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THE ROLE OF TUMOR SUPPRESSOR GENE p53 IN MALIGNANT TRANSFORMATION AND THE ROLE OF THE hREV1 GENE IN DAMAGE-INDUCED MUTAGENESIS OF HUMAN FIBROBLAST CELLS

presented by

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THE ROLE OF TUMOR SUPPRESSOR GENE *p53* IN MALIGNANT TRANSFORMATION AND THE ROLE OF THE *hREV1* GENE IN DAMAGE-INDUCED MUTAGENESIS OF HUMAN FIBROBLAST CELLS

By

Xi-De Wang

A DISSERTATION

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ABSTRACT

THE ROLE OF TUMOR SUPPRESSOR GENE *p53* IN MALIGNANT TRANSFORMATION AND THE ROLE OF THE *hREV1* GENE IN DAMAGE-INDUCED MUTAGENESIS OF HUMAN FIBROBLAST CELLS

By

Xi-De Wang

Alterations in oncogenes, tumor suppressor genes, or genes involved in DNA repair have been demonstrated to play causal roles in carcinogenesis. Activation of oncogenes or inactivation of tumor suppressor genes promotes cell growth, whereas a decreased rate of DNA repair or aberrant repair increases the mutation rate of the genome, including oncogenes and tumor suppressor genes. This dissertation concerns two aspects of carcinogenesis, i.e., the role of loss of function of *p53* in the malignant transformation of human fibroblasts in culture and the role of *hREV1* in human cell mutagenesis. The *p53* gene is the most frequently altered tumor suppressor gene in human cancers, and *hREV1* is considered to be an essential gene for mutagenesis induced by DNA damaging agents.

McCormick and colleagues found that carcinogens such as γ-radiation, ultraviolet (UV) radiation, benzo[a]pyrene diol epoxide (BPDE), or methylnitrosourea, can induce focus formation in the immortal, chromosomally-stable, non-tumorigenic human fibroblast cell strain MSU-1.1, and that a high percentage of the focus-derived cell strains have lost the function of *p53*. In addition, only the cell strains that lost *p53* could form tumors in athymic mice. To test whether loss of *p53* plays a causal role in allowing cells to form foci in

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culture and malignant tumors in mice, I transfected a wild-type *p53* gene into a focus-derived malignant cell strain that had lost *p53* function, and determined the effects of exogenous p53 expression on the cells' transformed phenotypes. The results indicate clearly that *p53* suppresses focus formation, anchorage-independence, and the ability to form tumors, strongly suggesting that loss of *p53* predisposes carcinogen-treated MSU-1.1 cells to transformation.

It has been determined that in *Saccharomyces cerevisiae*, the function of the *REV1* gene is required for mutagenesis induced by a variety of DNA damaging agents. To determine whether this is also the case for mutagenesis in human cells, Christopher Lawrence of the University of Rochester cloned a human gene homologous to the yeast *REV1* gene, and I determined whether this *hREV1* gene is required for human cell mutagenesis. To do so, I generated cell strains that express *hREV1* antisense RNA at high levels and used them to determine the effect of antisense RNA expression on the mutagenic effect of UV and of BPDE. The results showed that expression of antisense RNA greatly reduced the frequency of UV- or BPDE-induced mutants. I then expressed exogenous hRev1 protein in cells that express antisense RNA and found that it restored their capability of generating mutations in response to such DNA damage. These data strongly support the hypothesis that *hREV1* is required for virtually all damage-induced mutations. Amplification and sequencing of the *HPRT* cDNA from a series of independent BPDE-induced mutants derived from normal parental cells and from cells expressing exogenous hRev1 revealed that the kinds of base substitutions induced were virtually identical.

To my parents and the whole family for their love, encouragement and support

To my wife, Lin-Fang Zeng, for the love she dedicates to our little family

To my teachers and friends who care about us

ACKNOWLEDGEMENTS

I sincerely thank my mentor, Dr. Veronica M. Maher and Co-Director of the Carcinogenesis Laboratory, Dr. J. Justin McCormick, for providing me such a great opportunity to learn and to do research in the exciting field of cancer research. Their guidance, support, and encouragement are the most pivotal factors in my pursuance of the Ph.D. degree. Studying under their direction allowed me an opportunity to learn the ways of great scientists to design and execute the research, to manage the laboratory, and to strive to succeed in the field that they have chosen.

I would also like to indicate my appreciation for the advice and support from the other members of my graduate committee, Drs. Walter J. Esselman, Felipe Kierszenbaum, and John L. Wang.

My dissertation research could not have been done without the excellent environment of the Carcinogenesis Laboratory and of Michigan State University. I would like to thank the present and former members of the laboratory for their friendship and assistance. In particular, I would like to acknowledge Michele Battle, Katherine Bergdolt, Clarissa Dallas, Jackie Dao, Bethany Heinlen, Evan Kaplan, Susanne Kleff, Suzanne Kohler, Ziqiang Li, Hongyan Liang, Glenn McGregor, Terry McManus, Sandra O'Reilly, Beatrice Tung, Lijuan Wang, Yun Wang and Hong Zhang. I also thank Dr. Katheryn Meek and the staff in her laboratory for their advice and friendship. I also appreciate Dr. Winnie Chiang for critically reading part of the literature review and many professors, fellow

graduate students, undergraduate students and supporting staff at Michigan State University who taught me or helped me.

I am indebted to the members of my whole family as well. My wonderful wife, Lin-Fang Zeng, who enjoys the happiness and shares the difficulties of life with me, is my strongest support. I cherish with her the past, and the present, and I look forward to the future. Members of my family, including my parents, my brothers and sisters and their respective families, and members of my wife's family, including her parents and her brother, Yibin, and sister-in-law, Bei, all gave me their full support. I also appreciate the cooperation of my babies, Catherine Angie and William Jianing. Without the love and/or support of all these persons, I would not be where I am now.

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STS:

TG:

Ubc:

UV:

VEGF:

XPV:

LIST OF ABBREVIATIONS

a.a.:	amino acid
AP:	apurinic/apyrimidinic
BPDE:	(±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro-benzo[a]pyrene
EST:	expressed sequence tag
FAP:	familial adenomatous polypopsis
HNPCC:	hereditary non-polypopsis colon cancer
HPRT:	hypoxanthine phosphoribosyltransferase
LOH:	loss of heterozygosity
MMR:	mismatch repair
MMS:	methyl methanesulfonate
MNU:	*N*-methyl-*N*-nitrosourea
NER:	nucleotide excision repair
ORF:	open reading frame
Pol:	DNA polymerase
STS:	sequence tagged site
TG:	6-thioguanine
Ubc:	ubiquitin-conjugating enzyme
UV:	ultraviolet radiation
VEGF:	vascular endothelial growth factor
XPV:	xeroderma pigmentosum variant

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INTRODUCTION

Carcinogenesis is a multistep process, by which a cell accumulates a series of genetic changes until it finally becomes a cell capable of forming a malignant tumor. Three classes of genes are involved in this process: oncogenes, tumor suppressor genes, and DNA repair genes. My dissertation project studies two aspects of carcinogenesis: the role of loss of function of *p53*, a potent tumor suppressor gene, in the malignant transformation of human fibroblasts and the role of the *hREV1* gene in human cell mutagenesis induced by DNA damaging agents.

Chapter 1 is a review on the literature closely related to the research that I pursued in my dissertation project. The first section presents a review of carcinogenesis, including a brief description of the multistep model of carcinogenesis and a summary of the functioning of the tumor suppressor gene *p53*. The second section discusses in greater detail the *Saccharomyces cerevisiae* yeast *REV1* gene and its human homolog, *hREV1*; the mechanisms of translesion synthesis involving yeast Rev1, Rev3, Rev7 and their human homologs; and the RAD6/hRAD6 damage tolerance pathway to which these genes belong.

Chapters 2, 3 and 4, as well as the Appendix, summarize my dissertation research. Chapter 2 represents a manuscript to be submitted for publication in Cancer Research. It examines the relationship between loss of *p53* function and the ability of human fibroblasts of the MSU-1 lineage to express characteristics of

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malignant cells, i.e., ability to form foci, colonies in soft-agar, and/or tumors in athymic mice. Focus formation is a transformation phenotype in which cells in culture continue replicating, pile up, or form multilayers when the non-transformed cells form a confluent monolayer. Anchorage-independence, i.e., the ability of cells in culture to form colonies in semi-solid medium, reflects the ability of cells to grow without attachment. Athymic mice are used as animal models for assessing the tumorigenicity of human cells. It had been shown in the Carcinogenesis Laboratory that carcinogens can induce focus formation in MSU-1.1 cells, and that a high percentage of the focus-derived cells have lost the function of *p53*. It was also found that only cells that had lost *p53* could form tumors in mice. My research was devoted to testing whether loss of *p53* causes the cells to form foci in culture and malignant tumors in mice. The strategy I used was to transfect a wild-type *p53* gene into a focus-derived malignant cell strain that had lost both copies of the gene and determine whether expression of p53 could reverse the cell's transformed phenotypes. The results clearly indicate that *p53* suppresses focus formation, anchorage-independence, and the ability to form tumors. Terrence P. McManus, the second author, provided critical insight into the relationship between focus formation and loss of *p53* and carried out fundamental background studies on this important question.

Chapter 3 is a paper published in the Proceedings of the National Academy of Sciences USA in 2000. It describes the earliest study on the role of the human *REV1* gene in UV-induced mutagenesis. The yeast counterpart of *hREV1* was known to play an essential role in damage-induced mutagenesis and

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to function as an auxiliary factor required for DNA polymerase zeta to carry out synthesis past lesions that block replication. Drs. Peter Gibbs and Christopher Lawrence of the University of Rochester cloned the *hREV1* gene. My role was to test whether *hREV1* is required for mutagenesis induced by UV in human cells. Using an antisense RNA strategy, I found that cells expressing a high level of *hREV1* antisense RNA show a greatly reduced frequency of UV-induced mutants. The third author, Ziqiang Li, carried out several mutagenesis assays, and the fourth author, Terrence P. McManus, carried out the transfection of the human cells and isolation of the transfectant clones to be screened for expression of the antisense RNA.

Chapter 4 represents a manuscript to be sumitted for publication in Molecular and Cellular Biology. It summarizes further studies carried out to determine the role of *hREV1* in UV and BPDE-induced mutagenesis. The data indicate that antisense RNA-expressing cells exhibit a greatly reduced frequency of BPDE-induced mutants compared to normal cells and that the frequency of mutants induced by UV or by BPDE in the antisense RNA-expressing cells was significantly increased by expression of exogenous hRev1 protein. These data strongly suggest that hRev1 is required for both UV- and BPDE-induced mutagenesis. Sequencing of the *HPRT* cDNA amplified from 6-thioguanine-resistant clones derived from the normal parental cells and cells expressing the exogenous hRev1 protein show that *hREV1* is required for the majority of damage-induced base substitutions. Dr. Christopher Lawrence, the second author, provided critical insights into the possible function of the human homolog

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of yeast Rev1 and the polyclonal antibody 468 against hRev1. Dr. Yoshiki Murakumo, the third author, constructed and provided the plasmid for the expression of Flag-hRev1 protein.

The Appendix is a review article regarding the roles of yeast and human *REV1* and *REV3* in mutagenesis and translesion replication. It was prepared by Dr. Christopher Lawrence, and he presented a summary of the research in the 2000 Cold Spring Harbor Symposium on Quantitative Biology, entitled Biological Responses to DNA Damage. The text, which constitues the Appendix, has been published in the Cold Spring Harbor Symposium on Quantitative Biology Volume LXV. My contribution to this paper was to provide the data on the role of *hREV1* in UV mutagenesis in human cells.

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CHAPTER 1

Literature Review

I. Carcinogenesis is a multistep process

A. Characteristics of cancer

Cancer is a result of uncontrolled cell proliferation. Under normal conditions, the multiplication of cells is tightly regulated. Very occasionally, this exquisite control system breaks down, and a cell begins to grow and divide without responding to regulation. Ultimately, the indefinite proliferation gives rise to cancer. At least six essential alterations occur to cancer cells (Hanahan and Weinberg, 2000). These characteristics include self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion from apoptosis, unlimited replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

B. Evidence for the multistep nature of carcinogenesis

Several lines of evidence have led to the insight that carcinogenesis is a multistep or multi-event process. The incidence rate of most common human cancers, including cancers of the prostate, stomach, skin, rectum, pancreas and esophagus, increases dramatically with age (Miller, 1980). Apparently a longer period allows more events to happen. Patients who undergo radiation therapy often develop new cancers, but the tumors do not form immediately. Neoplasia of different levels of malignancy can be found in the colon (Kinzler and Vogelstein, 1996). In familial adenomatous polyposis (FAP) patients, one or a few, but not

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all, of the hundreds of adenomatous polyps eventually progresses to cancer. In sporadic colorectal tumors, malignant carcinoma and premalignant adenoma are frequently found adjacent to one another. Transfer of one oncogene was not sufficient to cause obvious changes in primary rodent fibroblasts, but co-transfer of two oncogenes caused abnormal growth of recipient cells, allowing formation of foci of piled-up cells, and these focus-derived cells formed tumors in mice (Land et al., 1983). Human papilloma viruses have many subtypes, but only those whose E6 and E7 proteins respectively bind Rb and p53 proteins are associated with cervical cancers in human (Munger et al., 1989). Three lines of systematic research supporting the mutlistep nature of carcinogenesis will be discussed in a greater detail below.

1. Colorectal tumorigenesis

a. Mutations found in colorectal tumors

Clinical and histopathology studies have identified in the colon of patients several types of neoplasia, including hyperproliferative epithelium (hyperplasia), adenoma (benign tumor), carcinoma-in-situ (malignant tumor) and invasive carcinoma and have suggested that most, if not all, carcinomas arise from preexisting adenomas (Toribara and Sleisenger, 1995). The availability of tissues at different steps provides much advantage in studying the genetic and molecular mechanisms underlying the multistep carcinogenesis.

The first gene identified to involve in colorectal carcinogenesis is the *APC* (Adenomatous Polyposis Coli) gene (Bodmer et al., 1987; Leppert et al., 1987). It was assigned to chromosome 5q21-22 in genetic linkage studies which compare

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the frequency of coinheritance of disease phenotypes and chromosomal markers (genotypes). Mutation of *APC* gene is responsible for the hereditary disease familial adenomatous polyposis (FAP). Loss of heterozygosity (LOH) in this gene was also found in 20% of sporadic colorectal tumors (Solomon et al., 1987). The *APC* gene was subsequently cloned by the positional cloning strategy (Groden et al., 1991; Kinzler et al., 1991). Studies indicated that more than 80% of the tumors that developed in FAP and non-FAP patients had at least one inactivating mutation in *APC* gene (Nishisho et al., 1991; Miyoshi et al., 1992; Powell et al., 1992).

The *APC* gene encodes a cytoplasmic protein of 2843 amino acids. Sequence analysis of this large protein has identified several domains and motifs that are important for APC function. These include homodimerization domain, the Asef-binding armidillo domain, domains for binding β-catenin, axin and microtubule, and nuclear import and export sequences (Sieber et al., 2000). The key tumor suppressor function of APC lies in its ability to bind and destabilize the free β-catenin protein (Smits et al., 1999). The mutation hot spots or mutation cluster region identified in *APC* gene of both FAP and sporadic colorectal cancer patients reside in the β-catenin binding and regulation domain of APC protein (Sieber et al., 2000). In the 15% of colon carcinomas that retain wild-type *APC*, mutations were found in β-catenin (Morin et al., 1997). In addtion, mutations in genes in the same pathway as β-catenin can also initiate tumorigenesis (for a review, see Bienz and Clevers, 2000). In normal cells, APC protein forms a complex with axin, which recruits β-catenin and facilitates the phosphorylation of

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β-catenin by glycogen synthase kinase 3β (GSK3-β). Phosphorylated β-catenin is targeted for deconstruction at the proteasome. GSK3-β can also phosphorylate APC and axin, and thereby enhance the binding of β-catenin to APC-axin complex and increase β-catenin degradation. The activity of GSK3-β is modulated by the Wnt-1 pathway. Upon binding of Wnt-1 peptide to its transmembrane receptor frizzled, the dishevelled protein is activated and inhibits the activity of GSK3-β. The inhibition of GSK3-β reduces the phosphorylation of β-catenin and thereby represses its degradation. The resulting accumulation of nuclear and cytoplasmic β-catenin up-regulates the transcription of several proto-oncogenes including cyclin D1 and c-myc by transcription factor Tcf (Sieber et al., 2000).

Mutation of a *ras* gene is another important factor in colorectal tumorigenesis. There are three members in the *ras* gene family: N-*ras*, H-*ras* and K-*ras*, located on chromosomes 1, 11 and 12, respectively (Barbacid, 1987). The *ras* gene encodes a small GTPase associated with the inner surface of the plasma membrane. Like all other GTPases, it exists in a GDP-bound, inactive form and a GTP-bound, active form. The GDP-bound form is the major form in unstimulated state. Guanine nucleotide exchange factors (GEF) catalyze the release of GDP from Ras, allowing GTP to bind. The GTP-bound Ras protein interacts with down stream effectors and activates them. Finally, the intrinsic GTPase activity of Ras, further enhanced further by GTPase activating proteins (GAP), hydrolyzes the GTP into GDP and inactivates Ras. In cells, Ras protein acts as a relay switch that transmits extracellular signal like epidermal growth

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factor (EGF) to intracellular signaling cascades. The best characterized cascade is the mitogen-activated protein kinases (MAPK) pathway involved in controlling cell proliferation, through the effector protein Raf serine/threonine kinase. Other effectors include lipid kinases such as phosphoinositide 3-phosphate lipid kinases (PI3Ks) and some guanine nucleotide exchange factors (GEF) (Marshall, 1996). The links between activated Ras protein and deregulated cell cycle are cell cycle regulatory proteins, such as cyclin D1 and cyclin-dependent kinase inhibitors, including p21 and p27 (Pruitt and Der, 2001).

Mutation of one or other of the *ras* proto-oncogenes or amplification of a *ras* gene (Bos, 1988) has been found in many types of human neoplasia. The majority of alterations are point mutations in codons 12, 13 and 61. The point mutations abolish the intrinsic GTPase activity and result in a constitutively activated Ras protein, while gene amplification causes an elevated level of Ras protein. In colorectal tumors, 40-50% have point mutations (Bos et al., 1987; Forrester et al., 1987), and among the *ras* gene family, K-*ras* is the most frequently mutated *ras* gene (Vogelstein et al., 1988).

More than 75% of colorectal carcinomas examined showed loss of chromosome 17p (Fearon et al., 1987). Chromosome mapping with twenty chromosome 17p markers localized the deleted region to 17p12 to 17p13.3 (Baker et al., 1989). This region contains *p53* (Isobe et al., 1986), which is a potent tumor suppressor gene mutated in more than half of human cancers (Greenblatt et al., 1994). Li-Fraumeni syndrome patients inherit loss of the

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function of one of their *p53* genes and retain only one functional copy of that gene. They are known to be predisposed to various cancers (Malkin et al., 1990).

In addition to chromosomes 5q and 17p, where the *APC* gene and *p53* gene are located, allelic loss of 18q is also frequently found in colorectal cancers (Vogelstein et al., 1989). The gene lost in chromosome 18q was originally believed to be the *DCC* gene (deleted in colorectal cancer), which encodes for a protein that shares a significant sequence similarity to cell adhesion molecules such as N-CAM (Fearon et al., 1990; Cho et al., 1994). However, recent evidence indicates that loss of an adjacent gene, *DPC4/SMAD4* (DPC: deleted in pancreatic cancer), localized to 18q21 and lost in about 90% of human pancreatic carcinomas (Hahn et al., 1996), is more likely to be the event playing a role in colorectal carcinogenesis (Thiagalingam et al., 1996). In addition, mutations in the *SMAD4* gene also cause Juvenile Polyposis Syndrome, a disorder characterized by a predisposition to hamartomatous polyps and gastrointestinal cancer (Howe et al., 1998). SMAD4 functions in the transforming growth factor β (TGF-β) signaling pathway. Upon the binding of TGF-β to its receptor, the SMAD4 protein forms a heteromeric complex with two other proteins, SMAD2 and SMAD3, and the complex translocates into the nucleus and interacts with transcription factors that transactivate inhibitory factors of cell cycle, such as p15 (Feng et al., 2000). Disruption of *SMAD4/DPC4* function abolishes TGF-β-induced growth control and apoptosis (Derynck et al., 1998)

b. Stages of the occurrence of mutations

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Benign tumors (adenoma) and malignant tumors (carcinoma) often coexist in colorectal cancer patients. Based on the size, grade of dysplasia and villous component of adenomas, Vogelstein et al. (1988) have further divided the adenomas obtained from patients into three subgroups, Class I, II and III. Class I tumors are small tubular adenomas with low-grade dysplasia. Class II adenomas are lesions that had not progressed to carcinoma, and Class III are adenomas that have given rise to small areas of invasive adenocarcinoma. Establishment of these three benign stages and one malignant stage allows the molecular charaterization of genetic changes occurring at each step.

About 50% of Class II and III adenomas have K-*ras* mutations, which is similar to the frequency of carcinomas. In addition, K-*ras* mutation occurs in 58% of adenomas larger than 1 cm, but only in 9% of those under 1 cm in size. Allelic loss of chromosome 5 is not detected in all Class I adenomas examined, but is found in 29% of either Class II or III adenomas and 36% of carcinomas. Loss of heterozygosity of chromosome 18q and 17p is not common in Class I and II adenomas (less than 13%). But it is common in carcinomas (about 75%). While loss of 17p sequence is found in 24% Class III adenomas, LOH of 18q is found in about 50% of Class III adenomas. These data revealed a possible order and timing of genetic changes in colorectal carcinogenesis. That is, *Ras* gene mutation and deletion of *APC* gene happen relatively early but probably neither of them is the first event. Alterations in 18q and 17p happen later, and deletion in 18q precedes the loss of 17p. These data indicate that carcinogenesis happens in several steps, and every step involves a genetic change.

2. Malignant transformation studies of the MSU-1 cell lineage

To investigate the mechanisms of malignant transformation of human cells, McCormick and colleagues (Morgan et al., 1991) developed an infinite life span human fibroblast cell strain, MSU-1.1. This was achieved in part by transfection of a v-*myc* oncogene into a diploid normal fibroblast strain (designated LG1) derived from the foreskin of a normal neonate. The vast majority of the descendants of this cloned transfectant eventually entered crisis and senesced. However, continued feeding and subculturing of the population yielded an infinite life span cell strain that had escaped senescence. This cell strain, designated MSU-1.1, has a stable, near-diploid karyotype composed of 45 chromosomes, including two marker chromosomes. It expresses endogenous telomerase activity, as shown by the Telomeric Repeat Amplification Protocol (TRAP) assay (McCormick et al., unpublished data), and grows in 5% serum as rapidly as early passage finite life span cells grown in 10% serum. Examination of populations stored frozen at earlier passages revealed the presence of a diploid precursor strain, designated MSU-1.0, which grows slowly. The MSU-1.0 cells cannot be transformed into malignant cells, whereas the MSU-1.1 cells can be. The possible role of the two marker chromosomes present in the MSU-1.1 cell strain in malignant transformation is under vigorous investigation.

In contrast to primary normal human fibroblasts, the nontumorigenic MSU-1.1 cells can be transformed into cells capable of forming tumors in athymic mice with a short latency by transfecting them with an activated (mutated) H-*ras* (Hurlin et al., 1989), N-*ras* (Wilson et al., 1990) or K-*ras* (Fry et al., 1990), or by

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exposing them to a single dose of γ-radiation (Reinhold et al., 1996; O'Reilly et al., 1998) or chemical carcinogen (Yang et al., 1992; Boley et al., 2000). Examination showed that the cells derived from tumors produced by cells overexpressing such oncogenes remained diploid (Hurlin et al., 1989; Fry et al., 1990), whereas the cells transformed to malignancy by carcinogen treatment exhibited chromosomal changes additional to those two marker chromosomes (Yang et al., 1992; Reinhold et al., 1996; O'Reilly et al., 1998; Boley et al., 2000). For example, a large fraction (≥40%) of independent cell strains derived from foci formed by MSU-1.1 cells treated with carcinogens had lost their p53 transactivation function (O'Reilly et al., 1998; Boley et al., 2000). In a focus-derived cell strain, designated MSU-1.1-γ1-2A1, one copy of chromosome 17 was lost. These data suggested that the loss of *p53*, a tumor suppressor gene, plays a causal role in transformation of MSU-1.1 cells by carcinogens. In summary, in this lineage of cells, as the cells become more tumorigenic, i.e., from the normal LG1 cells, the immortal MSU-1.0 cells, the MSU-1.1 cells with marker chromosomes, to tumorigenic cells transformed by oncogenes or carcinogens, more genetic alterations occur, and the changes can be gain-of-function of oncogenes or loss-of-function of tumor suppressor genes. This lineage of cells provides an excellent model of stepwise malignant transformation of normal human fibroblasts in culture.

