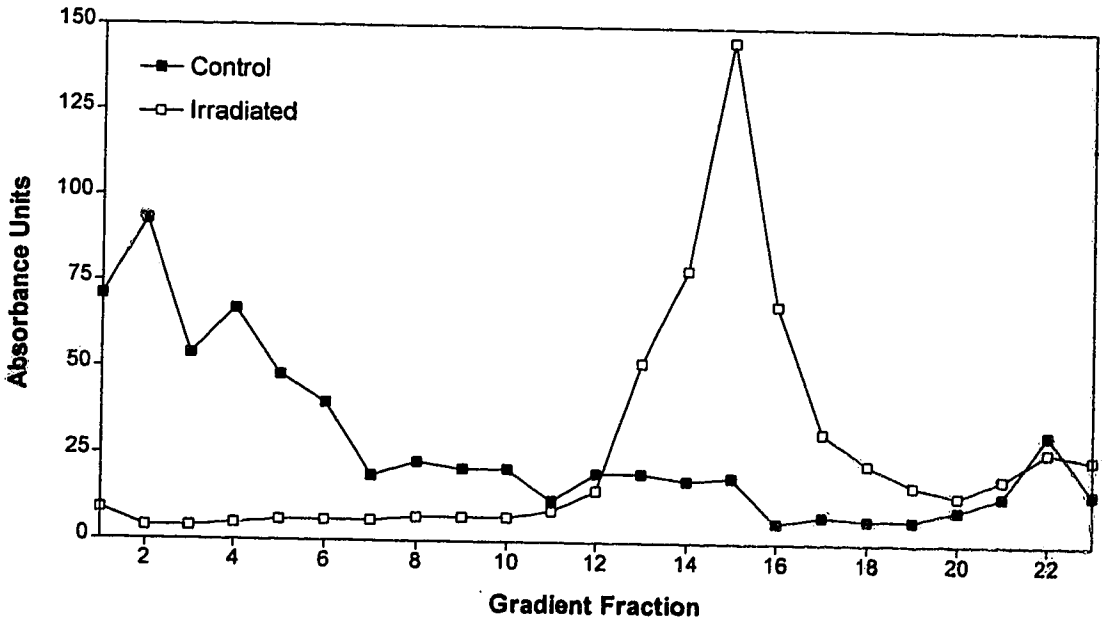

Figure 16. Graphical representation of the information contained within the dot-blot presented above. Values were generated using 1-dimensional analysis on an LKB Ultroscan Enhanced laser densitometer.

DISCUSSION

Recovery of DNA synthesis as an indicator of GGR status

To fulfill the research component of this thesis, the author has evaluated the DNA metabolic properties of non-cancerous fibroblast strains derived from informative LFS family members. It has been found that most of the LFS strains studied, like the NER deficient strains (XP, CS), are defective in the recovery of DNA synthesis following 5 J/m^2 of UV light. The slower recovery of DNA synthesis observed in these LFS strains suggested an underlying defect in UV-induced DNA damage processing. Since a defect in the recovery of DNA synthesis post-UV in NER deficient strains is attributable to a defect in the GGR subpathway, the LFS strains were also suspected to posses a defect in GGR. To ascertain whether GGR in *p53* mutant strains was indeed defective, a representative *p53* mutant strain (2800T) was chosen to use in the assay. As was predicted by the delay in the recovery of DNA synthesis, 2800T cells were clearly defective in GGR at 6 hours following 10 J/m^2 of UV light.

Recovery of RNA synthesis as an indicator of TCR status

With the observation of a global DNA repair defect in LFS fibroblasts, it became important to examine the extent to which the overall process of repair might be affected. To accomplish this objective, the same panel of LFS strains were screened for a defect in transcription-coupled repair using the recovery of RNA synthesis assay as an indirect indicator. The results from the recovery of RNA synthesis assay showed that four of the five LFS strains examined exhibited a slower recovery of RNA synthesis following 10 J/m^2 of UV light. 2800T was selected as a representative *p53* mutant strain in which to study TCR. The extent of TCR in 2800T fibroblasts by 6 hours post-UV was negligible when compared to the extent observed in normal controls. Taken together, these results indicate that DNA repair in LFS fibroblasts more closely resembles the repair characteristics seen in XP than in CS, since 2800T cells appear to be deficient in both GGR and TCR.