3. Transformation of normal cells with defined steps

Primary rodent cells can be easily transformed into malignant cells by expressing two oncogenes (Land et al., 1983 and Ruley, 1983), but efforts trying

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to convert primary human cells into tumorigenic cells by transfecting oncogenes have yielded little success. Very recently, Hahn et al. (1999) reported a successful tumorigenic conversion of normal human epithelial and fibroblast cells by successive ectopic expression of the telomerase catalytic subunit (hTERT), the simian virus 40 large-T antigen and an oncogenic allele of H-*ras*. This same set of genes can transform primary human mammary epithelial cells as well (Elenbaas et al., 2001). These data illustrate elegantly the mutlstep nature of carcinogenesis and indicate that a minimal of three events, i.e., telomere maintenance (by hTERT), inactivation of growth control (of p53 and pRb by SV40 large-T antigen), and constitutive activation of the mitogen-response pathway (via Ras) are sufficient to create tumor cells directly from primary human cells.

C. Cancer genes

Three classes of genes are mainly involved in carcinogenesis. They are oncogenes, tumor suppressor genes and repair genes. These genes will be briefly discussed.

1. Oncogenes

Oncogenes are genes that are able to transform cells in culture or to induce tumors in animals. They are often mutated forms of protooncogenes, i.e., cellular genes that function to encourage the normal cell growth (Bishop, 1995). The conversion of a protooncogene into an oncogene involves a gain-of-function mutation, which could be point mutation, gene amplification or chromosomal translocation. Oncogenes are often constitutively active, or expressed at an

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inappropriate level. The activation or overexpression of oncogenes promotes uncontrolled cell growth. Because of this gain-of-function mode of action, mutation in one copy of the oncogene, rather than mutations in both copies, is enough to produce its oncogenic effects.

The hallmark of cancer is uncontrolled cell growth. One of the most important mechanisms of controlling cell growth is the signal transduction cascade triggered by growth factors. This cascade often consists of extracellular growth factor, membrane-bound growth factor receptor, intracellular signal transducers and nuclear transcription factors. Indeed, many oncogene products participate in these signal transduction cascades (Lodish et al., 2000). For example, the *sis* oncogene, encodes a type of platelet-derived growth factor. Many human breast cancers overexpress Her2 oncoprotein, which is a receptor for epidermal growth factor and other related hormones. *Ras*, the first human oncogene identified, encodes a small GTPase, one kind of signal transducer involved in the MAP kinase pathway. Overexpression of transcription factors Fos, Jun, and Myc can induce transformation (Lodish et al., 2000).

2. Tumor suppressor genes

Tumor suppressor genes are genes that normally function to inhibit cell growth, and when they are mutated, cells will grow without a restraint. For a tumor suppressor gene to lose its function, both copies of this gene must be inactivated. The ways for inactivating tumor suppressor genes can be allelic loss, mutations or gene siliencing. At least four broad classes of genes are recognized as tumor suppressor genes (Lodish et al., 2000). They are inhibitors of cell cycle

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progression, such as RB, p21 and p16; receptors that function to inhibit cell growth, like TGF-β receptors, genes involved in checkpoint controls, e.g., ATM, BRCA1 and genes promoting apoptosis, like p53.

The retinoblastoma gene (*RB*) is the first tumor suppressor gene identified (Friend et al., 1986; Lee et al., 1987). Loss of function of this gene underlies the development of retinoblastoma, a malignant tumor arising in the undifferentiated retina of one or both eyes in early childhood. Since children with hereditary retinoblastoma develop tumor from only about one cell among 10^6 developing cells in the retina, the inherited mutation of *RB* is recessive to the wild-type allele. Loss of the function of the second allele is required for a tumor to form. The Rb gene is the prototype of tumor suppressor gene.

The Rb protein is a nuclear protein, which normally binds to the E2F family of transcription factors (E2F) and represses the transcription of genes required for G1 to S transition of the cell cycle by E2F (Weinberg, 1995). When Rb protein is phosphorylated by cyclin-dependent kinases complexed with cyclin proteins, the Rb is inactivated and the transcription by E2F is activated and the cell cycle progresses (Lundberg and Weinberg, 1998; Harbour et al., 1999). Inactivation of Rb removes the constraint on cell cycle progression imposed by Rb. Expression of exogenous Rb protein in retinoblastoma or osteosarcoma cell strains that had inactivated endogenous RB genes suppresses the growth, anchorage-independence and tumorigenicity of these tumor cells (Huang et al., 1988).

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Repair genes are genes involved in various DNA repair mechanisms functioning in human cells. They do not affect the cell growth directly but reduce the mutation rate of the entire genome, including oncogenes and tumor suppressor genes. In mammalian cells, DNA repair pathways repair ~100,000 modifications happening to each DNA molecule every day (Friedberg et al., 1995). Failure in these genome maintenance mechanisms causes cancer susceptibility in a variety of tissues (Hoeijmakers, 2001).

The importance of DNA repair in guarding against cancer is best illustrated in the Hereditary Non-Polyposis Colon Cancer (HNPCC), which accounts for 5-8% of all colon cancers. The first genetic locus linked to HNPCC was mapped to chromosome 2p15-16 (Peltomaki et al., 1993). The gene deleted in this region was found to be a human homologue of bacteria *MutS* (*hMSH2*), a gene required for mismatch repair (MMR) (Leach et al., 1993; Fishel et al., 1993). Mutations in other genes involved in human MMR were subsequently found, including *hMLH1* (Bronner et al., 1994; Papadopoulos et al., 1994), *PMS1* and *PMS2* (Nicolaides et al., 1994), all of which are homologues of the prokaryotic *mutL* gene. Indeed, 92% of tumors derived from 74 HNPCC kindreds showed microsatellite instability, a characteristic of MMR deficiency, and 70% of the 48 kindreds with such instability had mutations in the coding region of 5 MMR genes known at that time (Liu et al., 1996). Defects in the *hMLH1*, *hMSH2* and *hMSH6* are three most common germline mutations among HNPCC, with approximately 50%, 40% and 10% frequency respectively (Peltomaki, 2001).

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The MMR mainly deals with mismatches that have escaped the exonucleolytic proofreading of the replication machinery and insertion/deletion loops that are caused by slippage of DNA polymerase during replication (Jiricny, 1998). In bacteria, the lesions are recognized by the homodimeric MutS protein. Binding of MutL homodimer to MutS helps bringing the MutH protein, a strand discrimination factor and other necessary factors. The outcome of MMR is the removal of mistakes on the newly synthesized daughter strand. In humans, the heterodimer of hMsh2 and hMsh6 recognizes mismatches and heterodimer of hMsh2 and hMsh3 recognizes insertion/deletion loops. To mediate the interaction between recognition complex and other factors, hMlh1 forms a heterodimer with hPms2, hPms1 or hMlh3. The human MMR system is similar to the prokaryotic MMR, but has a higher complexity (Harfe and Jinks-Robertson, 2000).

Deficiency of MMR causes at least a 100-fold increase in mutation rate of microsatellite repeat sequences (Parsons et al., 1993), and enhances mutations in protooncogenes and tumor suppressor genes. In tumor cells with microsatellite instability, frameshift mutations have been found in tumor suppressor genes such as *TGFβRII* (Markowitz et al., 1995) and *PTEN* (Guanti et al., 2000). As models for human disease, mouse knockouts of *MSH2*, *MSH6*, *PMS2*, or *MLH1* showed a prediposition to diverse types of tumors (Harfe and Jinks-Robertson, 2000).

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D. The *p53* gene

1. From a tumor antigen to an oncogene, and from an oncogene to a tumor suppressor gene

The protein p53 was first identified as a ~53kD protein interacting with the large-T antigen protein encoded by the small DNA tumor virus simian virus 40 (SV40) (Lane and Crawford, 1979). Because the level of this p53 protein in the transformed cells was enhanced by SV40 infection or transformation (Linzer and Levine, 1979), and SV40 infection was indispensable for the induction and maintenance of the transformed state, p53 was believed to be a tumor antigen. Subsequent studies on p53 revealed that many tumor-derived cells or cells transformed in culture exhibited a higher level of p53 protein than normal cells (DeLeo et al., 1979) and that p53 protein expressed in SV40-transformed cells had a much longer half-life than p53 protein in normal cells (Oren et al., 1981). The fact that cloned *p53* genes could immortalize cells of finite lifespan in vitro (Jenkins et al., 1984) and could cooperate with the activated H-*ras* oncogene in transforming normal cells (Eliyahu et al., 1984; Jenkins et al., 1984) strongly suggested that *p53* was an oncogene. It is now well established that *p53* is a tumor suppressor gene. The *p53* genes that helped transform normal cells (Eliyahu et al., 1984; Jenkins et al., 1984) turned out to be mutant forms of *p53* (Hinds et al., 1989). It was shown that mutant p53 could interfere with the function of wild-type p53 by binding to or competing with the wild-type p53, and in that way cause stabilization of mutant p53 protein (reviewed in Blagosklonny, 2000). What is more, expression of wild-type p53 was found to inhibit oncogene-

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induced focus formation by primary rat embryo fibroblasts (Finlay et al., 1989; Eliyahu et al., 1989). Additional proof that *p53* acts as a tumor suppressor gene comes from genetic studies showing that the chromosomal region harboring the *p53* gene is lost at very high frequency in colorectal tumors (Vogelstein et al., 1988). It is now known that more than half of human cancers have mutations in the *p53* gene (Greenblatt et al., 1994), and that Li-Fraumeni syndrome patients, who inherit one mutant allele of *p53*, are predisposed to various tumors (Srivastava et al., 1990).

2. Functional domains of p53

The human p53 protein consists of 393 amino acids. It can be roughly divided into three major functional domains, the N-terminal transactivation domain, the central sequence-specific DNA-binding domain, and the C-terminal regulatory domain (Ko and Prives, 1996). The transactivation domain lies within amino acid residues 1-42 (Unger et al., 1992). This domain recruits basic transcription factors like TATA box-binding protein (TBP) (Seto et al., 1992; Truant et al., 1993) and TBP-associated factors (Thut et al., 1995; Farmer et al., 1996). The sequence-specific binding domain was mapped to the region between ~80-300 amino acids (Wang et al., 1993; Pavletich et al., 1993). The consensus sequence that this domain binds to consists of two inverted sequences of 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3', separated by 0-13 base pairs (el-Deiry et al., 1992). Binding of p53 to this sequence in the promoter region of downstream target genes initiates the transcription of these genes. Residues 300-393 is the C-terminal regulatory domain. In this domain, there are two

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subdomains. One, the tetramerization domain, lying within residues ~320-360 (Pavletich et al., 1993), can promote DNA binding and enhance transcription (Stenger et al., 1994). The other, consisting of the last 30 amino acid residues, acts to keep the DNA binding activity of the whole protein latent. This inhibition can be alleviated by deletion of the C-terminus, mild proteolysis of p53, addition of a monoclonal antibody against this region, or phosphorylation by casein kinase II (Hupp et al., 1992). In addition, p53 has a nuclear localization domain, which directs the transport of p53 protein into the nucleus. Compilation of more than 2,500 *p53* gene mutations that were found to have occurred in various human tumors and tumor cell lines revealed that the vast majority of mutations are clustered in the central sequence-specific DNA binding domain (Hollstein et al., 1994), suggesting that p53 exerts its tumor suppression function mainly as a transcription factor.

3. Activation of p53

In normal or unstressed cells, the level and the activity of p53 are both relatively low (Giaccia and Kastan, 1998). This is achieved mainly by two mechanisms, the action of Mdm2 protein and the allosteric inhibition by the C-terminal domain of p53. As a transcription factor, p53 transactivates the expression of its own negative regulator Mdm2 (Barak et al., 1993). The Mdm2 protein can form a complex with p53 protein (Momand et al., 1992) at the transactivation domain of p53 (Oliner et al., 1993) and thereby conceal the transactivation domain from basic transcription factors and reduce the level of transcription induced by p53 (Momand et al., 1992; Oliner et al., 1993). In

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addition, the Mdm2 protein also has a ubiquitin-ligase activity (Honda et al., 1997). Binding of Mdm2 protein to p53 causes ubiquitination of p53 (Fuchs et al., 1998) and therefore, targets p53 for degradation via the 26S proteasome (Haupt et al., 1997; Kubbutat et al., 1997). This latter activity of Mdm2 keeps the level of p53 protein low. The latent activity of p53 is achieved by the C-terminus, which normally folds back and keeps the sequence-specific DNA binding domain in a closed conformation. Together, the actions of Mdm2 and the C-terminus of the p53 protein keep the p53 function dormant.

The cryptic function of p53 can be activated when the cells are under stress. A number of factors can activate p53. These include ionizing radiation, UV radiation, hypoxia, cell adhesion and decreased ribonucleotides. Similarly, a number of mechanisms can activate p53, for example, phosphorylation, acetylation and glycosylation are employed. The complexity in the modulation of p53 has been reviewed in many articles (see for example, Ko and Prives, 1996; Giaccia and Kastan, 1998). The activation of p53 mainly involves stabilization of p53 protein by preventing Mdm2-mediated p53 ubiquitination and induction of the exposure of the sequence specific DNA-binding domain by changing the conformation of the C-terminus. As an example, in response to ionizing radiation, Ser15 of p53 protein is phosphorylated (Shieh et al., 1997; Siliciano et al., 1997), and Lys382 of p53 becomes acetylated (Sakaguchi et al. 1998). Phosphorylation of Ser15 leads to an inhibition in the binding of p53 to Mdm2 protein in vitro and in vivo (Shieh et al., 1997) and acetylation of Lys382 enhances the sequence-specific DNA binding of p53 (Sakaguchi et al. 1998). At

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least two kinases are able to catalyze the phosphorylation at Ser15, i.e., DNA-activated protein kinase (DNA-PK) (Shieh et al., 1997; Woo et al., 1998) and ATM, the product of the gene mutated in patients with ataxia telangiectasia (Siliciano et al., 1997; Banin et al., 1998; Canman et al., 1998). Conceivably, the presence of multiple serine, threonine residues on p53 protein, the availability of other protein kinases, such as checkpoint kinase (Chk1 and Chk2), ATM-rad3-related kinase (ATR), and the alternative ways of modification no doubt make the modulation of p53 activity much more complicated (for review, see Giaccia and Kastan, 1998).

In addition to being activated by stress factors, oncogenes, such as myc, can also activate p53, as a result of reduction of Mdm2 activity by turning on expression of $p14^{ARF}$, a negative regulator of Mdm2 (Prives, 1998). The cellular localization of p53 is another way of regulating p53 activity in the cell (Liang and Clarke, 2001).

4. Tumor suppression functions of p53

a. Growth arrest

As a transcription factor, p53 can activate the transcription of many genes. p53 executes its functions mainly through these downstream genes. *p21* is the most well studied down stream gene (el-Deiry et al., 1993). p21 protein is an inhibitor of many cyclin-dependent kinases (Cdk) (Xiong et al., 1993), especially for Cdk2, the kinase required for G1 to S phase transition (Harper et al., 1993). In doing this, p21 forms a quarternary complex with cyclin/Cdks and PCNA, the sliding clamp for DNA replication (Zhang et al., 1993). In response to DNA

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damage, p21 is upregulated by p53 and G1 arrest is induced (el-Deiry et al., 1994). Embryonic fibroblasts derived from p21 knock-out mice are defective in G1 arrest in response to DNA damage (Deng et al., 1995). The G1 arrest allows cells more time to carry out DNA repair and reduces the chance of introducing mutations into the genome during DNA synthesis at S phase. In addition, the increased function of p21 alone appears sufficient for tumor suppression. Introduction of p21 cDNA suppressed the growth of human brain, lung, and colon tumor cells in culture (el-Deiry et al., 1993). Adenoviral vector-transduced expression of p21 in a p53-deficient mouse prostate cancer cell line significantly suppressed the growth of the cells in culture and the ability of the cells to form tumors in mice (Eastham et al., 1995), and this was also the case for a mouse mammary tumor cell line (Shibata et al., 2001).

b. Apoptosis

A second mechanism by which p53 acts as a tumor suppressor is by inducing apoptosis, a form of programmed cell death. Apoptosis is characterized by distinct morphological phenotypes, including blebbing of the plasma membrane, condensation of the cytoplasm and nucleus, and cellular fragmentation into membrane apoptotic bodies, and by the biochemical hallmark of chromosomal degradation into monomers or multimers of 200 base pairs (Steller, 1995). In response to cellular stress, stabilization and activation of p53 activates the expression of a number of genes involved in apoptosis. Bax, an apoptosis-inducing factor, was the first one identified as participating in p53-dependent pathway (Miyashita and Reed, 1995). By using subtractive

hybridization or differential display to compare tumor cell lines lacking p53 and corresponding cells expressing endogenous or ectopic wild-type p53, it has been possible to identify a number of other genes that play a role in p53-dependent apoptotic pathway (Moll and Zaika, 2001). Examples include PERP, a member of the PMP-22/gas3 tetraspan membrane protein family, cloned after subtracting G1 arrest-associated p53-transactivated messages (Attardi et al., 2000), as well as p53AIP1 and p53DINP1, two factors induced when p21 protein is not induced (Oda et al., 2000; Okamura et al., 2001). PERP, p53AIP1 and p53DINP1 are presumed to be more specific to and more decisive in p53-dependent apoptosis than other factors. The effect of the apoptotic factors can vary depending on the types of tissues or cells, and/or the types of cellular stress. For example, mice lacking a functional *Bax* gene displayed hyperplasia in thymocytes and B cells, massive cell death in multinucleated giant cells and dysplastic cells, and aberrations in other cell lineages (Knudson et al., 1995). What is more, different factors can act in synergy. Bax deficiency only partially reduced the p53-dependent apoptosis in mouse primary fibroblasts expressing the adenovirus *E1A* oncogene, a cellular setting in which it has been shown that in response to DNA damage cells undergo apoptosis, but not G1 arrest (McCurrach et al., 1997). Apoptotic factors induced by p53 activate the caspase cascade and lead to the characteristic apoptotic phenotypes (Schuler and Green, 2001). In addition to transcription of apoptotic genes, mechanisms such as transrepression and direct protein-protein interaction involving p53 have been reported as well (Moll and Zaika, 2001).

c. Inhibition of angiogenesis

When a tumor reaches a certain size, it needs a greater supply of nutrients and oxygen to support its further growth. This is achieved by stimulating angiogenesis. Angiogenesis is controlled locally by stimulatory factors such as vascular endothelial growth fatcor (VEGF) and inhibitory factors such as thrombospondin (Iruela-Arispe and Dvorak, 1997). p53 appears to regulate angiogenesis as well. Li-Fraumeni patients inherit one normal allele and one mutant allele of *p53*. Fibroblasts derived from such patients would retain this characteristic at early passage, but would spontaneously lose the wild-type copy of *p53* in later stage (Yin et al., 1992). Concomitant with the loss of wild-type p53 in continued culture, the level of thrombospondin-1 (Tsp-1) protein secreted into the media was found reduced, and the anti-angiogenic activity of the media tested in vitro and in vivo was decreased as well (Dameron et al., 1994). The modulation of Tsp-1 expression occurred at the mRNA level and was caused by loss of p53 (Dameron et al., 1994). In addition, human bladder cancers with low expression level of Tsp-1 exhibited high microvessel density counts, and were significantly associated with loss of p53 as well (Grossfeld et al., 1997). These data suggest that p53 can inhibit tumor growth by transactivating inhibitory factor(s) of angiogenesis. To the same end, the expression of VEGF, the major angiogenic inducer, is negatively regulated by wild-type p53, and loss of p53 function exhibited an increase in the expression level of VEGF (Kieser et al., 1994; Mukhopadhyay et al., 1995; Bouvet et al., 1998; Pal et al., 2001). The p53 regulates the expression of VEGF at the transcription level (Kieser et al.,1994;

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Zhang et al., 2000c). Deletion of one of the four Sp1-binding elements in the human *VEGF* promoter region completely abolished the transcriptional repression activity of wild-type p53, but how p53 utilizes this Sp1 element in repressing transcription is not clear (Zhang et al., 2000c). Thus, tumor suppressor p53 appears to inhibit angiogenesis by increasing anti-angiogenic factor(s) and decreasing pro-angiogenic factor(s) simultaneously.

d. Senescence

p53 appeared to play a role in cellular senescence as well. Senescent cells are characterized by irreversible growth arrest, resistance to apoptosis, morphological changes, e.g., cellular enlargement, and biochemical alterations, e.g., expression of β-galatosidase with optimum pH of 6 (Itahana et al., 2001). Human fibroblasts in culture that had been immortalized with an inducible SV40 T-antigen underwent senescence following the de-induction of T-antigen, and the senescence could be rescued by re-expressing intact T-antigen from a second plasmid, but not by re-expressing a T-antigen deletion mutant lacking the p53-binding domain (Shay et al., 1991). By using an early passage, human diploid fibroblast stable transfectant cell strain, which expressed β-galatosidase under the control of p53-responsive element, Bond et al. (1996) found a steady increase of β-galatosidase activity and steady decrease in DNA synthesis as cells approached senescence, clearly indicating the involvement of p53 transactivation function in cellular senescence (Bond et al., 1996). Indeed, Sugrue et al. (1997) showed that induced overexpression of wild-type p53 in EJ bladder carcinoma cells lacking functional p53 triggered an onset of irreversible

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growth arrest within 2-3 days, with cells exhibiting morphological and biochemical senescent phenotypes. In addition to its essential role in G1 arrest, p21 is also a strong candidate for p53-dependent senescence (Fang et al., 1999; Sayama et al., 1999; Chang et al., 1999). Normal human diploid fibroblasts with the *p21* genes inactivated by two successive rounds of targeted homologous recombination escaped senescence during continuous growth in culture (Brown et al., 1997). Recently, Jung et al. (2001) showed that the NF-Y transcription factor, which is inactivated in senescent human fibroblasts and is essential for transcription of the cdk1 and cyclin B genes, which are also irreversibly repressed in senescent cells, was inactivated by p53 and its two homologues, p63 and p73, suggesting an alternative mechanism of p53-dependent senescence.

e. Role in DNA repair and homologous recombination

Several properties of p53 allow p53 to have a potential role in DNA repair and homologous recombination. As a transcription factor, p53 transactivates GADD45 gene expression in response to DNA damage (Kastan et al., 1992; Zhan et al., 1994). In addition to inhibiting entry of cells into S-phase by interacting with PCNA (Smith et al., 1994), GADD45 protein has activities related to DNA repair, including recognizing damaged chromatin, modifying DNA accessibility to other proteins (Carrier et al., 1999) and stimulating excision repair (Smith et al., 1994). Studies with fibroblasts heterozygous and homozygous for p53 mutations (Ford and Hanawalt, 1995), normal fibroblasts, and fibroblasts expressing human papillomavirus 16 E6 protein (Ford et al., 1998) showed that

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p53 is required for nucleotide excision repair of UV damage in non-actively-transcribing genomic regions. The p48 gene, whose expression is p53-dependent, and also GADD45 gene, has been implicated as playing a role in this p53-dependent global genomic nucleotide excision repair (Hwang et al., 1999; Smith et al., 2000).

Besides transactivating genes involved in DNA repair, p53 also interacts with proteins physically executing DNA repair. Direct involvement of p53 protein with the base excision repair machinery has been reported (Offer et al., 1999; Zhou et al., 2001). These investigators showed that p53 could interact with AP endonuclease and DNA polymerase β, and that association of p53 with DNA polymerase β stabilized the interaction between DNA polymerase β and abasic DNA. Base excision repair capacity of the cell extracts was much reduced when p53 was immunodepleted (Zhou et al., 2001). Interestingly, p53 protein also possesses a 3'-to-5' exonuclease activity (Mummenbrauer et al., 1996). This activity is carried out by a domain overlapping with the sequence-specific DNA binding domain, but it is differentially affected by factors that activate sequence-specific DNA binding activity (Janus et al., 1999b). The exact role of the exonuclease activity of p53 in vivo remains to be elucidated.

Transcription factor IIH (TFIIH) is a basal transcription factor for RNA polymerase II, and its function is also essential for nucleotide excision repair (NER) (Svejstrup et al., 1996). p53 protein has been found to interact with two TFIIH complex-associated NER factors, XPB and XPD, and a third NER factor, CSB (Wang et al., 1995). Although modulation of NER activity by p53 has been

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suggested (Wang et al., 1995), the role of p53 in NER in repair of actively-transcribing genes seems to be limited. Only defective global genomic NER activity was found in fibroblasts lacking p53 function (Ford and Hanawalt, 1995; Ford et al., 1998). These data suggest that association with TFIIH is a way for activation of p53 protein itself. In contrast to the wild-type p53-dependent apoptotic response in normal cells, primary fibroblasts derived from patients that belong to xeroderma pigmentosum complementation group B or D, i.e., XPB or XPD patients, but not XPA or XPC patients, have an attenuated p53-dependent apoptosis (Wang et al., 1996). Lymphoblastoid cell lines with XPD defect exhibited reduced and delayed apoptosis induced by doxorubicin, a DNA-damaging agent and topoisomerase II inhibitor (Robles et al., 1999). Indeed, the highly purified kinase components of TFIIH, the CDK7-cycH-p36 trimeric complex can phosphorylate the inhibitory C-terminus of p53 and activate the sequence-specific DNA binding activity of p53 in vitro (Lu et al., 1997). The ability of the C-terminus of p53 protein to bind various DNA structures, such as non-specific double-stranded or single stranded DNA, double-strand breaks, Holliday junctions and insertion/deletion mismatches may provide ways for p53 activation (Janus et al., 1999a).

p53 has been shown to exert an inhibitory role on homologous recombination. Wild-type p53 can interact with hRad51 protein and its prokaryotic counterpart, RecA protein, both of which are key factors of homologous recombination (Sturzbecher et al., 1996). Human p53 protein inhibits the activities of RecA required for in vitro recombination (Sturzbecher et al., 1996),

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and this suggests that p53 inhibits homologous recombination in eukaryotes as well. Indeed, inactivation of wild-type p53 function by a dominant negative form of p53 and various viral proteins causes a dramatic increase in the rate of homologous recombination as indicated by an engineered reporter plasmid (Mekeel et al., 1997). An elevated rate of homologous recombination between intrachromosomal direct repeat sequences was also observed in mouse cells expressing mutant p53 (Bertrand et al., 1997). Subsequent studies with cell strains expressing p53 defective in transactivation revealed that the involvement of p53 in homologous recombination does not require its transactivation function (Saintigny et al., 1999; Willers et al., 2000), suggesting that direct participation of p53 protein is involved.

II. The RAD6 pathway for dealing with DNA damage

A. Cellular responses to DNA damage

1. Sources of DNA damage

DNA is the genetic material for essentially all living organisms. Although it is relatively stable, it is still quite structurally dynamic and chemically active. The sources that cause alterations in DNA, or DNA damage can be generally classified into two categories, spontaneous or environmental. Spontaneous alterations include incorporation of incorrect nucleotides during replication, deamination of cytosine, adenine or guanine bases, loss of bases via depurination or depyrimidination, and damage caused by reactive oxygen species. DNA reacts easily with a variety of physical radiation or chemical compounds from the environment as well. Ionizing radiation like X-ray or γ-ray

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causes base damage and single- or double-strand breaks. Ultraviolet (UV) radiation induces dimerization of adjacent pyrimidines. DNA alkylating agents, like *N*-methyl-*N*-nitrosourea (MNU) and methyl methanesulfonate (MMS), drugs used for cancer chemotherapy, such as platinum derivatives or mitomycin C, and reactive metabolites of a myriad of other naturally occurring or man-made chemicals, such as aflatoxin and benzo[a]pyrene all react with and modify the chemistry of DNA in one way or the other (Friedberg et al., 1995). Fortunately, to maintain the integrity of the genome, organisms have evolved mechanisms to deal with these DNA damage.

2. DNA repair and damage tolerance

a. Three major pathways in dealing with DNA damage

A defect in pathways dealing with DNA damage often causes increased sensitivity to agents causing such damage. Thus, sensitivity to DNA damaging agents of a cell that has an inactivating mutation in a gene indicates that this gene may function in DNA repair. Comparison of such sensitivity between this mutant cell and a cell containing an inactivating mutation in this gene and a second gene can reveal the relationship of these two genes, i.e., either synergism or epistasis. If having mutations in two genes makes the cell more sensitive than cells with only one gene mutated, i.e., causes a synergistic effect, these two genes can be said to function in different pathways. If the second mutation does not increase sensitivity, that gene is epistatic to the other, i.e., these two genes are considered to function in the same pathway. By using this strategy, scientists have identified three major pathways dealing with DNA

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damage in yeast *S. cerevisiae*. They are: RAD3, RAD52, and RAD6 pathways (Friedberg et al., 1995). *RAD3*, *RAD52*, and *RAD6* are the most important genes in their own respective pathways because mutations in these three genes are epistatic in radiation-sensitivity to all the other mutants in the same pathway and exhibit the most extreme sensitivity in the same pathway as well. It is now well demonstrated that the RAD3 pathway is the excision repair pathway and the RAD52 pathway carries out homologous recombination. The RAD6 pathway is least understood, but in principle, it represents a damage tolerance mechanism that allows continuation of DNA replication without lesion removal (Friedberg et al., 1995). The yeast RAD3 and RAD52 pathways are largely conserved in humans, and will be described very briefly in the following sections, followed by a thorough description of the RAD6 pathway.

b. Excision repair

Excision repair refers to the excision of damaged or inappropriate bases or nucleotides and subsequent repair synthesis of correct bases or nucleotides. It includes three closely related but mechanistically distinct subpathways, i.e., base excision repair, mismatch repair and nucleotide excision repair. Base excision repair utilizes DNA glycosylase to excise the damaged or inapporiate bases, 5' AP endonuclease and DNA deoxyribophosphodiesterase to remove the deoxyribose-phosphate moiety, and DNA polymerase and ligase to fill the single nucleotide gap and to ligate the nicked DNA. Mismatch repair deals with mismatched base pairs and small insertion/deletion loops by specifically removing the single mispaired nucleotide or few affected nucleotides. The third

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one, nucleotide excision repair recognizes lesions that distort the helical struture of DNA and recruits a complex enzymatic machinery to excise an oligonucleotide of ~25-30nt containing the damage. The resulting gap is filled in by DNA polymerases and the repair is finished by DNA ligase. The excision repair system is evolutionarily conserved from prokaryotes to eukaryotes (Friedberg, et al., 1995).

c. Homologous recombination

Homologous recombination is the major pathway for repairing DNA double strand breaks caused by exogenous or endougenous agents (Johnson and Jasin, 2001). Homologous recombination takes place in a stepwise manner. First, binding of the Rad50/Mre11/Nbs1 complex to the 3' ends of the strand breaks exposes the 3'-OH groups, and promotes strand invasion. Then, filament assembly through Rpa, Rad51, Rad51-related proteins and Rad52 allows single strand exchange and DNA synthesis to occur. Finally, the Holliday junction is resolved by resolvase. The activation of homologous recombination requires the function of upstream protein kinases including ATM, a kinase mutated in ataxia telangiectasia and ATR (ATM- and Rad3-related). Defects in homologous recombination as a result of mutations in these upstream kinases predispose patients to lymphomas and other types of cancer (Hoeijmakers, 2001).

B. Damage tolerance: RAD6 pathway in *Saccharomyces cerevisiae*

1. Principal genes involved

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At least 15 genes have been assigned to the RAD6 pathway in yeast (Lawrence, 1994). *RAD6* and *RAD18* are the founding members of the pathway. Mutants for *RAD6* and *RAD18* genes are very sensitive to the cytotoxic effect of a broad range of DNA damaging agents, including UV, X-ray, γ-ray, 4-nitroquinoline-*N*-oxide, trimethoprim, bleomycin, and monofunctional and bifunctional alkylating agents (Friedberg, et al., 1995). A *rad6* mutant strain is unable to convert the low molecular weight DNA synthesized on UV-damaged DNA templates into normal high molecular weight form, and *rad18* strain is greatly inhibited in this process (Prakash, 1981). Unlike *rad6* mutants, which display a loss of UV- and chemical carcinogen-induced mutagenesis (Lawrence et al., 1974, 1976 and Prakash, 1974), *rad18* mutants show normal mutagenesis (Jones et al., 1988). The rates of spontaneous mutation and both spontaneous and induced mitotic recombination are increased in both *rad6* and *rad18* strains (Kern and Zimmermann, 1978). Biochemical analyses indicate that the yeast *RAD6* gene encodes a ubiquitin-conjugating enzyme (Jentsch et al., 1987) and the *RAD18* gene encodes a protein possessing DNA binding and nucleotide binding activities (Jones et al., 1988). The ability of yeast Rad6 and Rad18 proteins to form a heterodimer (Bailly et al., 1994 and 1997) suggest that Rad6 protein could be targeted to damage-containing DNA regions by Rad18 protein. In addition, the ubiquitin-conjugating activity of Rad6 protein is absolutely required for its function. The substitution of residue Cys88, an essential residue for the conjugating activity of Rad6 protein, with either alanine or valine completely abolished the functions of wild-type Rad6 in DNA repair and

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mutagenesis. The defects caused by this point mutation are equivalent to those conferred by *rad6* null mutants (Sung et al., 1990).

b. RAD5

Strains mutant for the *RAD5* gene are more sensitive to the cytotoxic effect of UV and other agents than wild-type strains, but the sensitivity conferred by a *RAD5* mutation is less than those caused by *RAD6* or *RAD18* mutations (Johnson et al., 1992). Epistasis analysis for UV-sensitivity assigns *RAD5* gene to the RAD6 pathway (Johnson et al., 1992). *RAD5* plays virtually no role in mutations caused by UV (Lawrence and Christensen 1978). Cloning of the *RAD5* gene reveals that this gene encodes a protein of 1,169 amino acids. The protein contains seven domains characteristic of helicases, one cysteine-rich motif that could form DNA binding zinc-finger domain, and one leucine zipper DNA binding motif (Johnson et al., 1992).

c. RAD30 and *PCNA*

Amino acid sequence alignment analysis of the complete genome of yeast *S. cerevisiae* revealed one gene, (SCD9461.8; YDR419W), whose gene product shares homology with bacteria UmuC, DinB proteins and eukaryotic Rev1 protein (Kulaeva et al., 1996). The gene was designated as *RAD30* when deletion of this gene was found to confer mild sensitivity to UV radiation (McDonald et al., 1997 and Roush et al., 1998). Epistasis analysis places *RAD30* in the RAD6 pathway (McDonald et al., 1997). *RAD30* disruption strains are more sensitive to the cytotoxic effect of UV than wild-type strains, but *rad30 rad6* and *rad30 rad18*

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double mutant strains are no more sensitive to UV than *rad6* or *rad18* single mutants. *RAD30* does not belong to the RAD3 or RAD52 epistatis group, since *RAD30* mutation is additive to either *RAD1*, a gene in the RAD3 group, or *RAD52* mutations in UV sensitivity. *RAD30* does not work in the same subpathways as *REV3*, *REV7*, *REV1* or as *RAD5* (McDonald et al., 1997). *rad30 rev3*, *rad30 rev7*, and *rad30 rev1* double mutants are all more sensitive to killing by UV than are single mutants for either *rad30*, *rev3*, *rev7*, or *rev1*. *rad30 rad5* double mutants are more sensitive to either single mutant as well. Interestingly, unlike *rev3*, *rev7* and *rev1* mutants, which are all deficient for UV-induced mutagenesis, *rad30* mutants show an increase in UV-mutagenesis, indicating that *RAD30* functions in an error-free manner in dealing with UV damage. In summary, *RAD30* appears to function in a *RAD6/RAD18*-dependent, *REV*-independent, error-free mechanism that does not involve *RAD5*. Biochemical analysis demonstrated that Rad30 protein is a translesion DNA polymerase that can replicate efficiently and faithfully past a thymine-thymine cis-syn cyclobutane dimer, incorporating two correct adenines opposite the misinstructional thymine-thymine dimer template (Johnson et al., 1999a). Rad30 has, therefore, been named DNA polymerase eta. Yeast Pol eta also has been shown to bypass cyclobutane dimers and (6-4) photoproducts formed at CC and TC sites in error-free manner (Yu et al., 2001). Kinetic analysis (Washington et al., 2001) and structural analysis (Trincao et al., 2001) also support the error-prone mode of action of yeast Pol eta. The DNA polymerase activity of Rad30 is essential for its biological function, since Rad30 protein carrying an inactivating Asp-Glu to Ala-

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Ala mutation in the highly conserved SIDE sequence cannot complement the *rad30* deletion mutation (Johnson et al., 1999c).

Proliferating cell nuclear antigen (PCNA), as a homotrimer, forms a ring-shape conformation and serves as sliding clamp or processivity factor for the major replicative DNA polymerase, Pol delta, during DNA replication and for DNA polymerase epsilon filling the gap in DNA during nucleotide excision repair (Shivji et al., 1992). The gene encoding *S. cerevisiae* PCNA is *POL30*. Because a null mutation of *POL30* is lethal to the yeast, the role of PCNA in the RAD6 pathway of DNA repair could not be determined until the mutant *pol30-46* was identified. Simultaneous mutations of two adjacent charged residues (Asp, Glu, Lys or Arg) to two alanines in the PCNA protein gave rise to two mutant proteins (pol30-9 and pol30-22) that allow normal growth and confer modest UV sensitivity (Ayyagari et al., 1995). Combination of the mutations in these two double mutants yielded the pol30-46 mutant (Torres-Ramos et al., 1996). This mutant exhibits a higher degree of UV sensitivity than either double mutant, but the degree of cytotoxicity, compared to wild-type yeast, is still mild. The pol30-46 mutant protein is as efficient as wild-type PCNA in being loaded by the clamp loader RFC protein and in mediating processive DNA synthesis by Pol delta or epsilon. Epistasis analysis clearly places *PCNA* into a *REV3*-independent RAD6 pathway, and not in the RAD3 or RAD52 pathways.

Amino acid sequence analysis reveals a potential PCNA-binding motif on the C-terminus of yeast Pol eta protein (Haracska et al., 2001a). Indeed, wild-type Pol eta protein forms a complex with PCNA since they are coeluted from

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size-exclusion columns. Also, addition of PCNA protein to the in vitro primer extension reactions greatly enhances the DNA synthesis catalyzed by Pol eta. On the other hand, evidence from a yeast two-hybrid system indicated that mutant Pol eta protein carrying either a deletion of the C-terminal eight amino acids or a substitution of two conserved amino acids loses the ability to bind PCNA protein. Although the mutant Pol eta proteins still retain robust DNA polymerase and T-T dimer in vitro bypass activities identical to wild-type protein, expression of either of these two mutant proteins in *rad5 rad30* double deletion mutants cannot increase the UV survival of the strain to the level that wild-type Pol eta protein achieves. These data suggest that interaction with PCNA is essential for Pol eta function in vivo. Therefore, it is very likely that Rad30 (Pol eta) functions with PCNA in one branch of error-free *RAD6*-mediated pathway.

d. Ub, MMS2 and *UBC13*

Ubiquitin is a small peptide of 76 amino acids. It is best known as a covalent degradation signal for cellular proteins. The ubiquitin molecules in the multiubiquitin chain for efficient degradation of substrate protein via the 26S proteasome are usually linked by Lys48-Gly76 isopeptide bonds (Hochstrasser, 1996). In a study looking at the possibility of multiubiquitin chain formation via alternate lysine residues, Spence et al. (1995) found that substitution of Lys-63 with arginine (UbK63R) causes disappearance of a family of abundant multiubiquitin protein conjugates. Interestingly, the overall protein turnover in the *UbK63R* mutant strain is not affected, as evidenced by the normal growth rate, normal levels of ubiquitin-protein conjugates, and fully efficient turnover of short-

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lived proteins in such mutant yeast (Spence et al., 1995). Cytotoxicity assays showed that the *UbK63R* mutation confers sensitivity to MMS and UV, indicating the presence of a defect in DNA repair in *UbK63R* mutant. Epistatic analyses further assign the *UbK63R* mutation to the RAD6 group instead of the RAD3 and RAD52 groups (Spence et al., 1995). These data implicate a role for Lys-63 as a linkage site in the formation of multiubiquitin chains in the RAD6 repair pathway.

The *MMS2* gene was first characterized in a yeast mutant (*mms2-1*) strain sensitive to methyl methanesulfonate (MMS) (Prakash and Prakash, 1977). *MMS2*, recently cloned from a yeast genomic library by its ability to complement the original *mms2-1* mutant phenotype (Broomfield et al., 1998), encodes a protein of 137 amino acids, which shares significant sequence homology to almost all known ubiquitin-conjugating (Ubc) proteins. However, since it lacks a cysteine residue critical for Ubc activity, Mms2 appears to be a ubiquitin-conjugating enzyme variant, i.e., a protein looks like Ubc but does not have the Ubc activity. *mms2* null mutants are sensitive to both MMS and UV. Epistasis analysis shows that mutant *MMS2* is additive in UV sensitivity to deletion mutants of *RAD4*, a gene for nucleotide excision repair, or of *RAD50*, a gene for recombination repair, but it is epistatic to both *rad6* and *rad18* deletion mutants. These data indicate that *MMS2* does not belong to the RAD3 and RAD52 pathways, but rather belongs to the RAD6 pathway. In addition, a mutation in *MMS2* does not impair the UV-mutagenesis and is synergistic to a mutation in *REV3* in both UV and MMS sensitivity, suggesting that *MMS2* functions in a *REV3*-independent pathway. Furthermore, because the spontaneous mutation

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rate of *mms2* mutant strains is elevated, and mutations in *MMS2* are epistatic to the mutant Rad6 lacking the first nine amino acids, which presumably causes a defect in the error-free but not the mutagenesis pathway, *MMS2* is considered to function in the error-free branch of RAD6 pathway (Broomfield et al., 1998).

In 1999, Hofmann and Pickart (1999) showed that a ubiquitin-conjugating enzyme, Ubc13 mediates the assembly of K63-linked polyubiquitin chains and that Ubc13 does this with the help of the ubiquitin-conjugating enzyme variant protein Mms2 (Hofmann and Pickart, 1999). Mms2 protein and Ubc13 protein form a complex with very high affinity. Genetic data in the same study also showed that a *ubc13* mutant yeast strain is sensitive to killing by UV, and single, double, and triple mutants of the *UBC13*, *MMS2*, and *UbK63R* genes display a comparable phenotype, suggesting that these three genes function together (Hofmann and Pickart, 1999).

The role of *UBC13* in RAD6 pathway was further defined by Brusky et al. (2000) using genetic epistasis analyses. Their data show that the *ubc13* mutation is epistatic to *mms2* and *rad6*, and that *ubc13* is synergistic to *rev3*, indicating that *UBC13* is in a pathway alternative to *REV3* but related to *MMS2*. The *ubc13* mutant is fully functional in UV-induced mutagenesis and displays up to a 30-fold *REV3*-dependent increase in the spontaneous mutation rate. All these results together demonstrate that *Ub*, *MMS2* and *UBC13* are members of the error-free branch of the RAD6 pathway.

e. Genes for mutagenesis: *REV3*, *REV7* and *REV1*

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In addition to *RAD6*, a number of other genes are involved in mutagenesis in yeast (Reviewed in Prakash et al., 1993; Lawrence, 1994 and Broomfield et al., 2001). Mutant *REV3*, *REV7*, *REV1* strains (collectively referred to as *REV*s) are slightly more sensitive to DNA damage than the wild-type yeast, but exhibit virtually no mutagen-induced mutagenesis. In *rev3* mutant strains, the spontaneous mutation rates are 50-75% lower than in *REV3*-wild-type strains. Mutants for other genes such as *REV6*, *NGM2*, *REV4* and *REV5* show varied levels of increased sensitivity and reduced mutagenesis. As will be further discussed below, yeast Rev3 and Rev7 interact and form DNA polymerase zeta (Nelson et al., 1996a), and Rev1 protein is an auxiliary factor for Pol zeta (Nelson et al., 1996b). Rev3, Rev7, Rev1, and probably other proteins employ a translesion synthesis mechanism to deal with a stalled replication fork, at a price of introducing mutations into the genome. Much less is known about the functions of *REV6*, *NGM2*, *REV4* and *REV5*.

f. Damage avoidance and mutagenesis subpathways

RAD6 mediates at least two subpathways (Lawrence, 1994; Xiao et al., 2000). The first one is the mutagenic pathway dependent on the functions of *REV3*, *REV7*, *REV1*, and possibly other genes. The functions of *REV3*, *REV1* or *RAD6* are required for more than 90% of base substitutions and frameshift mutations induced by UV (Lawrence and Hinkle, 1996). The mechanism of this pathway is translesion synthesis. The second pathway is a damage avoidance mechanism that requires the functions of *RAD5*, *MMS2* and other genes (Xiao et al., 2000), and contributes the major portion of the ability of yeast to tolerate DNA

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damage (Watkins et al., 1993). This mechanism is relatively error-free. In a screen for yeast strains defective in postreplication repair, i.e., the ability to convert low molecular weight DNA synthesized on UV-damaged DNA templates into normal high molecular weight DNA, Prakash (1981) found that *rad6* and *rad18* mutants are unable to carry out this repair, whereas *rev3* mutants are normal in this repair. The process of converting small DNA into large DNA might reflect the execution of this damage avoidance mechanism.

2. Damage avoidance mechanisms

a. Recombination-dependent mechanism in the error-free damage tolerance pathway

The mechanism(s) for the error-free damage avoidance pathway are not well understood. The conversion of low molecular weight DNA to high molecular weight DNA might be an intermediate step of this process (Prakash, 1981). Although homologous recombination is carried out by the RAD52 pathway, a pathway distinct from the RAD6 pathway, a recombination-dependent mechanism is believed to play a role in the error-free branch of the RAD6 pathway. Two models, i.e., strand exchange or template switching, have been proposed (reviewed in Broomfield et al., 2001). More evidence is needed in order to elucidate the mechanism(s) underlying in the damage avoidance pathway.

b. Coordination of two ubiquitin-conjugating enzymes Rad6 and Mms2/Ubc13

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It is now known that the RAD6 pathway has at least two ubiquitin-conjugating units. The Rad6 protein is a ubiquitin-conjugating enzyme (Ubc) that can attach ubiquitin to a substrate protein with or without the help of a ubiquitin ligase (Jentsch et al., 1987; Sung et al., 1991). Rad6 protein forms a complex with Rad18 protein. The second ubiquitin-conjugating unit is the stable Mms2-Ubc13 complex (Hofmann and Pickart, 1999). As discussed above, Mms2 protein itself is a Ubc variant and it is the Ubc13 protein in this complex that catalyzes ubquitination. The Mms2-Ubc13 complex can assemble multiubiquitin chain in the absence of a substrate protein (Hofmann and Pickart, 1999). How are Rad6, whose function is required for the whole pathway, and Mms2/Ubc13, whose function is required only for the error-free subpathway coordinated?

The function of *MMS2-UBC13* appeared to be dependent on *RAD5*, which is also genetically placed in the error-free subpathway, since mutation of *RAD5* is epistatic to mutation in *MMS2* in UV sensitivity (Ulrich and Jentsch, 2000; Torres-Ramos et al., 2002). In addition, yeast single mutant strains defective for *RAD5* or *MMS2*, and a double mutant strain defective in *RAD5* and *MMS2* exhibited similar reduced ability in converting low-molecular weight DNA into high-molecular weight DNA fragments after UV treatment (Torres-Ramos et al., 2002). By using yeast two-hybrid system and coimmunoprecipitation, Ulrich and Jentch (2000) found that Rad5 interacts with Ubc13 and Rad18 using two distinct domains. By using yeast three-hybrid system and colocalization assays, they further showed that interaction between Rad5 and Rad18 can bring Mms2-Ubc13, via association with Rad5, and Rad6 protein, via association with Rad18,

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into contact and promote the formation of a multimeric complex. Two models were proposed for the coordination of the activity of Rad6 and Mms2-Ubc13 (Ulrich and Jentsch, 2000). One, Rad6 might attach multiubiquitin chains pre-assembled by Mms2-Ubc13 to the substrate protein. The other, the substrate protein, conjugated with one ubiquitin moiety by Rad6, is further ubiquitinated by Mms2-Ubc13. The identification of one or more substrates of ubiquitination in RAD6 pathway will no doubt greatly help elucidate the mechanism of coordination.