Effects of a repair defect on post-UV survival

To determine whether the repair defect observed in 2800T fibroblasts results in a reduction in cellular viability following genotoxic assault, cell survival was measured at 6 and 7 days post-exposure to either 0, 5, 10, 15 or 20 J/m^2 UV light. The 2800T fibroblasts, when compared to GM38 fibroblasts, were 1.5 fold more resistant to the killing effects of UV light. This observation is somewhat surprising, because NER defective cells such as XP and CS are hypersensitive to UV-induced cell killing. For example, employing an assay similar to the one used here, XP group A fibroblasts were reported to be extremely sensitive to UV-induced inhibition of cell growth (Mirzayans and Waters, 1985). Although the nature of this unexpected observation needs to be investigated further, there exists at least two plausible explanations. 1) There is the possibility that p53 deficient cells may appear resistant to the cytotoxic effects of UV because they, unlike normal cells, lack the ability to initiate an apoptotic program (Little, 1994). This could explain the UV-resistance observed in 2800T fibroblasts because they carry a *p53* mutation at codon 234 which is believed to exert negative trans-dominant effects over the wild-type allele. 2) An alternative explanation might be that cells lacking functional p53 are unable to transcriptionally activate genes such as *WAF-1* and therefore will be defective for p53 mediated G1 arrest following a genotoxic assault. In this scenario, normal cells would enter G1 arrest while their p53 mutant counterparts would continue to proliferate, thus increasing their overall cell numbers. An increased number of cells per plate, when compared to normal controls would yield what resembles a UV-resistant phenotype. Although not presented as part of this thesis, we have demonstrated that *WAF-1* gene expression is not upregulated following genotoxic assault in the *p53* mutant strain 2800T.

Other DNA repair studies in p53 mutant fibroblasts

In the course of this study, two papers appeared that also deal with repair in p53 deficient LFS fibroblasts, the first by Wang *et al.* (1995), and the second by Ford and

Hanawalt (1995). At this point, I would like to describe some of the work presented in those papers and compare and contrast these studies with the findings presented here.

In the paper by Wang et al., (1995), the authors report protein-protein interactions between p53 and several TFIIH-associated repair factors, including XPB, XPD, and CSB. Furthermore, they showed that although both wild-type and mutant p53 bind to XPB and XPD, only the wild-type p53 is capable of inhibiting TFIIH helicase activity. This inhibition, the authors propose, may initiate DNA repair, perhaps through redirection of the TFIIH complex. To test that hypothesis, Wang and coworkers examined the rate of removal of cyclobutyl pyrimidine dimers from the DHFR gene in normal and LFS fibroblasts. They concluded that repair in both of the strains studied (2675T and 2673T which are derived from affected members of Family I) was significantly less at all time points examined (4, 8 and 24 hours). Although the data were not shown, the authors also state that recovery of RNA synthesis in these LFS strains was similar to that observed in normal controls. Although their results are in agreement with the results presented in this thesis for the repair of a transcribed gene, there seems to be some discrepancy regarding the extent of recovery of RNA synthesis. Wang *et al.* (1995) reported that RNA synthesis recovery in LFS strain 2675T was similar to that observed in normal controls; however, in the present study this strain was markedly defective. One explanation for this discrepancy could be that Wang and coworkers measured the recovery of RNA synthesis following exposure to lower fluences of UV light than what was used in this study. As shown in Figures 10 and 11, the defect in the the recovery of RNA synthesis in all LFS strains appears far less pronounced at the lower fluence of UV light (5 J/m^2)