3. Mutagenic mechanisms involving *REV*s

a. Molecular cloning of yeast *REV3*, *REV7* and *REV1* genes

The yeast *REV3* gene was cloned by the ability to restore UV-mutagenesis in a yeast *rev3* mutant strain. Sequence analysis predicts that Rev3 protein is a DNA polymerase. Since haploid yeast cells carrying a complete deletion of *REV3* are viable, the putative Rev3 polymerase is not essential for life (Morrison et al., 1989). In addition, the expression of *REV3* does not show cell cycle dependence, a pattern that many replication enzymes have, suggesting that Rev3, as a polymerase, has specialized function (Singhal et al., 1992). *REV7* gene was cloned by functional complementation as well in a *rev7* mutant strain (Torpey et al., 1994). The *REV1* gene of *S. cerevisiae* was isolated by its ability to complement the yeast *rev1-1* mutant, and its identity was verified by gene disruption using the cloned DNA sequence (Larimer et al., 1989). The *REV1* gene encodes a protein of 985 amino acid residues, with a 152-residue internal segment sharing 25% identity with bacteria UmuC protein, a protein that

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was recently determined to be a specialized polymerase that functions in translesion synthesis in *E. coli* during the SOS response. This protein has been re-named *E. coli* DNA Pol V (Tang et al., 1999).

b. Rev3 and Rev7 proteins form Pol zeta

The interaction between Rev3 and Rev7 proteins was first demonstrated by Nelson et al. (1996a) using the yeast two-hybrid system (Fields and Song, 1989). When LexA-Rev3 was used as bait to screen a GAL4 fusion library of yeast cDNA, only two positive clones were obtained, and both of these were in-frame fusions with *REV7* (Nelson et al., 1996a). When Rev7 protein was overexpressed in a yeast strain overexpressing Rev3 protein fused with the tag of Glutathione S-Transferase (Gst), i.e., Gst-Rev3, the Rev7 and Rev3 proteins were both present in the glutathione-sepharose fraction, indicating that yeast Rev3 and Rev7 form a complex (Nelson et al., 1996a). The DNA polymerase activity of the Gst-Rev3:Rev7 complex was 20-30 times the activity of Gst-Rev3 alone, as measured by in vitro primer extension assays. Based on this result, the Rev3:Rev7 complex was named DNA Pol zeta (Nelson et al., 1996a). Pol zeta is a nonprocessive polymerase that also lacks 3' to 5' exonuclease activity. But, in an in vitro assay, this polymerase was found to replicate past a thymine-thymine cis-syn cyclobutane dimer with an efficiency of ~10%. This major UV-induced photoproduct normally severely inhibits replication. In contrast, the efficiency of bypass replication past this photoproduct with yeast DNA polymerase alpha is less than 1% (Nelson et al., 1996a).

c. Studies on Rev1

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1) The Rev1 protein has a deoxycytidyl transferase activity

Purified recombinant Rev1 protein can transfer a dCMP from its precursor dCTP to the 3' end of a DNA primer in a template-dependent manner. Efficient transfer was observed opposite a template abasic site, but the efficiencies of transfer opposite a template guanine was only ~20%, opposite an adenine or uracil only ~10%, and opposite a thymine or cytosine, less than 1% (Nelson et al., 1996b). In the absence of Rev1, Pol zeta inserts a nucleotide opposite an abasic site and further extends it with an efficiency of 7%, but addition of Rev1 protein to the reaction increases the bypass efficiency to 30-40%. In contrast, no detectable bypass was seen with Pol alpha, in the absence or presence of Rev1 (Nelson et al., 1996b).

2) Rev1 as a dCMP transferase in abasic site bypassing

The role of Rev1 as a dCMP transferase in abasic site bypassing in vivo was initially recognized by Gibbs and Lawrence (1995). By transforming the yeast *S. cerevisiae* with a vector containing a single abasic site specifically located within a 28-nucleotide single-stranded region, and sequencing the replicated vectors from transformants, they found that the yeast prefers to insert a dCMP opposite the abasic site. In three different sequence contexts, the frequencies of dCMP insertion were 83%, 62% and 85%. A similar bias for cytosine insertion was also found with entirely single-stranded vectors (Gibbs and Lawrence, 1995). The yeast *rev1-1* strain, which expresses a Rev1 protein with a glycine to arginine mutation at position 193, and retains substantial dCMP transferase activity, showed a reduced ability to bypass abasic sites on

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engineered constructs, i.e., 7%, compared to 25% by wild-type yeast. Nevertheless, sequencing of those replicated constructs showed that 75% of them had a dCMP insertion opposite the original abasic site (Nelson et al., 2000). The above data indicate that the dCMP transferase activity of Rev1 is employed by yeast in bypassing abasic sites. However, Haracska et al. (2001b) reported a different observation. They studied the mutation spectrum of the chromosomal gene *CAN*^{S} induced by DNA alkylating agent MMS in yeast double mutant strains defective for AP endonucleases APN1 and APN2, and therefore incapable of base excision repair. MMS can methylate adenine at the N_3 position and guanine at the N_7 position. The removal of alkylated bases by an *N*-methyl purine DNA glycosylase results in abasic sites. These investigators found that ~70% of the mutations obtained were base substitutions, and ~30% were +1 or −1 frameshift mutations. Among these base substitutions, the majority (64%) were adenine, suggesting that dAMP is preferentially inserted across from the abasic sites. To further assess the role of the dCMP transferase activity of Rev1 in vivo, a Rev1 mutant protein lacking the dCMP transferase activity was generated by substituting the Asp 467 and Glu 468 residues in the highly conserved motif III consisting of serine, isoleucine, aspartate, and glutamate residues (SIDE) with alanines (Haracska et al., 2001b). Introduction of the *rev1* Ala^{467}-Ala^{468} mutant gene into *apn1 apn2 rev1* triple deletion mutant strain restored MMS-induced *can1*^{r} mutations to the level seen in the parental *apn1 apn2* double deletion mutant strain, indicating that the deoxycytidyl transferase activity of Rev1 is dispensable for mutagenesis induced by abasic sites (Haracska et al., 2001b).

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Although these data might reflect the difference of the sequence context of the abasic sites, i.e., an exogenous vector versus a chromosomal gene, other evidence is needed in order to clarify this discrepancy.

3) Evidence for a second function of Rev1

In addition to the bypassing of abasic sites, Rev1 function is required for mutagenesis induced by UV, ionizing radiation, and a variety of chemical mutagens (Lawrence, 1994). Since the latter events usually do not involve the insertion of dCMP, Rev1 may possess a function other than acting as a transferase. To obtain more evidence, Nelson et al. (2000) studied the bypass of T-T cis-syn cyclobutane dimers, T-T pyrimidine (6-4) pyrimidone adducts, and also abasic sites in yeast strains carrying *REV1* or *REV3* deletions or the *rev1-1* mutation. rev1-1 mutant protein retains substantial dCMP transfearse activity, but confers UV-sensitivity and impaired mutagenesis to yeast strains carrying this mutation. Double-stranded vectors containing one of these lesions located centrally within a 28-nucleotide single-stranded region were transformed into the mutant and wild-type strains, and the proportion of replicated plasmids resulting from translesion replication was calculated (Nelson et al., 2000). As expected, the bypass frequency of abasic sites in *rev1* or *rev3* deletion mutants was greatly reduced (1-3%), as compared to wild-type strains (25%). The bypass frequency in *rev1-1* mutant was significantly reduced as well (7%). Nevertheless, 75% of the mutations generated in *rev1-1* mutant resulted from a dCMP insertion. These data suggest at least in part, that function of Rev1 other than the transferase activity is needed for bypassing abasic sites. Interestingly, these investigators

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also found that *REV1* and *REV3* functions are required for the bypass of T-T (6-4) adducts but not T-T cyclobutane dimers. The wild-type strain bypassed 19% of T-T (6-4) adduct-containing vector molecules used, but *rev1* or *rev3* deletion mutants or *rev1-1* mutant bypassed only 1-2%. The data from the *rev1-1* mutant indicates that a function other than dCMP transferase activity is involved in bypassing T-T (6-4) adducts. The bypass of T-T cyclobutane dimers might depend on Pol eta.

Yeast Pol delta consists of three subunits encoded by the *POL3*, *POL31*, and *POL32* genes respectively. It has been shown that *POL3* and *POL31* are essential for viability, but the *POL32* gene is not and that *pol32* deletion mutants are UV-sensitive and defective in UV mutagenesis (Gerik et al. 1998). Haracska et al. (2001b) studied the role of replicative polymerase Pol delta in bypassing the MMS-induced abasic sites by determining the effect of the *pol32* deletion mutation on MMS-induced $CAN1^s$ to $can1^r$ mutations in the *apn1 apn2* double deletion mutant strain. Their data showed that *pol32* deletion mutants exhibit mild sensitivity to MMS, and complete abolishment of MMS-induced mutations, indicating a requirement of the Pol32 subunit and probably Pol delta for the mutagenic bypass of abasic sites. In in vitro primer extension assays, purified Pol zeta was unable to insert a nucleotide opposite this lesion, but Pol delta or Rev1 protein was able to insert a nucleotide but could not extend from the resulting primer. Efficient bypass of the AP site, however, could be achieved when Pol delta was combined with Pol zeta, or Rev1 was combined with Pol zeta. Rev1 did not stimulate AP bypass when it was combined with Pol delta. The bypassing

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efficiency with a Pol delta/Pol zeta combination (55%) was a little higher than that of a Rev1/Pol zeta combination (32%). The nucleotide preferentially inserted opposite AP sites by Pol delta in vitro was found to be dAMP, the same as the major nucleotide (64%) being inserted in vivo (Haracska et al., 2001b). Steady-state kinetic analyses show that Pol zeta was highly inefficient in inserting nucleotides opposite the AP sites, but was efficient in extending from nucleotides, particularly an A, inserted opposite this lesion. In light of these data, Haracska et al. (2001b) proposed that in eukaryotes, bypass of an AP site requires the sequential action of two DNA polymerases, wherein the extension step depends upon Pol zeta, and the insertion step involves either the predominant action of Pol delta, or the minor role of various translesional polymerases, like Rev1 or Pol eta, and that the predominant role of Rev1 in AP bypass is likely to be structural, i.e., to mediate the access of Pol zeta to the lesion.

C. Damage tolerance RAD6 pathway in humans

1. Damage avoidance mechanisms

a. hHR6A, hHR6B and hRAD18

Humans have a conserved RAD6 pathway. Human homologs for almost all known yeast genes of the RAD6 pathway have been found. For the founding member *RAD6*, two human homologs, *hHR6A* and *hHR6B*, have been identified (Schneider et al., 1990; Woffendin et al., 1991 and Koken et al., 1991), and they are located on Xq24-q25 and 5q23-q31, respectively (Koken et al., 1992). These two 152-amino acid Rad6 proteins share 95% sequence identity with each other

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and ~70% identity with the yeast Rad6 protein (Koken et al., 1991). Both of them are ubiquitously expressed in various tissues and cell types, with an elevated level in testis (Koken et al., 1996). Inactivation of the *hHR6B*-homologous gene in mice causes male infertility (Roest et al., 1996). In addition, both *hHR6A* and *hHR6B* can complement the damage tolerance functions of *RAD6* in *S. cerevisiae rad6* deletion mutants including damage avoidance and mutagenesis (Koken et al., 1991), suggesting that the function of *RAD6* is conserved. The human *RAD18* has been cloned as well (Tateishi et al., 2000 and Xin et al., 2000). Stable hRad18-hHR6A or hRad18-hHR6B protein complexes can be purified when hRad18 protein is co-expressed in yeast cells with either human Rad6 protein (Xin et al., 2000). Human fibroblasts which stably express a mutant hRad18 protein carrying a cysteine to phenylalanine substitution at residue 28 in the conserved Rad6-binding ring-finger motif become sensitive to the cytotoxic effect of UV, methyl methanesulfonate, and mitomycin C, and are defective in the replication of UV-damaged DNA (Tateishi et al., 2000).

b. hMMS2 and *UBC13*

A human homolog of the yeast *MMS2* gene (*hMMS2*) has been cloned as well (Xiao et al., 1998b). Like the yeast *MMS2* gene, the *hMMS2* gene also encodes a ubiquitin-conjugating variant protein. Interestingly, the hMms2 protein also shares >90% amino acid sequence identity with Croc-1, a protein previously identified by its ability to transactivate c-fos expression in cells in culture through a tandem repeat enhancer sequence. Because hMms2 and Croc-1 also share ~50% identity and ~75% similarity with the yeast Mms2 protein, and both the

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hMMS2 and the *CROC-1* genes are able to functionally complement the yeast *mms2* defects with regard to sensitivity to DNA damaging agents and spontaneous mutagenesis, the *hMMS2* and *CROC-1* genes are considered to be two homologs of yeast *MMS2*. hMms2 protein forms a complex preferentially with human Ubc13 (Moraes et al., 2001), just like its yeast counterpart does (VanDemark et al., 2001). Association of Mms2 protein with Ubc13 facilitates the ubiquitin chain assembly by Ubc13 (McKenna et al., 2001).

As discussed later, the *REV3*, *REV7*, *REV1* and *RAD30* genes all have human homologs. In summary, except for *RAD5*, all major yeast RAD6 pathway genes have identified human counterparts. Therefore, the RAD6 DNA repair pathway is very likely conserved in humans, and since some human genes have more than one homolog, the human RAD6 pathway might be more complex than the yeast system.

2. Translesion synthesis mechanisms

a. hREV3

1) Cloning of *hREV3* gene

The human homolog of the yeast *REV3* gene was cloned in 1998 by Xiao et al. (1998a) and independently by Gibbs et al. (1998). The latter group discovered an expressed sequence tag (EST) in the dBEST database that encoded a peptide with sequence homology to yeast Rev3 protein. The full length cDNA of the *hREV3* gene was then acquired by combining the EST sequence and 5' upstream sequence obtained by the method of 5' Rapid Amplification of cDNA Ends (RACE). The deduced hRev3 protein consists of

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3,130 amino acid residues and shares with the yeast Rev3 protein 29%, 29%, 39% identity in one N-terminal region of ~340 residues, one middle region of 55-residue and a C-terminal region of ~850 residues, respectively. In addition, the hRev3 protein contains all six motifs characteristic of eukaryotic DNA polymerases in the right order. The *hREV3* gene was also cloned independently by Lin et al. (1999a). The *hREV3* gene was originally considered to be located at chromosome 1p32-33 as a result of studies using fluorescence in situ hybridization with chromosome metaphase spreads (Xiao et al., 1998a). However, a second report of the same year (Morelli et al., 1998) localized *hREV3* gene on chromosome 6q21. A more recent study also provided evidence confirming that *hREV3* is located on chromosome 6q21 (Kawamura et al., 2001).

2) The level of hRev3 protein in human cells might be low

In addition to the start codon ATG for the main open reading frame, the *hREV3* gene has an out-of-frame ATG at position -58 nt. The translation initiated from the additional ATG terminates at a stop codon within the main *hREV3* open reading frame, and would generate a peptide unrelated to hRev3 protein (Gibbs et al., 1998). Moreover, the translation efficiency from these two ATG start codons are expected to be comparable since the sequence context of these two ATG's are almost equally good in supporting translation. An out-of-frame ATG is also found in the yeast *REV3* gene. Examination of the 5' untranslated region of the *hREV3* mRNA also revealed a segment with potential to form a stem-loop (hairpin) secondary structure (Lin et al., 1999a). The presence of an out-of-frame ATG and a hairpin structure in the 5' untranslated region suggest that the

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translation of full-length hRev3 protein is very inefficient, and the level of hRev3 protein in human cells may be very low.

3) Importance of the function of *hREV3* in mammals

The presence of the mRNA transcript is often used as an indicator of the functioning of a gene in certain tissues. By using RT-PCR, Lin et al. (1999a) examined 16 different human tissues and found that all expressed *hREV3* mRNA. In an independent study, Kawamura et al. (2001) reported that *hREV3* was ubiquitously expressed in all 27 normal human tissues tested and showed that the expression is not dysregulated in malignant human tissues.

To study the function of *REV3* in mammals, *mREV3* knock-out mice were generated independently by three groups at almost the same time (Wittschieben et al., 2000; Bemark et al., 2000; and Esposito et al., 2000). Wittschieben et al. (2000) replaced two *mREV3* exons containing conserved DNA polymerase motifs with a cassette encoding G418 resistance and beta-galactosidase, under the control of the *mREV3* promoter. They found that disruption of *mREV3* caused mid-gestation embryonic lethality, with the frequency of embryos declining markedly between 9.5 and 12.5 days post coitum. *mREV3* knock-out embryos were smaller than their heterozygous littermates and showed retarded development. Tissues in many areas were disorganized, with significantly reduced cell density. mRev3 expression, traced by beta-galactosidase staining, was first detected during early somitogenesis and gradually expanded to other tissues of mesodermal origin, including extraembryonic membranes. Embryonic death coincided with the period of more widely distributed mRev3 expression. No

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haematopoietic cells, fibroblast cells and embryonic cells could be derived in *mREV3(-/-)* embryos (Esposito et al., 2000). These data indicate that *REV3* is essential for embryonic development in mammals. The exact mechanism causing embryonic lethality is not known.

4) The role of *hREV3* in mutagenesis

The requirement of *hREV3* gene for mutagenesis was demonstrated with human cells (Gibbs et al., 1998). Human fibroblasts expressing high levels of an *hREV3* antisense RNA to a 5' portion of *hREV3* mRNA grow normally, are not significantly more sensititve than normal cells to killing by UV, but show greatly reduced UV-induced mutagenesis. The human gene, therefore, appears to carry out a function similar to that of its yeast counterpart (Gibbs et al., 1998). *hREV3* also plays a role in somatic mutations in the immunoglobulin and bcl-6 genes (Zan et al., 2001; Diaz et al., 2001). *hREV3* was constitutively expressed in human B cells. Upon B cell receptor engagement and coculture with activated CD4+ T cells, these lymphocytes upregulated Pol zeta, and mutated the *Ig* and *bcl-6* genes. Inhibition of the *hREV3* by specific phosphorothioate-modified oligonucleotides decreased *Ig* and *bcl-6* hypermutation and UV damage-induced mutagenesis, without affecting cell cycle or viability (Zan et al., 2001). Following immunization with antigens, transgenic mice that expressed antisense RNA to a portion of *mREV3* gene could induce vigorous antibody response, switch to IgG and form germinal center, but showed a delay in the generation of high affinity antibodies and a decrease in the accumulation of somatic hypermutation in the V_H genes of memory B cells (Diaz et al., 2001). These data suggest that

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mammalian *REV3* is required not only for the mutagenesis induced by DNA damaging agents, but also for natural process that requires mutagenesis such as IgG maturation.

b. hREV7

In 2000, a candidate human homolog of *S. cerevisiae REV7* (*hREV7*), was identified in a yeast two-hybrid screen by using fragments of *hREV3* as bait (Murakumo et al., 2000). The *hREV7* gene product displays 23% identity and 53% similarity with yeast Rev7. Northern blot analysis showed that *hREV7* mRNA was ubiquitously expressed in various tissues, with the highest level in testis followed by thymus, spleen, and peripheral blood leukocyte, indicating the importance of the function of *hREV7* in humans. The *hREV7* gene was localized to chromosome 1p36, a region that had been previously shown to have high frequency of loss of heterozygosity in some types of human tumors (Moley et al., 1992; Simon et al., 1995). However, Northern blot analysis using a human multiple tissue blot did not reveal any alterations in the length of *hREV7* transcript. Furthermore, sequence analysis of *hREV7* cDNA in 50 tumor-derived cell lines and 33 clinical tumor samples did not reveal any mutations. The hRev7 protein shares 23% identity and 54% similarity with the human mitotic checkpoint protein hMad2 (MAD: mitotic arrest-deficient). What is more, an interaction between hRev7 and hMad2 proteins was identified in in vitro pull-down assay. The significance of the interaction between hRev7 and hMad2 remains to be elucidated, and also, it has not yet been demonstrated that the hRev7 homolog conserves the function of yeast Rev7 in mutagenesis.

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c. hREV1

1) Cloning of *hREV1* gene

The *hREV1* gene was cloned independently by two laboratories at virtually the same time (Lin et al., 1999b; Gibbs et al., 2000). Lin et al. (1999b) screened human bone marrow and leukocyte cDNA libraries with a human expressed sequence tag (EST) as probe and derived the *hREV1* cDNA sequence by aligning the clones generated, based on the overlapping sequences. Gibbs et al. (2000) started by identifying an EST encoding a peptide with similarity to the C-terminus of yeast Rev1 protein, followed this by sequencing of the clone, and then obtained the remaining cDNA by the method of 5' rapid amplification of cDNA ends (Gibbs et al., 2000). The *hREV1* genes identified in these two laboratories are the same. The gene encodes an expected protein of 1,251 amino acid residues, compared with 985 residues in the corresponding yeast protein. Evidence supporting the hypothesis that this human gene is a homolog of yeast *REV1* comes from comparing these two proteins. They share 41%, 20%, 31% and 83% identity in two N-terminal regions of approximately 100 residues, one region of approximately 320 residues, and one central motif of 13 residues, respectively (Gibbs et al., 2000). The calculated molecular weight of the hRev1 protein is ~140kD.

2) Presence of an out-of-frame ATG on *hREV1* open reading frame

The *hREV1* cDNA has a 5' out-of-frame ATG codon at position –35nt upstream of the initiator ATG codon of the main open reading frame (ORF). This ATG initiates a small reading frame that terminates at a TGA stop codon

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overlapping the main ORF ATG (Gibbs et al., 2000). Translation from the 5' out-of-frame ATG would lead to synthesis of an unrelated peptide. The presence of this alternate open reading frame predicts that the level of hRev1 protein in cells is likely to be low.

3) Chromosomal location and functional importance

By using the *hREV1* cDNA as a probe, Lin et al. (1999b) isolated two *hREV1* genomic clones from a human placenta genomic DNA library. One of these two clones contained a sequence tagged site (STS) localized between 2q11.1 and 2q11.2 on the cytogenetic ideogram. The STS was approximately 20kb upstream from the 5' end of the *hREV1* gene, as estimated by polymerase chain reaction. Therefore, the *hREV1* gene is located between 2q11.1 and 2q11.2. The mRNA of *hREV1* gene was present in all 16 human tissues examined, suggesting that the function of *hREV1* is ubiquitous in humans (Lin et al., 1999b). In an independent study, Murakumo et al. (2001) reported that the testis has a much higher level of *hREV1* mRNA than does other normal tissues examined and that *hREV1* expression is not abnormal in human cancer cell lines.

4) hRev1 as a dCMP transferase

The yeast Rev1 protein has an intrinsic deoxycytidyl transferase activity, which inserts a dCMP at the 3' end of a DNA primer opposite a template G or an apurinic/apyrimidinic (AP) site (Nelson et al., 1996). To determine whether the hRev1 protein possesses this activity, Lin et al. (1999b) performed in vitro primer extension experiments with purified recombinant hRev1 protein. Their results

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showed that human Rev1 protein specifically inserts a dCMP opposite a template G, but does not insert any deoxynucleotide opposite a template A, C or T, suggesting that hRev1 is a dCMP transferase. In addition, the hRev1 protein is able to efficiently and specifically insert a dCMP opposite a DNA template abasic site or a uracil residue, but cannot extend the synthesis if the next template is not a G. The hRev1 protein, and its yeast counterpart, are members of a growing family of translesion DNA polymerases that includes umuC (Tang et al., 1999) and dinB/dinP (Wagner et al., 1999) of *Escherichia coli*, Rad30 (Johnson et al., 1999a) of the yeast *Saccharomyces cerevisiae*, and XPV/Rad30A (Johnson et al., 1999b and Masutani et al., 1999a,b), Rad30B (McDonald et al., 1999), and DINB1 (Gerlach et al., 2001) of humans. Although hRev1 protein contains the conserved domain required for the polymerase activity of other members in the family, it does not possess such polymerase activity. Instead, it has a template-dependent transferase activity. To determine the domains that are responsible for the transferase activity, Masuda et al. (2001) generated various deletion and point mutation mutants of the hRev1 protein. Primer extension analyses showed that hRev1 proteins that carried mutations in the conserved domain of polymerases lost the deoxycytidyl transferase activity and DNA binding ability, suggesting that the structure of the active site of the deoxycytidyl transferase closely resembles the active site of other members of the family.

5) Function of *hREV1* is required for human cell mutagenesis

In yeast, the function of *REV1* is absolutely required for virtually all damage-induced mutagenesis (Lawrence, 1994). My research for the Ph.D.

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degree involved the role of *hREV1* in human cell mutagenesis. My hypothesis was that *hREV1* is required for mutagenesis. To test this, I employed an antisense RNA strategy to decrease the function of hRev1 in human fibroblasts. The data presented in Chapter 3, Chapter 4, and Appendix of this dissertation indicate that *hREV1* is required for mutagenesis induced by UV and BPDE, and that hRev1 is involved in the generation of all base substitutions induced by BPDE (Gibbs et al., 2000; Lawrence et al., 2000; Wang et al., manuscript in preparation).

d. Interactions of hRev3, hRev7 and hRev1 proteins

A yeast two-hybrid system was extensively used to study the interactions of the hRev1, hRev3 and hRev7 proteins (Murakumo et al., 2000, 2001). In this system, a bait protein or fragment of a protein is fused with the GAL4 DNA binding domain and another protein, or fragment of a protein, under study is fused with the GAL4 transcription activation domain. If the bait protein and the protein under study bind each other sufficiently, the hybrid transactivator will activate the expression of two reporter genes, i.e., the gene that allows the yeast cells to grow in medium without histidine and the gene coding for β-galactosidase. These two reporter genes allow qualitative and quantitative evaluation of the degree of binding between the bait protein and the protein under study, by using colony growth assay and β-galactosidase assay, respectively. Using this strategy, Murakumo et al. (2001) found that hRev3 binds hRev7. The minimal domain for hRev3 to bind hRev7 is within amino acid sequence 1847-1892 and the minimum region for hRev7 to interact with hRev3 is

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within 21-155aa. These investigators also showed that the hRev1 and hRev7 proteins interact with each other. The minimum sequences of hRev1 and hRev7 proteins for such binding are amino acid sequences 1130-1251 and 21-155, respectively. Interestingly, the hRev7 protein binds hRev3 and hRev1 protein with the same region. hRev7 protein was also shown to form homodimer, and the region of hRev7 for homodimerization is located between amino acid residues 21 and 155, the same region required for its interaction with hRev1 or with hRev3. The authors used pull-down assays and coimmunoprecipitation assays to confirm the interactions identified by yeast two-hybrid system (Murakumo et al., 2001). However, in their in vitro pull-down assays, these investigators failed to detect hRev1, hRev3, and hRev7 proteins forming a stable complex. Nevertheless, the possibility that hRev3, hRev7, and hRev1 function together in human cells cannot be ruled out just because the complex of three proteins could not be identified in one kind of in vitro pull-down assay.

Sequence comparisons between human and yeast Rev proteins indicated that the regions homologous to the interaction domains of hRev3 and hRev7 are also present in yeast Rev3 and Rev7 proteins, suggesting that DNA polymerase zeta complex is functionally conserved from yeast to human. However, the C-terminal region of hRev1, which is important for interaction with hRev7 protein, shows no homology with yeast Rev1, suggesting that hRev1 may somewhat different from Rev1 in its enzymatic properties. Based on their results, Murakumo et al. (2001) proposed a model in which, prior to DNA damage, hRev7 exists as a homodimer, and hRev1 or hRev3 exists in the form of monomers in human cells,

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and that when DNA damage occurs and translesion synthesis activity is needed, hRev7 dissociates from the homodimers and forms the complex with hRev1 or hRev3, allowing them to execute their functions. More studies are needed in order to gain more insight into this pathway.

e. Polymerase eta, homolog of yeast *RAD30*

The human homolog of yeast *RAD30* is found to be the gene that is mutated in the cells of patients with the variant form of xeroderma pigmentosum (XPV) (Masutani et al., 1999a, b). Cells derived from XPV patients are sensitive to the cytotoxic effect of UV and show an elevated UV-induced mutagenesis (Maher et al., 1976). In vitro studies with XPV cell extracts revealed that XPV cells are less efficient than normal cells in bypassing most bulky lesions, including UV photoproducts (reviewed in Cordonnier and Fuchs, 1999). By using a SV40 origin-based plasmid containing a site-specific T-T cyclobutane dimer, Masutani et al. (1999a) isolated a protein from HeLa cells capable of complementing the T-T dimer bypassing defect of the XPV cell extracts. A full length cDNA was subsequently identified from a HeLa cDNA library using DNA probes deduced from four partial amino acid sequences of this protein (Masutani et al., 1999b). Sequence homology searching revealed that this protein shares an overall 19.6% identity and 31.9% similarity with yeast Rad30 protein (Masutani et al., 1999b), which had just been identified as DNA polymerase eta (Johnson et al., 1999a). Thus, the XPV gene is a human homolog of yeast RAD30. The XPV gene was designated as *hRAD30A* as a second homolog, *hRAD30B*, to yeast *RAD30* was found later (see below). Because XPV protein

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(Pol eta) inserts dAMP across T-T dimers very efficiently (Wang et al., 1991; Masutani et al., 2000 and Johnson et al., 2000b), i.e., in an error-free manner, the function of yeast *RAD30* is conserved in humans.

f. Additional lesion-bypassing DNA polymerases

1) Pol iota

The yeast *RAD30* gene was first identified by Woodgate and his colleagues (McDonald et al., 1997). In an attempt to isolate human homolog(s) of the yeast *RAD30*, the same group identified a novel homolog (McDonald et al., 1999) different from the first homolog, *hRAD30A*, coding for human Pol eta. The second homolog was designated as *hRAD30B*. Northern blot analyses showed that *hRAD30B* is expressed in many human tissues, but at a low level (McDonald et al., 1999). In vitro primer extension experiments with purified recombinant hRad30B protein revealed that it possesses a DNA polymerase activity, and therefore, hRad30B protein was named Pol iota (Tissier et al., 2000a). Although Pol iota replicates DNA in a template-dependent manner, it frequently incorporates wrong nucleotides, even on undamaged DNA (Tissier et al., 2000a). This polymerase can insert nucleotides opposite lesions, including UV photoproducts (Tissier et al., 2000b), abasic sites, 8-oxoguanine and *N*-2-acetylaminofluorene-adduct in vitro (Zhang et al., 2001).

2) Pol kappa

Sequence homology searching for a human peptide with homology to *E. coli* DinB protein (*E. coli* Pol IV), a UmuC homolog involved in untargeted

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mutagenesis, identified hDinB (Gerlach et al., 1999; Ogi et al., 1999). The *hDINB1* gene was found to encode a DNA polymerase (designated Pol kappa), which synthesizes DNA with extraordinarily low fidelity (~1 error per 200 nucleotides) (Zhang et al., 2000b; Ohashi eta l., 2000). Pol kappa can bypass an abasic site, an *N*-2-acetylaminofluorene (AAF)-adduct, or an 8-oxoguanine in an error-prone manner, but it does not bypass a cis-syn or (6-4) thymine-thymine dimer or a cisplatin-adduct (Zhang et al., 2000a; Ohashi eta l., 2000). In addition, human Pol kappa effectively bypasses a template (-)-trans-anti-benzo[a]pyrene-N_2-dG lesion in an error-free manner (Zhang et al., 2000a). These data implicate an important role for Pol kappa in the mutagenic bypass of certain types of DNA lesions. Recently, Pol kappa was found to be overexpressed in lung cancer (O-Wang et al., 2001).

g. Summary of the kinds of lesions bypassed by error-prone DNA polymerases

Table 1 summarizes the ability of error-prone DNA polymerases in bypassing various kinds of DNA lesions. This is mainly based on the data obtained from in vitro primer extension assays. Genetic evidence is used when biochemical data are not available. It should be noted that the ability of a purified polymerase found in vitro might not be used in vivo, and that in some circumstances, more than one DNA polymerase could act together.

Table 1. Properties of lesion-replicating DNA polymerases[a]

											Notes

Table 1. Properties of lesion-replicating DNA polymerases[a]

	Undamaged DNA	Abasic site	8-oxo-guanine	T-T cyclo-butane dimer	T-T (6-4) dimer	BPDE-Guanine	AAF-Guanine	Notes
Type of lesion	N/A	Apurination/ Apyrimidination	Oxidative base damage	UV photoproducts		Bulky adducts		
Rev1	dCMP transferase	Inserts a C, no extension		Required for in vivo mutagenesis				Rev1, Rev3 (+Rev7) are required for mutagenesis induced by a variety of mutagens
Rev3[b] (+Rev7)	Distributive	Blocked, but can extend from nucleotides inserted by Rev1 or Pol δ		Bypasses with 10% efficiency	Required for in vivo mutagen-esis			
Rad30A (Pol eta)	Processive	Inserts A/G, no extension	Inserts C/A, extends	Inserts AA, extends (error-free)	Inserts G, no extension	Inserts A>T>G/C, extends	Inserts C, extends (error-free)	Specialized for bypassing TT dimers
Rad30B (Pol iota)	Prefers G opposite T, but no extension from GT mismatch	Inserts G>T>A>C, no extension	Significantly blocked	Largely blocked	Inserts A, no extension		Inserts C, no extension	An extremely distributive and error-prone polymerase
hDinB1 (Pol kappa)	Moderately processive	Inserts A, but needs a T for extension (–1 deletion)	Inserts A, extends	Blocked	Blocked	Inserts C, extends (error-free)	Inserts T/C>>A>G, extends	

[a] mainly based on the reviews by Wang, 2001; Lawrence and Maher, 2001).
[b] data on yeast Rev3, as data on hRev3 are not available.

h. Lesion-replicating polymerases could act sequentially in bypassing lesions

To bypass a replication-blocking lesion and allow continuation of DNA synthesis, the polymerase(s) must accomplish two steps, the insertion of a deoxynucleotide opposite the lesion and the extension of synthesis from the incorporated deoxynucleotide (Woodgate, 2001). This two-step bypassing can be carried out by one DNA polymerase. For example, human Pol eta inserts AA across from T-T dimer and extends from the inserted AA very efficiently (Masutani et al., 1999b). In some circumstances, at least in vitro, error-prone DNA polymerases have been shown to cooperate in bypassing lesions that block DNA synthesis (Johnson et al., 2000a). Human Pol iota is error-prone in DNA synthesis even on undamaged DNA. It can efficiently insert a deoxynucleotide opposite an abasic site or the 3' T of a T-T (6-4) photoproduct, but can not extend the synthesis from inserted deoxynucleotides (Johnson et al., 2000a). In eukaryotes, including yeast and humans, DNA polymerase zeta is essential for virtually all damage-induced mutagenesis (Lawrence and Maher, 2001). Although Pol zeta bypasses a T-T dimer with a frequency of 3%-10% (Johnson et al., 2000a; Nelson et al., 1996), it does not bypass a T-T (6-4) photoproduct and abasic site (Johnson et al., 2000a). However, efficient bypass of a T-T (6-4) photoproduct and an abasic site was achieved by the combination of Pol iota and Pol zeta (Johnson et al., 2000a). Indeed, yeast Pol zeta extends from mismatched primer-template ends on undamaged or damaged DNA very efficiently (Johnson et al., 2000a). Other examples of polymerase coordination

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include bypassing of abasic sites by Rev1 and Pol zeta (Nelson et al., 1996b), and bypassing of bulky adducts by hRev1 and Pol kappa (Zhang et al., 2002), if Rev1 or hRev1 is considered a DNA polymerase as suggested by Haracska et al. (2002). Although the cooperation between lesion-replicating polymerases has only been demonstrated in vitro so far, it is possible to be employed in vivo.

3. Relevance of the damage tolerance pathway to carcinogenesis in humans

Cancer is a disease charaterized by uncontrolled cell proliferation, which is caused by mutations in oncogenes and/or tumor suppressor genes. Evidence suggests that the human RAD6 pathway plays an important role in tumorigenesis.

Mutations in the *hRAD30A* gene cause the variant form of xeroderma pigmentosum (XPV) (Masutani et al., 1999a, b). XPV patients are predisposed to skin cancer (Mamada et al., 1992). In vitro transformation studies also showed that XP variant cells are more sensitive than normal cells to UV-induced transformation to anchorage independence (McCormick et al., 1986).

As discussed above, Pol kappa is a low-fidelity polymerase with a moderate processivity when replicating undamaged DNA. In vitro primer extension assays also showed that recombinant Pol kappa can replicate DNA containing lesions such as an abasic site, a *N*-2-acetylaminofluorene guanine adduct or 8-oxoguanine. Transient expression of this polymerase in cultured mouse cells caused a 10-fold increase in the mutation frequency of the endogenous *HPRT* gene. In an attempt to explore the potential involvement of

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Pol kappa in carcinogenesis, O-Wang et al. (2001) compared the expression level of Pol kappa in tumors and adjacent nontumorous tissues by Northern blot, semiquantitative RT-PCR, and Western blot analyses. Among 29 pairs of tumor and normal specimens from patients with stages I to IIIb non-small cell lung cancer, including 13 adenocarcinomas, 15 squamous cell cancers, and 1 adenosquamous carcinoma, 21 tumor samples were found to have overexpression of Pol kappa. The elevated Pol kappa expression was likely due to an activated transcription, because gene amplification was not found in tumor samples exhibiting higher expression of Pol kappa, as shown by Southern blot analysis. These data suggests that Pol kappa may contribute to lung tumor development by accelerating the accumulation of mutations.

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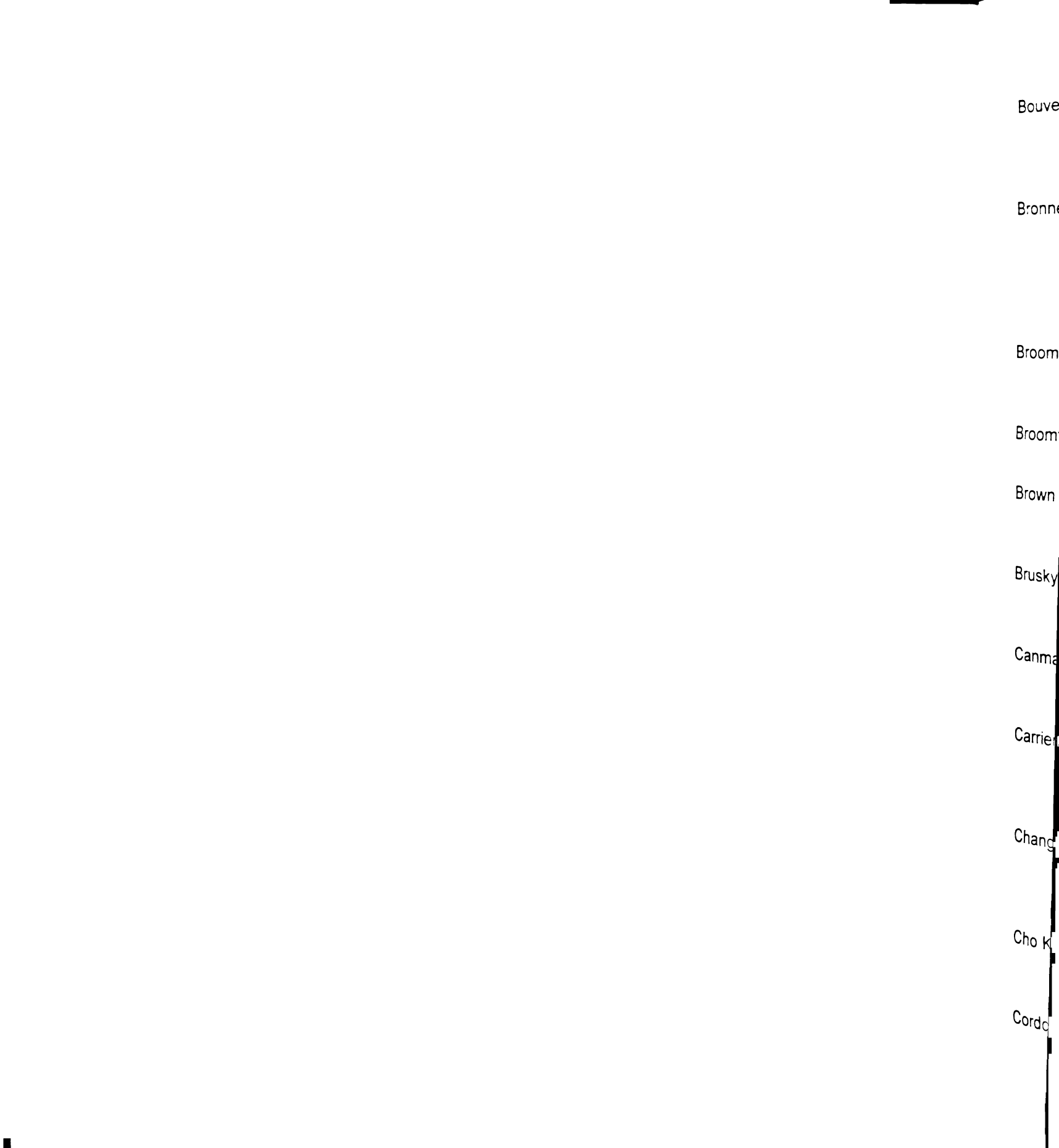
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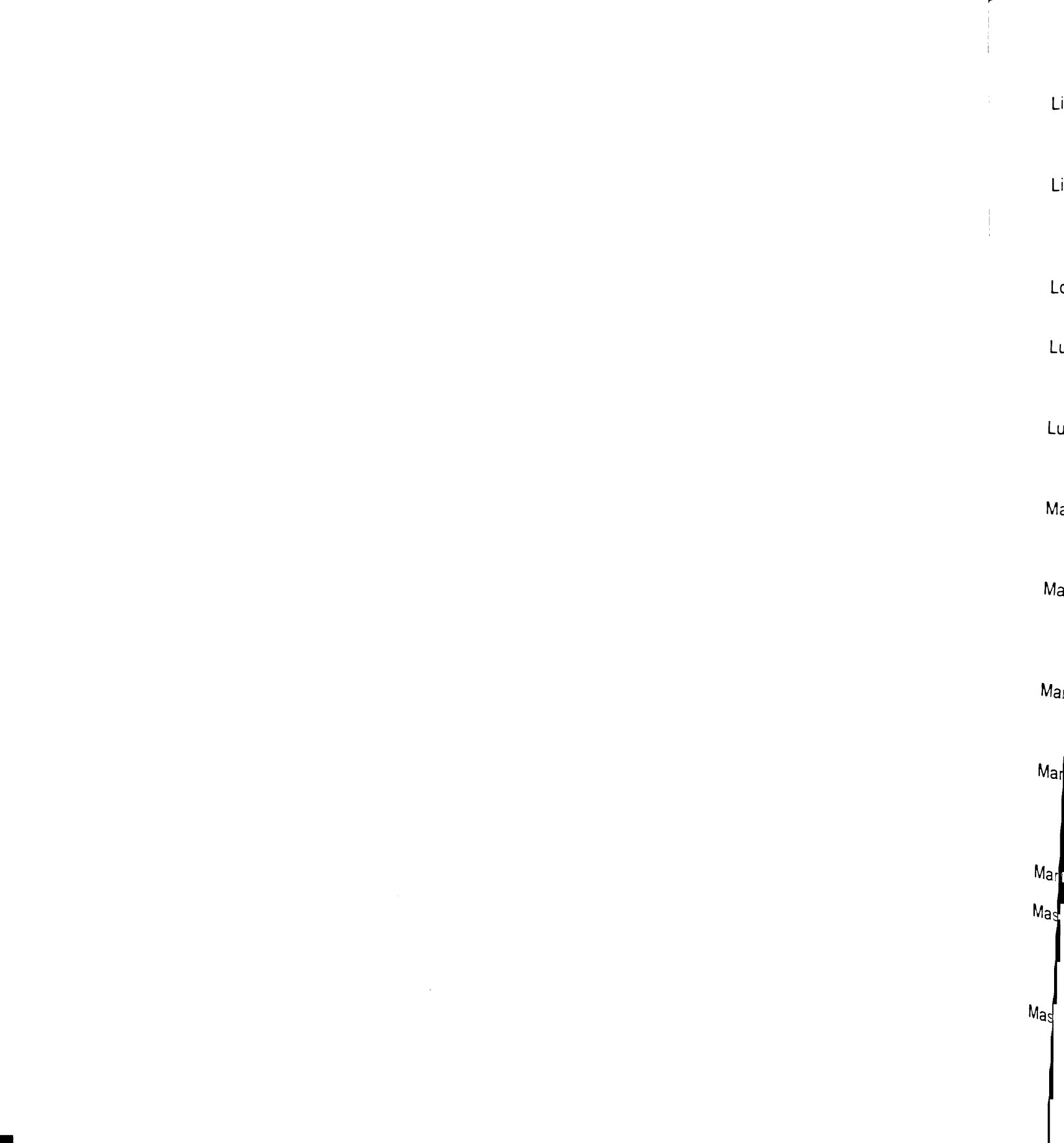
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CHAPTER 2

Re-expression of Wild-type p53 in a Focus-derived Human Fibroblast Strain that Can Produce Tumors in Athymic Mice Prevents Focus Formation, Anchorage Independence, and Tumorigenicity, but Does Not Affect Growth in Culture[1]

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[3] The abbreviations used are: FBS, fetal bovine serum; MNU, *N*-methyl-*N*-nitrosourea; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBST, Tris-buffered saline containing Tween-20; TD, tumor-derived; tTA, tetracycline-controlled transactivator; TRE, tetracycline-responsive element; UV, ultraviolet radiation.

Abstract

More than half of human tumors lack functional *p53.* When human fibroblast cell strain MSU-1.1 was treated with a single dose of carcinogen and assayed for focus formation, a significant fraction of the focus-derived cell strains were found to have lost functional *p53.* When cells from the foci were propagated and injected into athymic mice, only those lacking p53 function formed tumors. To determine whether loss of *p53* plays a causal role in allowing cells to form foci, and whether restoration of p53 to these cells would prevent them from forming tumors, we transfected a wild-type *p53* gene into MSU-1.1-γ1-2A1, a focus-derived cell strain that does not contain *p53* DNA and produces malignant tumors in athymic mice (Reinhold et al., Radiat. Res., 69:707, 1996). Two independent transfectants that expressed wild-type p53 protein at a normal level, i.e., comparable to that of the original, non-transformed parental strain MSU-1.1, were identified. One of the cell strains replicated at a rate comparable to that of cell strain MSU-1.1, and the other at a rate 50% lower. However, their frequency of focus formation and the quality of the foci were very significantly reduced,

compared to those of the focus-derived strain from which they were derived. The ability of the two wild-type *p53* transfectant cell strains to form colonies in semi-solid medium (anchorage-independence) or tumors in mice was very significantly decreased, and the cells derived from the few tumors that did form no longer expressed p53 or expressed it at very low level. The data indicate that expression of exogenous wild-type *p53* in carcinogen-transformed, focus-derived, malignant human fibroblasts can significantly inhibit focus formation, anchorage-independence, and tumor formation. Expression of functional *p53* at a normal level in these focus-derived cell strains either directly prevented them from forming tumors, or prevented them from acquiring the genetic change(s) needed to become tumor-forming cells.

Introduction

More than half of the human tumors tested have lost functional *p53*, a tumor suppressor gene (1) and Li-Fraumeni patients, who inherit one mutant allele of the *p53* gene, are predisposed to soft tissue sarcomas and various other kinds of cancer (2). In response to DNA damage, p53 protein triggers growth arrest of cells in the G1 phase of the cell cycle by transactivating p21 (3), a universal inhibitor of cell-cycle-dependent kinases (4). This arrest allows cells in G1 additional time to carry out DNA repair before the onset of DNA synthesis. In badly damaged cells, p53 triggers apoptosis by transactivating Bax, an apoptotic factor (5). When expression of wild-type p53 is lost, cells are much more likely to acquire mutations (6), including those involved in malignant transformation. Reintroduction of wild-type *p53* into human tumor-derived cell lines has been

shown to cause growth arrest or apoptosis, depending in part on the level of expression of the transgene (7,8). It can also trigger senescence (9).

To investigate the mechanisms of malignant transformation of human fibroblasts, McCormick and colleagues (10) developed an infinite life span, near-diploid, chromosomally-stable, nontumorigenic human fibroblast cell strain, MSU-1.1, from the foreskin of a normal neonate. MSU-1.1 cells can be malignantly transformed into cells capable of forming tumors in athymic mice by transfection of an overexpressed activated (mutated) H-*ras* (11), N-*ras* (12) or K-*ras* (13) oncogene, or by a single exposure to γ-radiation (14,15) or a chemical carcinogen (16,17). Cells from tumors produced by cells overexpressing a transfected *ras* oncogene did not exhibit any change in karyotype. The carcinogen-treated cells were allowed a 7 to 8 day expression period following exposure to carcinogen before being assayed for ability to form foci. Analysis of independent focus-derived cell strains for the transactivation function of p53 revealed that 40 to 60% had lost all p53 transactivation function. When clonally expanded and assayed for the ability to form tumors, a significant fraction (45% to 70%) of the focus-derived cell strains formed fibrosarcomas after a short latency (15-17). All of these focus-derived cell strains had lost the transactivating function of p53. These results strongly suggested that loss of *p53* function allows focus formation by these MSU-1.1 cells and that loss of *p53,* although not sufficient in itself to cause such focus-forming cells to form tumors in the athymic mice, nevertheless plays an essential role in the cells acquiring the additional changes needed to progress further.