The second paper, by Ford and Hanawalt, examined global and transcription-coupled repair in human cell strains harboring mutations in one or both alleles of the *p53* gene. These workers reported that the homozygous mutant *p53* strains had a much lower rate and overall extent of repair in the genome as a whole than either the heterozygous mutants or control

strains. The authors also mention that although the heterozygous mutant strains had a slower rate of repair at early times, they achieved normal levels of repair by 24 hours. After discovering a defect in global repair in these LFS strains, Ford and Hanawalt examined the effects of a *p53* mutation on transcription-coupled repair in the homozygous mutant strains alone. They reported that both double mutant strains showed normal rates of repair of the transcribed strand, but appeared to be markedly defective at the repair of the non transcribed strand. Unfortunately, the authors neglected to include the strains which were heterozygous for *p53* mutations in their TCR assay. These strains could have yielded some very informative results, since they more accurately reflect the premalignant condition present in LFS patients.

In the present study, the p53-deficient strain 2800T, bearing a somatic mutation at codon 234, proved to have a level of global genome repair, at 6 hours post-UV, which was significantly lower than the level observed in the normal control strain. Interestingly, the heterozygous mutant LFS strain used in the Ford and Hanawalt paper responded identically in a GGR assay to the 2800T strain used here. Again, these results further strengthen the findings presented in this thesis.

In summary, the results presented in the preceding section on global and transcription-coupled repair in p53 deficient LFS fibroblast strains point to a common aspect which is shared by these two subpathways. Since DNA repair in the genome as a whole and DNA repair within transcribed genes are both defective in 2800T, it is likely that p53 plays a central role in repair as well as in G1 arrest and apoptosis.

Effects of metabolic labelling on DNA repair studies

One experimental aspect of the effect of p53 on DNA repair which has yet to be explored in any detail is the nature of repair in the absence of any metabolic labelling with

radioactive DNA precursors. This question stems from the fact that most DNA repair assays rely on the labelling of genomic DNA prior to UV light exposure. Under these conditions cells are receiving sufficient exposure to an internal radiation source prior to the actual experimental treatment which is upregulating the expression of *p53* and therefore perhaps biasing the results. In an attempt to circumvent this problem, a protocol was developed which can be used in conjunction with the standard alkaline sucrose sedimentation assay. This approach relies on labelling the DNA for detection purposes, after alkaline sucrose sedimentation, as opposed to performing the traditional metabolic labelling prior to UV exposure. To accomplish this, fractions which were normally collected on filter paper prior to counting were transferred to a nylon membrane via a dot-blot apparatus for subsequent probing. DNA on the membrane was hybridized with random primed genomic DNA and then exposed to an autoradiographic film. The relative amount of DNA in each fraction can be determined by measuring the dot intensity using a laser densitometer. The absorbance readings generated by this technique can be analyzed using the same type of program that was used on the scintillation counter data. Although this technique is still in its infancy, it may provide an excellent way to circumvent the prelabelling of DNA in this type of repair assay.

Future research objectives

The current study not only resolved some issues regarding the DNA metabolic properties of LFS cells, but it has also opened the door to several new areas of experimentation. The insight gained from the study of repair in a p53 deficient background was invaluable in formulating what would be the next series of experiments necessary to address the greater issue, which is the role that p53 plays in the process of DNA repair. To further dissect the involvement of p53 in repair, an experimental approach is outlined below which should provide new insights into p53 function.

i) Further characterize the repair defect observed in LFS

There is much more to be learned about the involvement of p53 in repair through further study of cells derived from affected LFS members. Clearly, not all of the cell strains studied showed a defect in the recovery of DNA and RNA syntheses. Furthermore, some of the strains responded differently to different doses of UV light. Together, these heterogeneous responses cloud the interpretation of the data, making the formulation of a unifying explanation difficult. In addition to addressing some of these unexplained observations, the number of families as well as the number of individuals per family could be increased to provide a broader perspective of repair in LFS. Since different LFS families harbor different germline mutations, sequencing of the *p53* gene in these families may be informative. Mutations in different domains of the protein could conceivably explain much of the heterogeneity in response to UV that was observed in the current study. It may be equally interesting to look at *p53* mRNA and protein stability, because a shorter half-life than normal could also explain the differences seen between families. Similarly, in the absence of a disruptive *p53* mutation, it might be useful to examine the expression and functionality of a number of downstream factors which could affect DNA repair or G1 arrest.