To test the hypothesis that loss of *p53* is sufficient to enable human fibroblasts to form foci and significantly increases their chance of becoming malignant, we transfected a wild-type copy of *p53* into cell strain MSU-1.1γ1-2A1, which was derived from a focus induced by irradiating parental MSU-1.1 cells with 4.35Gy ^{60}Co (14). The focus-derived MSU-1.1γ1-2A1 cells were shown to have lost one copy of chromosome 16 and chromosome17. Immunoprecipitation studies showed that they do not express any p53 protein. When the cells were propagated and injected into athymic mice, they formed tumors with a diameter of 1 cm within 3-7 weeks (14). Therefore, these focus-derived MSU-1.1γ1-2A1 cells were used as the target cells to determine whether restoration of wild-type p53 expression at a normal level could significantly inhibit focus formation, anchorage-independence, and/or the ability to form malignant tumors. The results obtained from two independent *p53* transfectant strains exhibiting a normal expression level of p53 protein and retaining a normal growth rate in culture indicate that loss of p53 plays a causal role in allowing cells to form foci, to form large-colonies in semi-solid medium (anchorage-independence), and to form tumors in athymic mice.

Materials and methods

Cell strain and cell culture

The target cell strain used for transfection of wild-type *p53* was MSU-1.1γ1-2A1, derived from a focus formed by the original infinite life span parental human fibroblast cell strain designated MSU-1.1 (10) that had been irradiated with a single dose of cobalt 60 at 4.35 Gy and assayed for focus formation (14).

The focus-derived cell strain was shown in the original study (14) to be capable of forming tumors in athymic mice. Unless otherwise noted, cells were cultured in Eagle's minimal medium (Gibco, Gaithersburg, MD), pH 7.2, supplemented with 0.2 mM L-aspartic acid, 0.2 mM L-serine, 1.0 mM sodium pyruvate, and 10% (v/v) supplemented calf serum (HyClone, Logan UT). The medium also contained penicillin (100 U/ml), streptomycin (100 μg/ml), and hydrocortisone (1 μg/ml). Cells were cultured in 5% CO_2 humidified incubators at 37^0C.

Plasmid construction

Plasmid pBPSTR1-WT*p53*.19 was constructed by excising the 1.8 kb WT*p53* DNA fragment from pc53-SN (provided by Dr. B. Vogelstein) by *BamH* I digestion and ligating it into pBPSTR1 that had been linearized at the multiple cloning site by *BamH* I digestion. pBPSTR1 contains a tetracycline-responsive promoter upstream of the multiple cloning site, as well as a tTA expression cassette for enhancement of tetracycline responsiveness, and the gene coding for resistance to puromycin as the selectable marker.

Transfection

Cells plated at 10^5 per 60 mm-diameter tissue culture dishes were transfected ~18 h later with plasmid DNA linearized at the unique *Sca* I site. Plasmid DNA, 1.5 μg per dish, was introduced in lipofectamine according to the manufacturer's instructions (Gibco, Bethesda). Tetracycline (2.0 μg/ml) was included in the transfection solution. After 48 h, the medium was changed to medium containing puromycin (1.0 μg/ml) to select for transfectants. Tetracycline

(2.0 μg/ml) was added every 2-3 days. The cells were given fresh medium containing puromycin (1.0 μg/ml) every 5 days.

Growth curves

Cells growing logarithmically were dislodged with trypsin and plated at a density of 1×10^5 cells per 100 mm-diameter dish on day 0. On day 1, 2, 3, 4, 5, 6 and 7, the cells in three dishes were dislodged with trypsin, suspended in isoton solution and counted by using an electronic counter (Coulter, Hialeah, FL) to determine the total number of cells per dish.

UV^3-irradiation

Cells in exponential growth in 100 mm-diameter dishes at 40-50% confluence were aspirated, washed with PBS, aspirated again and irradiated with 5 J/m^2 UV (254nm) at an incidence dose of ~0.3 J/m^2/sec. Cells were given fresh medium immediately after irradiation.

Preparation of cell lysates

To assay expression of p53 protein, the protocol described previously by Qing et al. (18) was used. Briefly, exponentially growing cells were washed with cold PBS and lysed on ice with lysis buffer composed of 50 mM Tris-HCl, pH7.2, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 0.1 mM sodium vanadate, 2 mg/ml aprotinin. The cell lysates were collected with a rubber scraper and centrifuged at 14,000 rpm on a microcentrifuge (Eppenforf, Westbury, NY) at 4^0C. The supernatant was collected, an aliquot was taken for protein

quantification, and the rest was stored at -80^{0}C. The protein concentration of the lysates was determined using the Coomassie protein assay reagent (Pierce, Rockford, IL). To assay expression of p21, cells were dislodged with trypsin, counted with a Coulter counter, centrifuged at 2000 rpm on a microcentrifuge for 10 min, resuspended in PBS and centrifuged again. To lyse the cells, the cells pellets were lysed with lysis buffer containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.05% bromophenol blue and 62.5 mM Tris, pH 7.2. For 1× 10^6 cells, 40 μl of lysis buffer was used. The lysates were heated at 95^{0}C for 15 minutes, allowed to cool on ice, briefly centrifuged to collect condensation, and stored at -80 ^{0}C.

Western blot analysis

Cell lysates were loaded on a 10% SDS-PAGE gel (40 μg of protein for p53 and 10 μl lysate for p21), and the gel was run at constant 50 mA. The proteins were electrotransfered onto an Immobilon-P membrane (Millipore, Bedford, MA) for ~18 h at constant 50 mA. The membranes was blocked at 4^{0}C overnight in 5% (w/v) non-fat dry milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.2, 137 mM NaCl) containing 0.1% (v/v) Tween-20 (TBST), and incubated for 2 h at room temperature with the appropriate mouse monoclonal antibodies, i.e., anti-p53 antibody (Clone Pab1801) or anti-p21 antibody (sc-817) (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 0.5-1.0 μg/ml. The membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO) that had been diluted 1:20,000 in TBST solution supplemented with 5% milk. Enhanced chemiluminescence

(SuperSignal HRP substrate, Pierce, Rockford, IL) was used to detect the protein bands, and a Molecular Phosphor Imager system (BioRad, Hercules, CA) was used to quantitate them. The actin level, assessed with goat polyclonal anti-actin antibody (sc-1616) (Santa Cruz Biotechnology, Santa Cruz, CA), was used to normalize the levels of p53 and p21 proteins.

Focus reconstruction assay

Cells in exponential growth, together with 2×10^5 feeder cells, were suspended in Eagle's medium supplemented with 20 mM HEPES (Sigma, St. Louis, MO) and 0.5% FBS (Summit Biotechnology, Fort Collins, CO) and plated into 100 mm-diameter dishes that had been plated with 5×10^4 normal, non-transformed MSU-1.1 cells in the same medium ~16h earlier. Feeder cells used were MSU-1.1 cells that had been treated with 30 Gy γ-radiation. They could not divide, but could increase the colony forming ability of the live cells in the first several days after plating. Sixteen dishes were plated for each cell strain to be tested. The cells were incubated at 37 ^{0}C in the same 5% CO_2 humidified incubator, and the medium was replaced every 4 days. At that time, the dishes were also scanned for foci using a focused beam of light from beneath the dishes. Some dishes were stained to verify the growth of foci as needed. Representative foci were photographed before and after fixation with methanol and staining with methylene blue.

Assay of anchorage-independent growth

Cells were assayed for the ability to form colonies in semi-solid medium, i.e., 0.33% agarose (Seaplaque, FMC, Rockford, ME). 5000 exponentially growing cells in 1.5 ml of McM medium (19) supplemented with 20 mM HEPES (Sigma, St. Louis, MO), 0.33% (w/v) agarose and 2% (v/v) FBS (Summit Biotechnology, Fort Collins, CO) were plated on top of a solidified bottom agarose layer made up of McM medium supplemented with 2% (w/v) agarose and 2% (v/v) FBS. The top agar was allowed to solidify, and then covered with 3.5 ml of McM medium containing 2% FBS, 20 mM HEPES, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were incubated at 37 ^{0}C in a 3% CO_2 humidified incubator, and the 3.5 ml of medium was replaced every week for 3 wk. The parental MSU-1.1 cell strain, which does not form colonies in soft agar and a derivative H-*ras*-transformed tumorigenic cell line, MSU-1.1 A210, which forms large-sized colonies in soft agar, were used as negative and positive controls, respectively. After 3 wk, the medium overlaying the agar was removed and glutaraldehyde (2.5%) was added to fix the colonies in the top agar layer. The dishes were stored at 4 ^{0}C until analyzed. The size of colonies was measured under microscope using a calibrated ruler placed in the eyepiece of the microscope. Representative fields of colonies were photographed.

Tumorigenicity test

Athymic BALB/c mice of 4-5 weeks of age were injected subcutaneously in the left front and right hind flank regions with 1×10^6 cells suspended in 0.2 ml of serum-free Eagle's medium. The mice were monitored weekly for tumor growth, and the size of tumors was measured using a caliper. When a tumor

reached 1 cm in diameter, the mice were sacrificed, and the tumor was removed. Cells from a portion of the tumor were returned to culture, and the rest of the tumor was fixed with formalin for histological examination. Because MSU-1.1 cells express a transfected neomycin resistance gene (10), making them resistant to G418, tumor-derived cells were propagated in medium containing G418 (Gibco, Bethesda, MD) to eliminate any possible contaminating mouse cells. All mice were sacrificed four months after injection.

Results

Determining whether functional wild-type *p53* could be successfully re-introduced and expressed at a normal level into cell strain MSU-1.1γ1-2A1

Exogenous expression of wild-type *p53* in cancer cell lines has been shown to induce growth arrest or apoptosis (7,8), or to promote cellular senescence (9,20). Such effects indicated that it might be very difficult to obtain cells that expressed a transfected wild-type *p53* gene, but were still capable of growing normally in cell culture. In an attempt to control expression of the transfected wild-type *p53* in order to avoid these deleterious effects, we used a construct that was designed to be tetracycline-regulatable and transfected it into the focus-derived cell strain MSU-1.1γ1-2A1. For the sake of brevity, this strain will be referred to as 2A1 from now on. The constructed vector, pBPSTR1-WT*p53*.19, is designed so that when tetracycline is absent, the tTA binds to the TRE and activates the expression of the transgene *p53*. In the presence of tetracycline, tTA should be unable to bind the TRE, and therefore the expression of the transgene is shut down. Focus-derived cell strain 2A1 was transfected with

the plasmid in medium containing tetracycline, and puromycin-resistant transfectants were selected in the presence of tetracycline. A total of 55 puromycin-resistant clones were expanded. Lysates for Western blot analysis were prepared from cells cultured for 7 days in the absence of tetracycline. Of the 55 cell strains assayed, two, designated MSU-1.1-2A1-88A and MSU-1.1-2A1-85, were found to express p53 protein. For the sake of brevity, these two strains will be referred to from now on as cell strains 88A and 85. As shown in Figure 1, the recipient cell strain 2A1 does not express p53; transfected cell strain 88A expressed p53 protein at a level comparable to that found in the parental MSU-1.1 cell strain, and the level of p53 in transfected cell strain 85 was ~2-fold higher than that seen in parental MSU-1.1 cells. Therefore, cell strains 88A and 85 were suitable for determining the role of loss of p53 in carcinogen-induced human cell transformation. Additional studies with strain 88A revealed that p53 expression in these cells could not be modulated by tetracycline (data not shown). Therefore it was not possible to examine the effects of p53 expression on transformation using the same strain cultured in the presence or absence of tetracycline. Lack of modulation of gene expression using this expression system has also been reported elsewhere (21).

Evidence that the level of expression of exogenous wild-type p53 did not greatly affect the rate of growth of the two cell strains

As noted above, expression of exogenous p53 in human cells has been reported to induce growth arrest (7-9). Therefore, we compared the rate of growth of strains 88A and 85 with that of their parental cell strain, 2A1, which

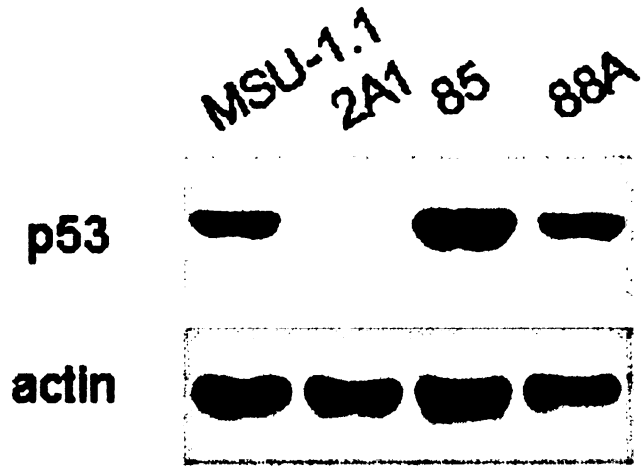


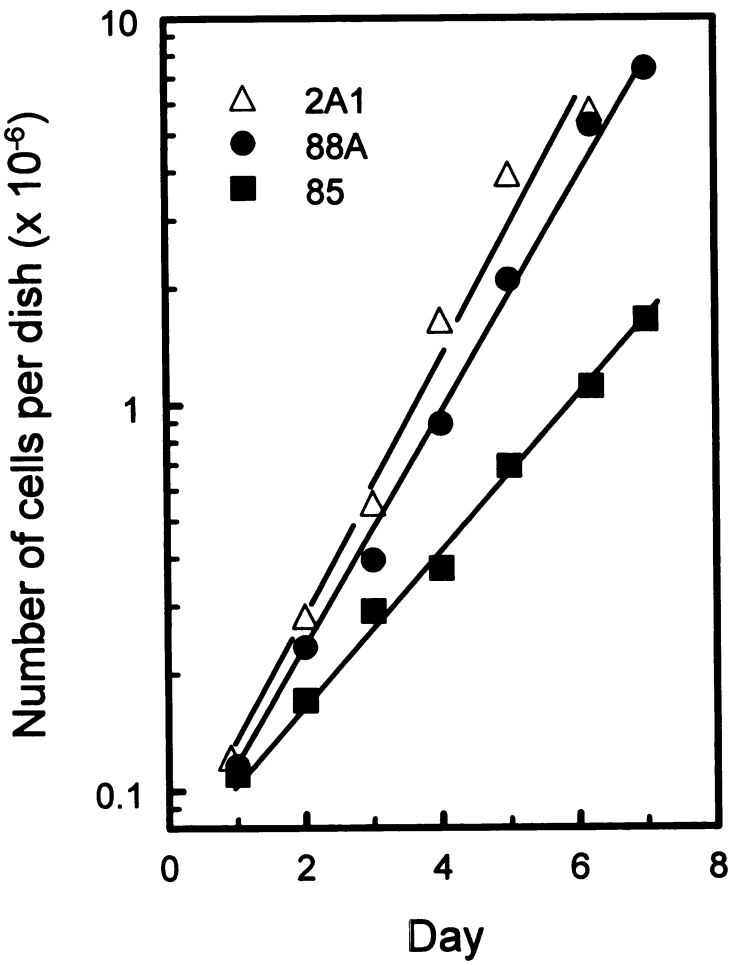
Figure 1. Western blot analysis of cell strains 88A and 85 for the expression of p53 protein. Cell strains MSU-1.1 and 2A1 were used as a positive and a negative control respectively. For each strain, 100 μg of lysate was loaded. The p53 protein was probed with monoclonal anti-p53 antibody (Pab1801). Actin was used as a loading control.

does not express p53. As shown in Figure 2, the population doubling time of strain 88A was 24 h, which is very similar to that of the non-transfected strain 2A1, i.e., 22 h, and the population doubling time for strain 85, which expresses p53 at a slightly higher level, i.e., 35 h. These results, indicating that the level of p53 expression in these two strains in the absence of tetracycline did not have a significantly deleterious effect on their rate of cell growth, allowed us to use them to examine the effects of p53 expression on other properties of the cells.

Evidence that the exogenous p53 in the transfected strains was functional

To determine whether the transfected wild-type *p53* in strains 88A and 85 was functional, we tested using Western blotting whether UV radiation increased the level of expression of p53 in the cells and also that of p21 protein, the product of a gene for which p53 is a transcription factor (22). Such a test of the functionality or activation of p53 has been commonly used (23,24). Parental strain MSU-1.1, which expresses wild-type p53, was used as a positive control, and focus-derived cell strain 2A1 was used as a negative control. As shown in Figure 3, the relative basal level of p21 protein in each of the four strains corresponded to the basal level of p53 protein in the same cell strains. For example, 2A1 cells did not express either p53 or p21; strain 88A had a lower basal level of p53 and of p21 than parental cell strain MSU-1.1; and strain 85 had a higher basal level of p53 and of p21 than strain MSU-1.1. In response to UV, strain 2A1, which lacks expression of p53, showed no upregulation of expression of p21, whereas strain 88A, as well as MSU-1.1 cells, exhibited increased

Figure 2. Expression of exogenous p53 in cell strains 88A and 85 does not significantly affect the rate of cell growth. Cell strains 88A (●), 85 (■), and cell strain 2A1 used as the recipient for transfection of p53 (Δ) were plated at 1×10^5 per 100 mm-diameter plates on day 0. On each of the following seven days, cells on 3 p100's of each strain were dislodged with trypsin, combined, and counted with an electronic counter. The average number of cells on each dish for each strain was calculated. The data point of 2A1 (Δ) on day 1 has been offset slightly for clarity. The population doubling time of strains 2A1, 88A, and 85 were ~22h, ~24h, and ~35h, respectively.


10
1
0.1
Number of cells per dish (x 10-6)
2A1
88A
85
0
2
4
6
8
Day

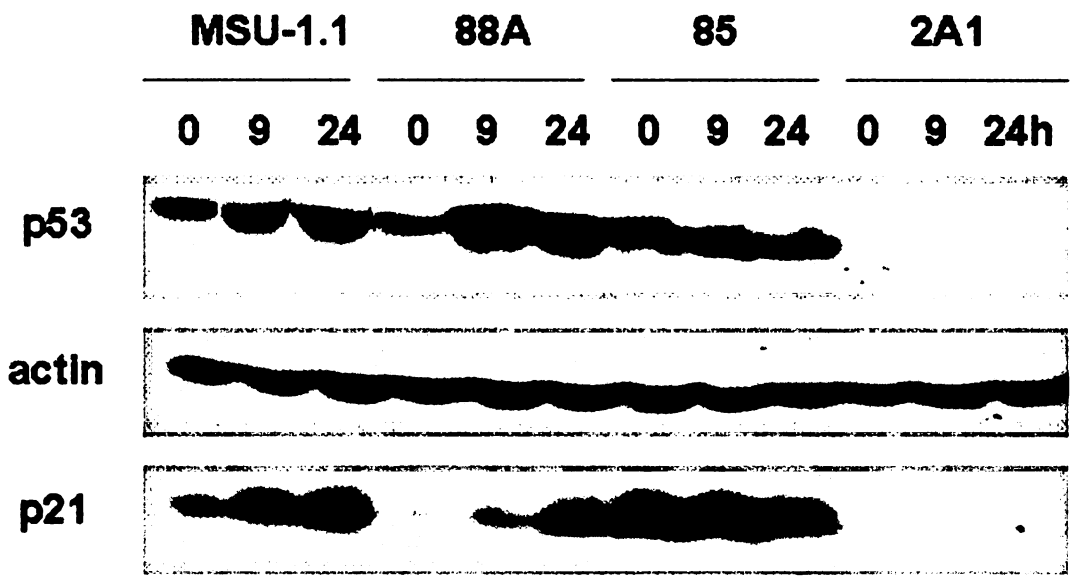


Figure 3. Expression of p53 and p21 proteins in cell strains 88A and 85 in response to UV irradiation. Cell lysates were prepared from exponentially growing cells prior to UV treatment (labeled as 0) or 9 or 24 hours (labeled as 9 or 24) after UV treatment at 5J/m^2. Western blot analysis was performed using anti-p53 (Pab1801) or anti-p21 (sc-817) mouse monoclonal antibodies. Cell strain MSU-1.1, which contains wild-type p53, and cell strain 2A1, which lacks p53 expression, were included as a positive control and a negative control, respectively. Actin was the loading control.

expression of both p53 and p21. The level of both p53 and p21 protein in strain 85 did not change significantly as a result of UV treatment. These data, showing the p53-dependent expression of p21 protein in cell strains 88A and 85, and the lack of expression of p53 and p21 in the non- transfected strain 2A1, strongly suggest that the function of p53 had been restored in strains 88A and 85.

Effect of expression of exogenous p53 on focus formation by the focus-derived cell strain 2A1

Focus reconstruction assays are used to evaluate the ability of cells to proliferate, form a multilayer, and pile up on top of confluent monolayer of lawn cells. The 2A1 cell strain was derived from a focus formed by the parental strain MSU-1.1 after γ-irradiation (14). To test whether p53 expression in the *p53* transfectant cell strains 88A and 85 inhibited focus formation, focus reconstruction assays were carried out, plating the same number of clonable cells on the lawn of cells in each dish for all strains tested. Pilot studies showed that plating 80 cells of strains 88A and 2A1 and 500 cells of strain 85 per 100 mm-diameter tissue culture dish, in presence of 2×10^5 feeder cells per dish gave a plating efficiency of 60-70 clones on each dish. To minimize possible variation in focus formation by cells to be tested that might reflect the status of the lawn cells used, we assayed the various cell strains plated onto two different populations of MSU-1.1 cells, i.e., SB62-7 cells, a non-cloned population, and SB62-7.9 cells, a clonal population derived from it. The former cells grow a little faster than the latter, but otherwise the lawn cells grow in an identical manner. After 2 to 3 wk, foci formed by the parental focus-derived 2A1 cells on top of the

monolayer of lawn cells could easily be seen using a focussed beam of light. In contrast, with p53-expressing cell strains 88A and 85, foci could only be differentiated from the background lawn cells after 4 wk. Strain 2A1 formed distinct foci, while strains 88A and 85 formed only weak foci. Compared to the foci formed by 2A1 cells, those formed by cell strains 88A and 85 contained fewer crossing layers in a much more limited area (see Figure 4). As shown in Table 1, which presents the numbers of foci formed by 88A, 85 and 2A1 cells on the two separate lawns, the frequency of focus formation of strain 88A was five times lower than that of the parental 2A1 cells, and that of strain 85 was ten times lower. The reduction in both the frequency of focus formation and the quality of foci in two independent strains, 88A and 85, clearly indicates that expression of wild-type p53 at a normal level inhibits focus formation by these cells.

Expression of p53 also reduced the anchorage-independence

To obtain additional evidence of the inhibiting effect of p53 on human cell transformation, we tested the anchorage independence of cell strains 2A1, and its two derivative strains, 88A and 85, i.e., their ability to form colonies in semi-solid agarose. Anchorage-independence, another important characteristic of malignantly-transformed cells, has been widely used to determine whether a particular cell strain has been transformed into malignant cells (15,25). For this assay, MSU-1.1, the infinite life span, non-tumorigenic parental strain from which the 2A1 cells arose, and cell strain A210, an H-*ras*-transformed, tumorigenic derivative of MSU-1.1 cells, were included as the negative and positive controls,

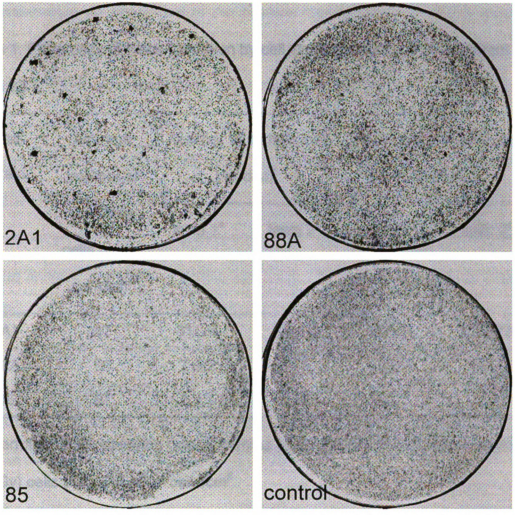


Figure 4. A representative set of foci formed by cell strains 2A1, 88A and 85. Eighty 2A1 and 88A cells or 500 85 cells, together with 2×10^5 feeder cells, were plated into p100 tissue culture dishes that had been plated with 50,000 normal cells ~16h earlier: Cells were refed and checked every 4 days. Dishes were stained with methylene blue.

Table 1. Frequency of focus formation by cell strains 88A, 85, and the parent strain 2A1

Cell strain	Average number of clonable cells plated per dish[a]	Number of distinct foci per dish[b]		Percentage of cells able to form foci[b]	
		lawn 1[c]	lawn 2[c]	lawn 1[c]	lawn 2[c]
2A1	67	31 ± 4	32 ± 6	46 ± 6	47 ± 10
88A	59	7 ± 2	6 ± 2	12 ± 4	10 ± 4
85	67	3 ± 1	2 ± 1	4 ± 1	3 ± 2

[a] Determined from assaying colony forming ability of cells plated with γ-irradiated (30Gy) cells plated at 4000 cells/cm^2.