ii) Determine the mechanisms by which p53 influences DNA repair

The findings implicating p53 in the process of DNA repair have opened a new field of study into the functional nature of the interaction between p53 and repair proteins. Several experimental approaches designed to shed light on the nature of this interaction have been outlined. The first potentially confounding variable is the effect of prelabelling on *p53* gene expression. Experiments could be conducted to determine the amount and significance of *p53* upregulation on repair in normal human fibroblasts (and perhaps compare the results with those from identical experiments done on LFS cells). Should the effects of prelabelling significantly alter the outcome of the repair measurements, new protocols like the one mentioned above should be routinely employed.

a) The p53 transcriptional activation domain

There are many ways in which p53 could influence repair, since this fascinating protein transcriptionally activates a variety of different genes, and has been shown to bind directly to a plethora of functionally unrelated proteins. As discussed earlier, there are at least three mechanisms, which could explain how p53 exerts its influence on repair. Initially, experimentation should be focused on testing these three mechanisms. Outlined below are some of the experimental approaches one might use to determine which, if any, of these mechanisms is correct. The experimental approaches to be used in testing mechanisms I and II are very similar, and would involve negating p53's transcriptional activation of specific genes in normal cells. By using either antisense RNA directed against the desired genes or antibodies against proteins like Gadd45 or p21, it might be possible to simulate the DNA metabolic properties observed in LFS fibroblasts. Similarly, by either using microinjection or transfection assays it might be possible to restore the DNA metabolic properties in LFS cells to normal levels (again using either Gadd45 or p21). It would be ideal to include PCNA in these studies as well; however, given its essential role in replication, the results might be very difficult to interpret. In addition to gaining invaluable information regarding the role of p53 in repair, this line of experimentation could also clarify p53's role in G1 arrest, by merely changing the endpoints in the assay.

An alternative approach, which would permit the screening of a much larger number of candidate genes, would be to compare expression levels of p53 regulated genes in both normal and LFS cells following a 10 J/m^2 dose of UV radiation. In theory, any differences in gene expression between normal and LFS cells could account for the observed repair defect, and would therefore necessitate further study.

To conclusively demonstrate that it is the *p53* mutation in the LFS strains which confers these DNA metabolic defects and not some other irregularity, an expression vector

carrying a wild-type copy of the *p53* gene could be transfected into an LFS strain, with the expectation that it would restore a normal phenotype to the cells. Only LFS stains exhibiting germline *p53* mutations would be considered, to facilitate interpretation of the results. These results would be supported by the converse experiment, showing that expression of the mutant p53 (with its trans-dominant effect) in normal cells confers a DNA metabolic defect.

To determine what function if any that the mutant p53 serves in LFS cells exhibiting the trans-dominant effects, knockout strains could be engineered. This experiment would have one of three possible outcomes: 1) the knockout cells are not viable, indicating that even the mutant protein performs some essential function in the cell 2) there is no noticeable changes in either DNA metabolic properties or G1 arrest, suggesting that the trans-dominant effects of the mutant allele abrogates all normal functions of the wild-type allele, or 3) there are measurable changes in one of the above mentioned pathways, indicating that some functions, but not all, are adversely affected by the mutant allele.

b) The p53 protein binding domain

Site specific mutagenesis in the p53 protein binding domain, as opposed to its transcriptional activation domain, may demonstrate that p53 exerts its effect on repair through protein-protein interactions. If this were the case, proteins like PCNA, XPB, XPD, CSB and others that directly participate in DNA repair would become preferred candidates for further study. At present, there are still many basic questions which need to be addressed before any concrete conclusions regarding p53's role in repair can be drawn. This section was designed to provide a framework with which to begin experimentation.

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