[b] Data presented as mean ± standard deviation. Six dishes were counted for each cell strain.

[c] Lawn 1: SB62-7; lawn 2: SB62-7.9.

respectively. As shown in Figure 5, 2A1 cells formed large-sized colonies, with size distribution similar to that of the positive control cell strain A210, whereas 88A and 85 cells, with the function of wild-type *p53* restored, showed greatly reduced ability to form colonies in soft agar. As shown in Table 2, the 2A1 cells formed colonies with a diameter greater than 70μm at a frequency of >20%. In contrast, only ~1% of 88A cells and ~0.5% of 85 cells formed colonies with a diameter of 70μm or more. Unlike 2A1 cells, which could produce colonies with a diameter of ≥105μm with a frequency of ~10%, the 88A and 85 cells never did so. The number and size of colonies formed in soft agar by 88A and 85 cells did not differ from those formed by non-transformed parental MSU-1.1 cells. These data indicate that induced expression of exogenous p53 in the focus-derived strain 2A1 strongly suppressed its anchorage-independence. The decrease in anchorage-independence of these *p53*-transfected cell strains correlated well with the decrease in their focus forming ability.

Effect of expression of exogenous p53 on the tumorigenicity of cell strains 88A and 85

A significant fraction of the cell strains derived from foci induced by treating MSU-1.1 cells with benzo(a)pyrene diol epoxide (16) or ionizing radiation (15) can form tumors in athymic mice. This is true for cell strain 2A1 generated by selection for focus formation. When the cells derived from the focus were propagated and injected into athymic mice, they proved to be highly tumorigenic (14). Because expression of p53 so greatly reduced the focus-forming ability of cell strains 88A and 85, we determined whether p53 expression could also

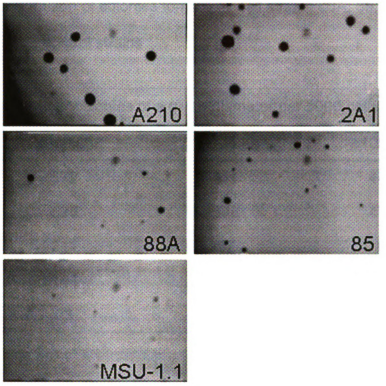


Figure 5. Photos of representative field of colony formed by cell strains 88A, 85 and 2A1 in soft agar. MSU-1.1, the immortal and non-tumorigenic original parental strain, and cell strain A210, an H-*ras*-transformed, tumorigenic derivative of MSU-1.1 cells, were included as the negative and positive controls, respectively. The photos were taken under microscope with 6× magnification.

Table 2. Size distribution of colonies formed by cell strains 88A, 85, the parent strain 2A1, and two control strains

Cell strains	Number of colonies of a given diameter per 5000 cells assayed:		
	35-70 μm	70-105 μm	≥105 μm
A210	572	911	445
2A1	525	599	476
88A	211	57	0
85	144	20	0
MSU-1.1	23	0	0

reduce the tumorigenicity of the cells. Cell strains 88A and 85 were injected subcutaneously into athymic mice. As a control, 2A1 cells were also injected into mice at the same time. As expected, cell strain 2A1 proved to be highly tumorigenic, forming tumors in all 18 sites of injection. In contrast, the incidence of tumor formation by cell strains 88A and 85 was greatly reduced. Cell strain 85 did not form tumor at any of the 18 injection sites; strain 88A formed tumors at only 6 out of 18 injection sites. The frequency of tumor formation by 88A cells was consistent in three independent injection experiments, with 1-3 tumors out of 6 injection sites. What is more, the length of the time required for the 88A cells to form tumors that reached 0.5 cm in diameter was ~6wk, i.e., 1 wk longer than required for 2A1 cells. These data indicate that expression of exogenous wild-type p53 protein eliminated or markedly reduced the tumorigenicity of the cells.

Histological examinations of tumor slides showed that all the tumors formed by 2A1 cells and 88A cells were high grade round spindle cell sarcomas. Cells from the largest tumors formed were propagated and designated 88A-Tumor Derived (TD)1, -TD2, -TD3, -TD4 and 2A1-TD1, -TD2, and analyzed for the expression of p53 protein by Western blotting. As expected, the 2A1-TD1 and 2A1-TD2 did not express any p53 protein. What is more significant, the cells derived from the tumors that had not been expected, i.e., 88A-TD1, 88A-TD2 and 88A–TD4 had lost expression of p53 protein, and those from strain 88A-TD3 showed a greatly reduced level of p53 protein. These data strongly suggest that the tumors formed following injection of 88A cells that had been expressing a transfected *p53* gene resulted from the growth in the animals of cells that had

Table 3. Tumorigenicity of cell strains 88A, 85, and the parental strain 2A1

Cell strains	Tumor incidence[a]	Tumor latency[b] (wk)	p53 expression in tumor-derived cells[c]
2A1	18/18	~ 5	no
88A	6/18	~ 6	no or markedly reduced
85	0/18	N/A	N/A

[a] Number of sites with tumor per number of sites injected.

[b] The length of the time required for cells to form tumors of 0.5 cm in diameter.

[c] As determined by Western blotting shown in Figure 6.

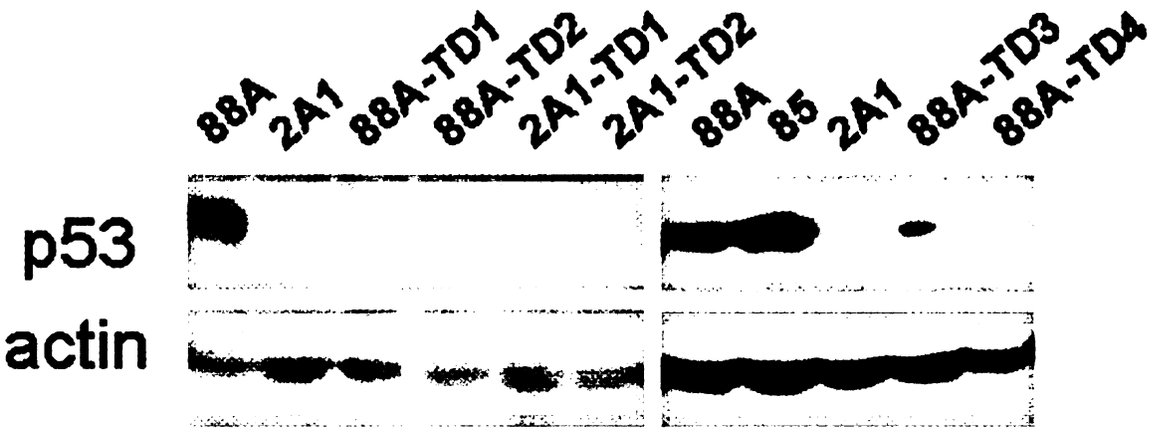


Figure 6. Lack of or markedly reduced expression of p53 protein in cells derived from tumors formed by 88A cells. Cells from four largest tumors formed by injected 88A cells, designated as 88A-Tumor Derived (TD)1, -TD2, -TD3 and -TD4, were propagated and evaluated for the expression of p53 protein by Western blot analysis. Lysates of 88A and 85 cells, of 2A1 and 2A1-TD1, -TD2 cells, were used as positive controls and negative controls, respectively. p53 protein was detected using an anti-p53 monoclonal antibody (Pab1801), and actin was used as the loading control.

lost expression of wild-type p53 or in which expression of p53 had been greatly reduced. Because all the injections were made using a common population of cells that had been propagated as a pool, such loss or reduction of expression of the transfected p53 is not likely to have occurred before the cells were injected into the athymic mice, but rather reflects selection for a growth advantage in the mice of cells with reduced levels of p53. Transgene (*p53* cDNA)-specific amplification by polymerase chain reaction in 88A-TD1 cells and 88A-TD2 cells showed that these two tumor-derived strains had lost their transfected DNA (data not shown). The absence or reduced frequency of tumor formation by cell strains 85 and 88A, and the lack of or low level of p53 expression in tumors that formed following injection of cell strain 88A, strongly suggest that p53 suppresses tumor formation and that loss of p53 expression plays a causal role in human cells becoming malignant.

Discussion

In the studies of the malignant transformation of MSU-1.1 by γ-irradiation (15) or by MNU (17), a significant fraction (>40%) of the focus-derived cells were found to have lost the function of *p53*. Of the 13 independent focus-derived strains assayed that had been induced by a single exposure of MSU-1.1 cells to ^{60}Co irradiation, 11 showed loss of p53 transactivation activity. Assays of 40 independent focus-derived cell strains induced by MNU showed that 15 (~40%) lacked functional *p53*. These data suggest that *p53* plays an important role relative to focus formation. In these transformation studies referred to above, it was also found that a high percentage (62% and 29%, respectively), but not all,

of the focus-derived cell strains assayed that had lost *p53* function were capable of forming tumors in athymic mice. However, all the focus-derived strains that did form tumors lacked p53. This led to the hypothesis that loss of *p53* enables human fibroblast MSU-1.1 cells to form distinct foci on a monolayer of normal cells, and that, although loss of *p53* is not sufficient to convert the focus forming cells into malignant cells, it enables or predisposes the fibroblasts to acquire an additional change(s) necessary for them to become malignant, i.e., able to form malignant tumors in athymic mice. If so, restoration of wild-type p53 expression in a focus-derived cell strain should inhibit focus formation and tumor formation by the cells. It was to test these hypotheses that we transfected wild-type *p53* cDNA into a focus-derived cell strain, 2A1, that had been shown to lack *p53*. Our study with cell strains 88A and 85 show that restoration of wild-type *p53* inhibited the focus formation, anchorage-independence and tumorigenicity of these cell strains.

p53 suppresses tumor by a number of mechanisms, including growth arrest (7), apoptosis (8) and senescence (9). These mechanisms were discovered when wild-type p53 protein was overexpressed in cancer cell lines, and a common feature of these mechanisms is the inability of the p53 transfectants to replicate. In our study, out of the 55 strains tested, we identified two independent strains that express p53 protein, and these two strains express p53 at a level comparable to the nontumorigenic parental strain MSU-1.1. Our failure to identify strains that overexpress the exogenous protein probably reflects the deleterious effect on cell growth of overexpression of exogenous wild-type

p53 protein. The two strains, 88A and 85, expressing a normal level of p53, grew normally. Strain 88A replicated as fast as the parental strain 2A1 in culture; strain 85 had 50% longer doubling time. The fact that the growth rate of cell strains 88A and 85 was virtually normal facilitated our study of their transformed phenotypes. The effect of overexpression of wild-type p53 protein on cell growth appears to vary with cancer cell type. For example, the human glioma cell line U87MG has wild-type *p53* function. The growth of this cell line, as measured by clonogenic survival, is only reduced by 25% even when 20-25 times more exogenous wild-type p53 protein is expressed (26). In an independent study, the growth of this same cell line did not change when exogenous p53 protein was introduced (27).

Our data showing that expression of wild-type *p53* inhibited focus formation strongly suggest that loss of *p53* function results in focus formation. This result agrees with a previous study reported by Maclean et al. (28) that inactivation of the wild-type p53 by SV40 large T antigen causes focus formation by fibroblasts derived from a patient with Li-Fraumeni syndrome. Wild-type *p53* also inhibits focus formation induced by transfected oncogenes. In the study of transformation of primary rat embryo fibroblasts, Eliyahu et al. (29) found that wild-type *p53*, but not mutant *p53*, greatly reduces the number of foci induced by combinations of bona fide oncogenes, such as *myc* plus *ras* or adenovirus *E1A* plus *ras*. *p53* also suppresses focus formation induced in primary rodent epithelial cells by *E1A* and *E1B* oncogenes. Interruption of p53 function by adenovirus E4orf6 protein promotes the transformation of these cells by *E1A* and *E1B* oncogenes (30). Studies carried out in this laboratory, including the present

study and those by O'Reilly et al. (15) and Boley et al. (17), identify the inhibitory role of p53 on focus formation of human fibroblasts induced by various carcinogens. This information provides insight into the tumor suppressor function of *p53* from a new perspective. It should be noted that factors other than loss of p53 also induce focus formation. For example, MSU-1.1 cells possessing wild-type *p53* genes can still be induced to form foci by transfection of activated (mutated) oncogenes including *ras* (11-13). It is important to note that in transformation studies of MSU-1.1 cells induced by γ-radiation (15) or by MNU (17), focus-derived cell strains that retained their wild-type p53 function were also identified. Furthermore, a substantial percentage of human cancers have been determined not to have a mutation in the *p53* gene (11).

Both of the cell strains expressing transfected wild-type p53 showed reduced anchorage-independent growth potential and the ability to form tumors in athymic mice. This is not surprising because the ability of cells to grow in semisolid medium has been closely correlated with tumorigenicity in animal models (31). The correlation between anchorage-independence or tumorigenicity and loss of wild-type *p53* function has been reported as well. For example, as an animal model for human urothelial carcinomas, an immortalized nontumorigenic rat urothelial cell line, MYP3, expressing wild-type p53, was used for studying the effect of loss of p53 function on induction of malignant phenotypes. The investigators found that expression of *p53* antisense RNA resulted in a significant reduction in p53 protein level, a stimulation of anchorage-independent growth, and an acquired ability to form malignant tumors in athymic mice (32). In the

present study, cell strain 88A formed tumors in several sites of injection, but in the four tumor-derived cell strains that we tested for the expression of the transgene, its expression level very significantly reduced. A similar loss of an exogenous *p53* transgene has been reported in human colorectal carcinoma cell lines (33). The loss of p53 expression or greatly reduced level of p53 expression in tumors formed by cell strain 88A strongly suggests that loss of p53 function is essential for the tumorigenicity of these cells.

The p53 protein, composed of 393 amino acid residues, can be divided roughly into three functional domains, i.e., the amino-terminal activation domain, the central core sequence-specific DNA binding domain and the multifunctional carboxy-terminal domain (34). The tumor suppressor functions of *p53* such as growth arrest, apoptosis and senescence require the transactivation function of p53. It has also been reported that p53 can suppress transformation using a function other than transactivation, as p53 mutants lacking domains required for transactivation are still capable of suppressing transformation, albeit at lower efficiency than the wild-type protein (reviewed in 34). In addition, p53 can prevent tumor formation by interfering with angiogenesis of human fibroblasts by upregulating expression level of thrombospondin-1, a potent inhibitor of angiogenesis (35) or down-regulating vascular endothelial growth factor (VEGF) expression (36,37). At present, we do not know for certain how p53 suppresses foci and tumors in our system. It could be a combination of more than one of the mechanisms mentioned above, or a novel mechanism.

In summary, our data show that wild-type p53 inhibits focus formation as well as tumor formation in athymic mice by carcinogen-treated, focus-derived MSU-1.1 fibroblasts, and that p53 suppresses the transformation without altering the rate of cell growth in culture.

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CHAPTER 3

The Function of the Human Homolog of *Saccharomyces cerevisiae REV1* Is Required for Mutagenesis Induced by UV Light

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Abbreviations used are: RACE, rapid amplification of cDNA ends; Pol, DNA polymerase; EST, expressed sequence tag; *HPRT*, hypoxanthine phosphoribosyltransferase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF206019).

Abstract

In *Saccharomyces cerevisiae*, most mutations induced by a wide range of mutagens arise during translesion replication employing the *REV1* gene product and DNA polymerase ζ. As part of an effort to investigate mammalian mutagenic mechanisms, we have identified cDNA clones of the human homologs of the yeast *REV* genes and examined their function in UV mutagenesis. Previously, we described the isolation of a human homolog of yeast *REV3*, the catalytic subunit of Pol ζ, and here report the identification and sequence of a human homolog of yeast *REV1*. This gene was isolated by identifying an expressed sequence tag encoding a peptide with similarity to the C terminus of yeast Rev1p, followed by sequencing of the clone and retrieval of the remaining cDNA by 5' rapid amplification of cDNA ends. The human gene encodes an expected protein of 1,251 residues, compared with 985 residues in the yeast protein. The proteins share two amino-terminal regions of ≈100 residues with 41% and 20% identity, a region of ≈320 residues with 31% identity, and a central motif in which 11 of 13 residues are identical. Human cells expressing high levels of an *hREV1* antisense RNA grew normally, and were not more sensitive to the cytotoxic effect of 254 nm UV radiation than cells lacking antisense RNA. However, the frequencies of 6-thioguanine resistance mutants induced by UV in the cells expressing antisense *hREV1* RNA were significantly lower than in the control ($P = 0.01$), suggesting that the human gene has a function similar to that of the yeast homolog.

Introduction

Information about the mechanisms that generate mutations in eukaryotes is likely to be useful for understanding human health concerns, such as genotoxicity and cancer (1). At the present, these mechanisms have been investigated most intensively in budding yeast, *Saccharomyces cerevisiae*, and in this organism almost all induced mutations arise during translesion replication, a process that promotes elongation past sites of unrepaired lesions that might otherwise block this event (1, 2). The major pathway for translesion replication in yeast employs DNA polymerase (Pol) ζ (3) together with Rev1 protein (4), an enzyme that has two functions. These include a deoxycytidyl transferase activity that incorporates dCMP opposite abasic sites in the template and a second, as yet poorly defined, activity that is required for replication past a wide variety of lesions as well as abasic sites (J. R. Nelson, P.E.M.G., A. M. Nowicka, D. C. Hinkle and C.W.L., unpublished observations). In addition to this general pathway, yeast also possesses a specialized pathway for translesion replication employing Pol η (5), a distant homolog of Rev1 protein that is encoded by *RAD30* (6, 7). Although the substrate range of this enzyme has not yet been fully defined, it is likely that it entails only a few types of lesion, which Pol η appears to bypass with relatively high accuracy. Pol η appears to contribute very little to overall mutagenesis compared with Pol ζ/Rev1 both because *RAD30* mutants have little effect on mutagenesis and because mutants lacking the Pol ζ pathway are substantially deficient in mutagenesis induced by almost all mutagens.

DNA repair and damage tolerance mechanisms in yeast often provide good models for these processes in mammals, and evidence of several kinds suggests that this is likely to be the case with translesion replication. We (8) and others (9, 10) have identified and sequenced cDNA clones of a human homolog of the yeast *REV3* gene, which encodes the catalytic subunit of Pol ζ, and a very similar gene has been described in the mouse (11). More particularly, we have further shown that UV-induced mutagenesis is markedly reduced in human cells expressing high levels of a *REV3* antisense RNA, suggesting that the human gene is also likely to be used in translesion replication (8). In addition, human cells have been shown to possess two homologs of the yeast *RAD30* gene (12-15). As further evidence for a yeast-like mechanism for translesion replication in humans, we report here the isolation and sequence of a cDNA clone of a human homolog of yeast *REV1*, together with evidence that it too is used in UV mutagenesis. A recent report also describes this gene, and provides evidence indicating that it possesses a deoxycytidyl transferase activity (16).

Materials and methods

cDNA synthesis, 5' Rapid Amplification of cDNA Ends (RACE), and cloning

Total RNA or polyadenylated RNA from human brain (Stratagene) was used as a template for cDNA synthesis, using Superscript II Reverse Transcriptase (Life Technologies, Gaithersburg, MD) and primers specific for the candidate *hREV1* cDNA clone (pHIBAC55; American Type Culture Collection). After removal of residual primer, an oligo(dC) tail was added to the cDNA with terminal transferase, and the cDNA selectively amplified by PCR, using elongase

(Life Technologies) or *Taq* polymerase, a nested *hREV1*-specific primer, and a commercial primer with 3' complementarity to the oligo(dC) tail. The PCR products were separated on agarose gels, and material from the trailing edge of the DNA smear was purified for use as a substrate for a second amplification with *Taq* polymerase; in this instance, both primers had 5' sequences containing multiple dU residues. The products were again separated on agarose gels, and the largest visible DNA species were purified and treated with uracil *N*-glycosylase in the presence of the pAMP-1 vector (Life Technologies). The resulting annealed DNAs were used to transform *Escherichia coli* DH5α cells. For assembling clones with a nearly complete insert sequence, PCR used two *hREV1*-specific primers, each of which contained a restriction enzyme recognition site in the 5' sequence. Subsequent digestion with the appropriate restriction nucleases enabled ligation with similarly digested vector DNA. A plasmid (pMINo-10) containing the entire ORF and 3'-untranslated region, but with a truncated 5' end, was constructed in pSPORT1 (Life Technologies).

DNA purification and sequence analysis

Plasmid DNAs were isolated by alkaline lysis, and in many cases further purified on midi columns (Qiagen, Chatsworth, CA) for sequence analysis. The initial clone, pHIBAC55, and clones from the first two rounds of 5' RACE were sequenced entirely by the dideoxy method with Sequenase II. Later clones were sequenced by using dye terminator chemistries (Perkin-Elmer Applied Biosystems). Primers for sequencing and for PCR were developed as required from the known *hREV1* sequence as it became available. Both strands of each

clone were sequenced, and all regions of the cDNA were sequenced in at least three clones (except for the 5' 26 nucleotides, where only one clone was available) to establish a consensus sequence for *hREV1* free of reverse transcription or PCR-generated errors. Attempts to confirm the sequence at the extreme 5' end of the mRNA with data from human expressed sequence tag (EST) sequences were unsuccessful, because none carried sequence at the 5' end of the cDNA.

Preparation of cells that express *hREV1* antisense RNA

A plasmid designed to express antisense *hREV1* RNA was constructed by inserting a 4,117-bp *REV1* fragment into pTet-Puro in the antisense orientation. The pTet-Puro plasmid, described previously (8), contains a puromycin-selectable marker and places the gene of interest under the control of the TetP promoter (17, 18), with the gene being transcribed in the absence of tetracycline (Tet OFF system). The 4,117-bp sequence contained the complete *hREV1* ORF, the entire 3' untranslated region, and a short oligo(A) sequence. Initial experiments, using a 972-bp fragment containing 75 bp of the *hREV1* 5' untranslated sequence cloned in the antisense orientation into pTet-Puro, proved incapable of suppressing *hREV1* function. This was probably caused by an interaction between the antisense RNA and 28S rRNA, and indeed the *hREV1* 5' untranslated sequence has regions of strong similarity to part of the rRNA sequence. The *Xho*I and *Sal*I fragments from pEcNo-4 containing the biologically effective 4,117-bp sequence were cloned into the *Sal*I site of the vector pTet-Puro, to give the *hREV1* anti-sense expressing plasmid, pR1P27-AS. The

orientation of the insert in this clone was determined by restriction enzyme analysis and by sequencing across the ligation boundaries.

Plasmid pR1P27-AS was transfected into 7AGM cells, which were derived from MSU-1.2, a near diploid, nontumorigenic, karyotypically stable human fibroblast cell line obtained originally from foreskin material of a normal neonate (19). Strain 7AGM was engineered to contain the tetracycline-controlled transactivator (tTA), which can activate transcription of genes of interest controlled by the Tet-responsive element. The pTet-tTAk plasmid used to introduce the tTA element carried the gene for histidinol resistance (20). pR1P27-AS transfectants were selected for resistance to puromycin and screened for the level of expression of *hREV1* antisense by Northern blot analysis.

Northern blot analysis

The conditions used for comparing the level of expression of *hREV1* antisense in the transfectant cell strains were described previously (21). Briefly, 15 µg total RNA was electrophoresed on a denaturing formaldehyde gel, transferred to a Hybond-N membrane by a downward capillary transfer technique, and fixed by UV crosslinking. The template DNA for preparing the *hREV1* antisense DNA probe was excised from the pR1P27-AS plasmid using *Eco*RI restriction. A ≈2,900-bp fragment from the 5' end of the insert *hREV1* cDNA was radiolabeled by random-primer labeling (21). The probe to be used for the loading control was similarly prepared from PCR-amplified cDNA of the hypoxanthine phosphoribosyltransferase (*HPRT*) gene of human cells. Northern hybridization was performed at 42°C overnight in 50% formamide, containing

SSPE [NaCl (0.75 M)/$NaH_2PO_4 \cdot H_2O$ (0.05 M)/EDTA (pH 8.0) (0.5 mM)] and the other components listed in ref. 22, and the blot was washed as described (21). Variation in RNA loading per lane was evaluated by probing with *HPRT* cDNA.

Determination of the cytotoxic and mutagenic effects of UV radiation

Cells were routinely cultured in medium containing 10% supplemented calf serum (HyClone), hydrocortisone (1 μg/ml), penicillin (100 units/ml), and streptomycin (100 μg/ml). The medium used was McM medium (22) or Eagle's MEM, modified by addition of L-aspartic acid (0.2 mM), L-serine (0.2 mM), and pyruvate (1 mM). The cytotoxic effect of the 254-nm UV radiation was determined from the survival of colony-forming ability, as described (23). Briefly, cells were plated into a series of dishes at cloning densities (100-1,000 cells per 100-mm-diameter dish, depending on the cloning efficiency of the strain and the expected survival). When the cells had attached and flattened out, the medium was removed, and the cells were rinsed and irradiated as described (24), using the designated doses. The mutagenic effect of UV was determined from the frequency of *HPRT*-defective, 6-thioguanine-resistant cells. Briefly, for each experiment, cells in exponential growth were plated into a series of 150-mm-diameter dishes at a density of 0.5-2 × 10^6 cells per dish. For each dose of UV, sufficient dishes were used to ensure at least 1 × 10^6 surviving cells. At the same time, cells were plated at cloning density into a series of 100-mm-diameter dishes to be used for determining the cytotoxic effect of each dose. Again, when the cells had attached and flattened out, the medium was removed, and the cells were rinsed and irradiated. For each experiment, 1 × 10^6 cells were mock-treated

as a control. Fresh medium containing serum was returned to the cells immediately after irradiation, and the cells were refed with culture medium after 24 h. The cells plated at cloning densities were allowed 14 days to form colonies, with one additional refeeding after 7 days. The cells irradiated at high density for mutation induction were allowed to replicate for 4-5 days. They were then detached from the dishes, pooled, and 1-2 × 10^6 plated again and allowed to continue replicating for 4 to 5 additional days to allow depletion of preexisting *HPRT*. The unused population for each dose was stored in liquid N_2 for future use. After an 8- to 9-day expression period, at least 1 × 10^6 cells from each population were selected for resistance to 6-thioguanine at a density of 500 cells/cm^2, as described (23). A portion of the cells from each population was also plated in nonselective medium at a density of 100 cells per 100-mm-diameter dishes to assay the colony-forming ability of the cells at the time of selection. This value was used to correct the observed frequency of mutants for the cloning efficiency of the cells at the time of selection (23).

Results and discussion

Human cells possess a homolog of yeast *REV1*

A candidate human *REV1* cDNA clone was identified by screening the dbEST database of the National Center for Biotechnology Information, using the BLAST algorithm. This screen yielded a single EST (GenBank T08134) among the approximately 52,000 entries listed at that time, and the corresponding clone pHIBAC55 was obtained from the American Type Culture Collection. The 2.13-kb human DNA insert in this plasmid was fully sequenced and found to consist of

1,813 bp of the 3' end of an ORF, 296 bp of 3' untranslated DNA, and 20 bp of poly(A). The remainder of the ORF, together with at least some of the 5' untranslated region, was obtained by using five rounds of 5' RACE, with the fifth round extending the sequence to a point beyond which no further elongation was obtained. To eliminate possible errors of reverse transcription and amplification, a consensus sequence was established from at least three independent clones at each round of 5' RACE.

The *hREV1* ORF (GenBank accession no. AF206019) encodes an expected protein of 1,251 residues (Fig. 1*A*) with significant identity to yeast *REV1* (Fig. 2) and to candidate *REV1* genes from other species, including *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Schizosaccharomyces pombe*, and *Drosophila melanogaster* (data not shown). Significant identity exists in two amino-terminal regions, each ≈100 residues in length, that exhibit 41% and 20% identity with their corresponding yeast sequences, a region of ≈330 residues with 31% identity, and a centrally located motif that is highly conserved in Rev1 proteins and also found in *E. coli* dinB. A total of perhaps nine sequence motifs are suggested by alignments with the Rev1 proteins from *S. cerevisiae*, *S. pombe*, *C. elegans*, *D. melanogaster*, and *A. thaliana* (Fig.1*B*). Motif I lies within a region that shows weak homology to the terminal region of the BRCA1 gene (BRCT), although the similarity is greater with the Rev1 protein from budding yeast than with the human protein and the possible functional significance is unclear. However, of greater functional significance, motif I in the human protein contains G76 (highlighted in Fig. 1*A*), homologous to G193 in

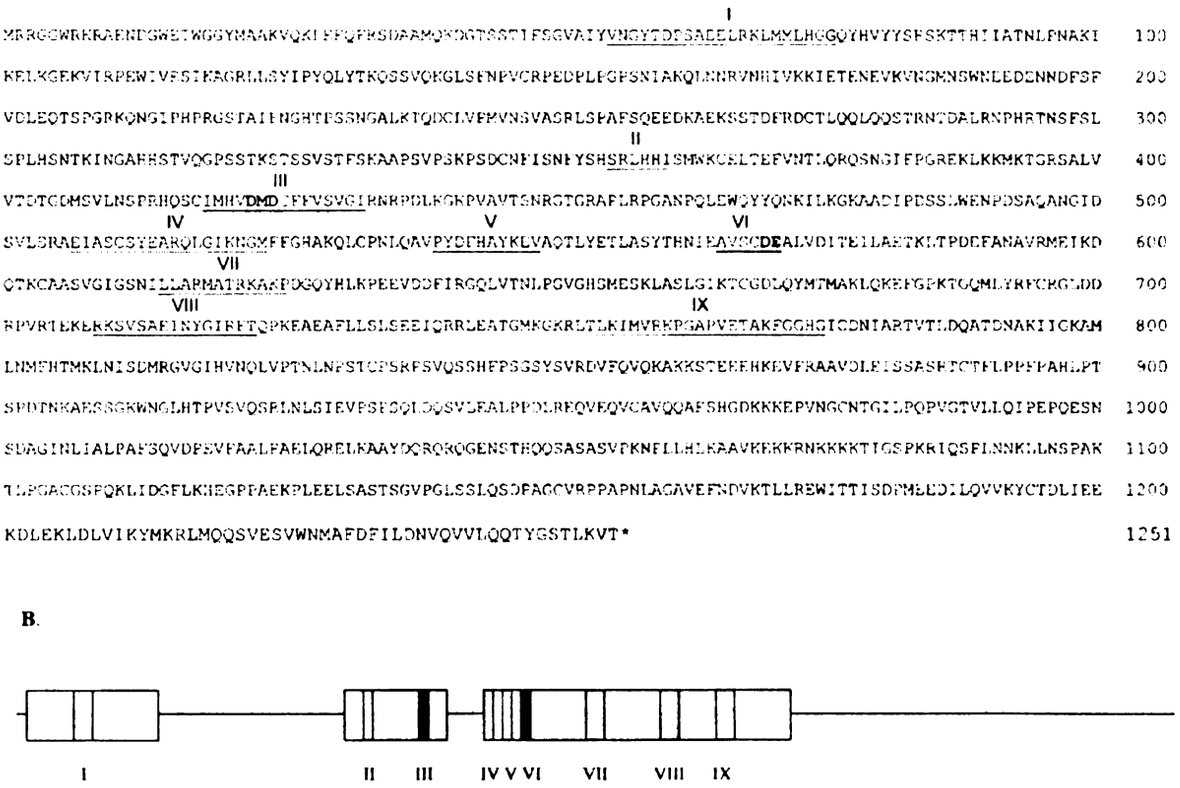

Figure 1. (*A*) Sequence of the translation product of *hREV1* mRNA. The underlined sequences numbered I-IX are possible sequence motifs, and the highlighted residues in motifs I, III, and VI are known to be important for function. (*B*) Depiction of regions of similarity to *C. elegans* and *D. melanogaster* sequences (open boxes) together with the location of the predicted motifs. The darker filled boxes indicate the location of motifs III and VI.

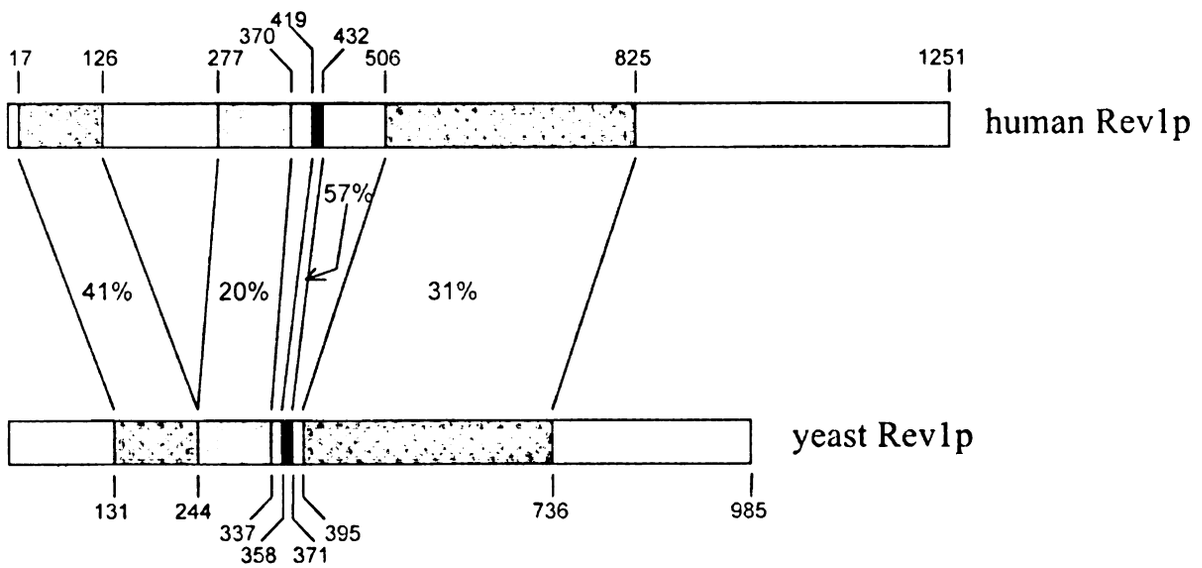

Figure 2. Schematic representation of the alignment of *hREV1* and yeast *REV1* protein sequences. Regions with significant identity are shaded, and the percent identity is indicated.

yeast and the site of the G193R mutation found in the yeast *rev1-1* mutant (25); the Rev1-1 protein retains a significant fraction of its deoxycytidyl transferase activity, but has lost almost all of its general bypass function (J. R. Nelson, P.E.M.G., A. M. Nowicka, D. C. Hinkle and C.W.L., unpublished observations). Conversely, the highly conserved DXD and DE sequences found in motifs III and VI, respectively, appear to be concerned with catalysis (5, 26), and a Pol η D156A, E157A tandem double mutation lacks polymerase activity (5).

The sequence 5' to the ATG codon at the start of the main ORF contains an out-of-frame ATG sequence at nucleotide -35, initiating a small reading frame that terminates at a TGA stop codon overlapping the main ORF ATG. Further, the sequence context of the out-of frame ATG is almost as good as that of the ORF ATG. This feature suggests that the *hREV1* message is translated very inefficiently, and predicts that hRev1 protein levels are likely to be low (27). A number of the clones analyzed encoded a protein of 1,250, rather than 1,251, residues, in which the sequence CAGCAG at nucleotides 1041-1046 was replaced by CAG. A similar pair of sequences was also found in the homologous region of the mouse *REV1* sequence (data not shown); in both cases, they may result from slippage at the 3' splice site of an intron. The protein sequence given in Fig. 1*A* is identical with that of ref. 16, but differs by one synonymous mutation (Ile1150, ATT vs. ATC), that may represent a polymorphism. Finally, a sequence yielding an exact match to the 3'-terminal 60% of the ORF of *hREV1* is described in GenBank as encoding a protein that interacts with α3A integrin (28); the significance of this observation is unclear.

Although determining the sequence of the ORF presented few problems, establishing the sequence of the 5' untranslated region of the mRNA was less straightforward, and it is unlikely that it is complete. Because the 5' sequence is GC-rich, attempts to extend primers into this region resulted in multiple premature termination events in clones with intron sequences, as identified both by the presence of canonical 3' splice junction sequences and by the loss of the ORF. Other attempts recovered clones showing rearrangements of the cDNA sequence that presumably occurred during either synthesis or amplification. To confirm that the GC-rich sequence indeed constituted the 5' untranslated sequence, sense-strand primers were designed specific to each of the possible upstream sequences, together with a common antisense primer. Only the GC-rich specific primer yielded an appropriate amplification product (data not shown). Additional evidence supporting this conclusion is provided by the sequences of three mouse ESTs (accession nos. AA4202230, AI019222 and AI481088), which show strong similarity to the region of *hREV1* flanking the putative initiation codon. In contrast, sequence identified by an alternative upstream primer was clearly an artifact, because it matched sequence of an mRNA from human cortex (HUMMRNAC, accession no. L10374).

UV-induced mutagenesis is much reduced in human cells expressing *hREV1* antisense RNA

In yeast, the *REV1* gene is required for ≈95% of the base pair substitutions induced by UV (19), and *rev1* mutants are more readily killed by this radiation, although this hypersensitivity is relatively modest (30). Unlike *REV3*

however, the *REV1* gene appears to be required to a much smaller extent for the production of frame-shift mutations induced by UV. The extent varies among the different genetic sites investigated, however, and at three of them >80% of the UV-induced frameshifts depended on *REV1* function (29, 31). Results with other mutagens, although less extensive, appear to be similar to those with UV, both with respect to base pair substitutions and frameshifts (32). To examine whether human *REV1* is also required for UV mutagenesis, we have used the antisense method used previously with *hREV3* (8), in which high levels of an antisense RNA are expressed under the control of the TetP promoter. To this end, a series of independent puromycin-resistant colonies obtained by transfection of parental 7AGM cells with *hREV1* antisense-expressing plasmid, pR1P27-AS, were isolated and expanded to $\approx 10 \times 10^6$ cells. A portion was used for Northern blot analysis for antisense expression, and the rest were cryopreserved. Two unequivocally independent clones, designated 7AGM-12B-R1 and 7AGM-17C-R1, found to express antisense RNA at a level much higher than the level of natural transcript from the endogenous *hREV1* gene (Fig. 3), were chosen for study. The level of expression of antisense *REV1* RNA in these two strains, corrected for slight differences in RNA loading per lane, was very similar (Fig. 3).

The frequency of 254-nm UV-induced mutants and the sensitivity to UV cell killing in these two cell strains was compared with that in their nontransfected parental cell strain, 7AGM. In all but one experiment, the parental cell strain and one or other or both of the derivative strains 7AGM-12B-R1 and 7AGM-17C-R1 were compared simultaneously. As shown in Fig. 4*A*, these two cell strains

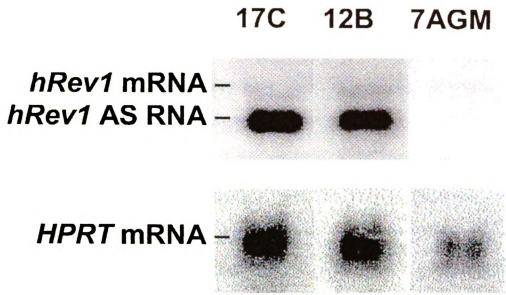

Figure 3. Northern blot analysis of the level of expression of *hREV1* antisense RNA in the cell strains. RNA extracted from the two derivative cell strains, 7AGM-17C and 7AGM-12B, which had been transfected with plasmid pR1P27-AS containing the 4,117-bp sequence of the *hREV1* RNA in an antisense orientation, and from their nontransfected parental cell strain, MSU-1.2-7AGM, and analyzed for expression of *hREV1* antisense RNA (*hREV1* AS RNA) and/or the endogenous *hREV1* sense RNA. The latter mRNA, which is ≈4.4 kbp in length, can be seen as a faint band just above the antisense band in the first two lanes, and in the third lane. The lower band, approximately 1.4 kbp in length, is the endogenous *HPRT* mRNA, which was used to normalize the amount of RNA loaded per lane. There was no significant difference between the two derivative cell strains in the level of expression of the antisense RNA.

expressing *hREV1* antisense RNA were not more sensitive than their parental strain to the cytotoxic effect of UV. The line with the slightly steeper slope is a least squares fit to the data from the parent strain 7AGM and also from strain 7AGM-17C-R1; the other line represents the least squares fit to data from strain 7AGM-12B-R1. Although the survival curves did not show a significant difference between these two strains and their parental strain, the frequency of 6-thioguanine-resistant mutants induced in the two strains expressing *hREV1* antisense RNA was significantly lower ($P = 0.01$) than that seen with their parental cell strain (Fig. 4*B*). The 7AGM-17C-R1 cells showed a ≈64% decrease; the 7AGM-12B-R1 cells showed a ≈94% decrease in frequency.

The results shown in Fig. 4*B* for the latter strain were obtained from two independent experiments, using 0, 11, 13, and 15 J/m^2 of UV in one experiment, and 0, 12, and 14 J/m^2 of UV in the other. No mutants were seen in the unirradiated control population. The slope of the line for this strain in Fig. 4*B* was fitted to the five data points from these two experiments. Although the slope is virtually parallel with the *x* axis, the line cannot, in reality, intersect the *y* axis above zero because the data represent the increase in frequency of mutants above the background frequency in the population. Cell strain 7AGM-17C-R1 was tested in three experiments. In each, the UV fluences used were 0, 11, 13, and 15 J/m^2. The values shown for each UV fluence represent the average of the three determinations. Nevertheless, the slope of the line shown in Fig. 4*B* for this strain was fitted to the nine individual data points. The background frequencies of mutants per 10^6 cells in the unirradiated population for these three experiments,

i.e., 0, 0, and 5, were almost as low as those observed with cell strain 7AGM-12B-R1. The values shown in Fig. 4*B* for parental cell strain 7AGM were taken from four experiments. Except for a single determination at 10 J/m^2, the data are the average from two or three independent determinations in which the same UV fluence was used. Again, the values shown for each fluence, except 10 J/m^2 UV, represent the average of the multiple determinations. Nevertheless, the slope of the line was fitted to the ten individual determinations obtained. The background frequency of mutants per 10^6 cells in the experiments with this strain were 4, 10, 13, and 17. To allow a comparison of the surviving fractions, the number of mutants seen, the total cells assayed for mutants, and their corresponding cloning efficiency at the time of selection for 6-thioguanine resistance, an example of the data obtained from an experiment for each strain is shown in Table 1.

The data in Fig. 4*B* indicate that, in cells expressing of *hREV1* antisense, the frequency of UV-induced mutants was significantly reduced. Unlike what is found with yeast, however, there was no evidence for increased sensitivity to the cytotoxic effect of UV radiation in either of the two cell strains expressing the *hREV1* antisense, and in which, presumably, the level of Rev1 protein is reduced by virtue of the antisense RNA. Such a lack of hypersensitivity may well result from the redirection of DNA damage into another pathway, one in which mutations are less likely to occur, or it may reflect a decreased importance of translesion replication for survival in these human cells, compared with yeast cells.

Figure 4. Percent survival (*A*) and frequencies of UV-induced 6-thioguanine resistant mutants (*B*) in the parent cell strain 7AGM and the *hREV1* antisense RNA-expressing cell strains 7AGM-17C-R1 and 7AGM-12B-R1, plotted against fluence of 254 nm UV. The data are the average of results from four experiments with the parent strain, 7AGM; three experiments with 7AGM-17C-R1 cells; and two experiments with 7AGM-12B-R1cells. The background frequencies of mutants, which have been subtracted to obtain the frequencies induced above the background frequency by UV (23), are cited in the text. The mutant frequencies observed for untreated control and each UV fluence were corrected for the cloning efficiency of the cells determined after an 8-day expression period, i.e., at the time they were plated into selection medium containing 6-thioguanine. The average cloning efficiencies for control and UV-irradiated cells were: 7AGM cells, 33.3% ± 1.4% (SEM for four independent experiments); 7AGM-17C-R1 cells, 26.7 ± 1.3% (SEM for three independent experiments); and 7AGM-12B-R1 cells, 31.8% ± 0.9% (SEM for two independent experiments).

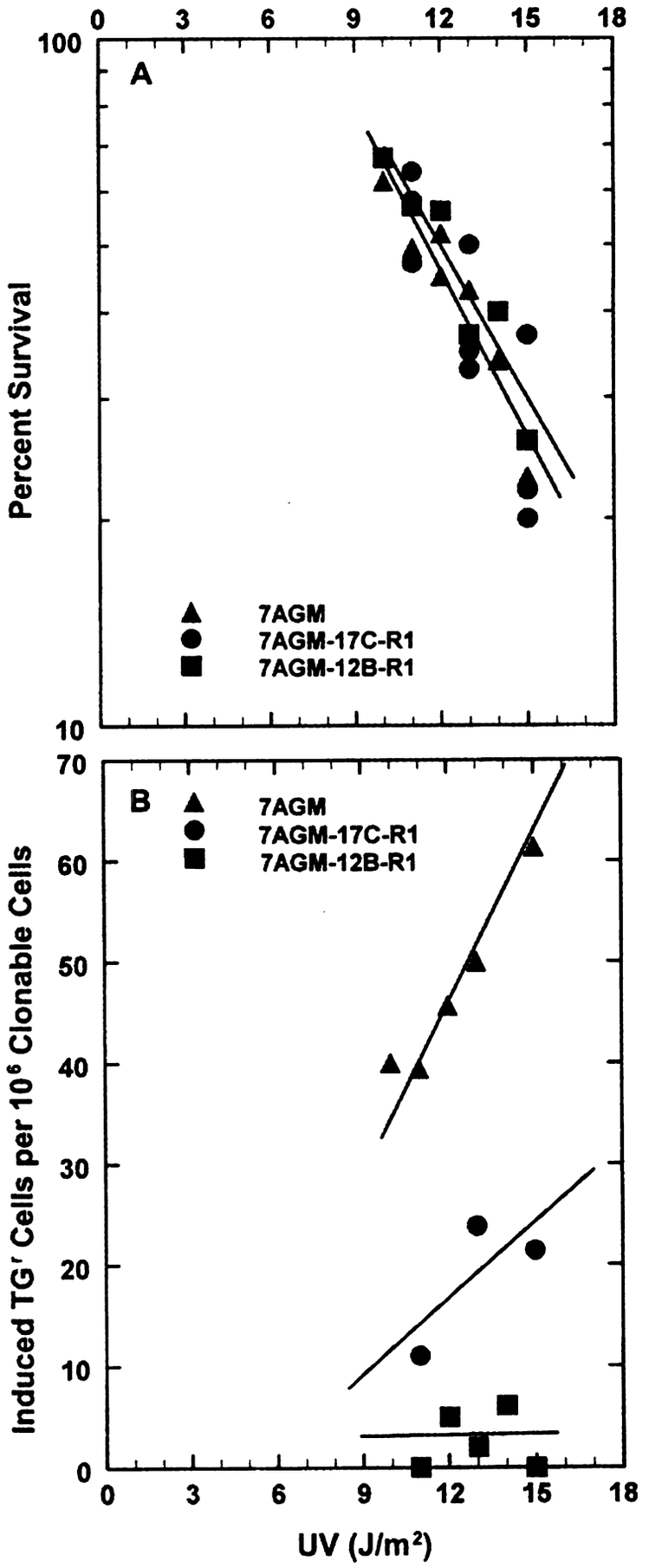
A
Percent Survival
100
10
0
3
6
9
12
15
18
7AGM
7AGM-17C-R1
7AGM-12B-R1
B
Induced TG^r Cells per 10^6 Clonable Cells
70
60
50
40
30
20
10
0
UV (J/m^2)

Table 1. Example of UV cytotoxicity and mutagenicity in parental cell strain MSU-1.2-7AGM and two derivative cell strains expressing *hREV1* antisense RNA

Cell strain	UV (J/m^2)	Percent survival	Cells selected ($\times 10^6$)	Mutants observed	Cloning efficiency (%)	Mutant frequency ($\times 10^6$)	Induced frequency ($\times 10^6$)
7AGM	0	100	1	4	40	10	0
	10	62	1	18	36	50	40
	12	52	1	22	40	55	45
7AGM-17C-R1	0	100	1.8	2	24	5	0
	11	47	1.8	6	24	14	9
	13	33	1.8	12	21	32	27
	15	20	1.8	7	21	19	14
7AGM-12B-R1	0	100	1	0	33	0	0
	11	57	1	0	32	0	0
	13	35	1.5	1	32	2	2
	15	26	1.5	0	30	0	0

Acknowledgements

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CHAPTER 4

Expression of Exogenous hRev1 in Human Cells Devoid of Mutations as a Result of Antisense *hREV1* Restores their Ability to Be Mutated by UV or Benzo[a]pyrene Diol Epoxide

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Runnng title: ROLE of hRev1 IN UV- AND BPDE- INDUCED MUTAGENESIS

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Abstract

Expression of antisense RNA against *hREV1* gene in human fibroblasts in culture reduced the frequency of 6-thioguanine-resistant mutants induced by ultraviolet (UV)($_{254\ nm}$) radiation up to 20-fold, i.e., to 5% of that induced in the parental cell strain (Gibbs et al., Proceedings of the Natl. Acad. Sci. USA, 97:4186, 2000). To determine whether the decrease in mutagenicity in the antisense RNA-expressing cell strains resulted from decreased levels of hRev1 protein, we prepared a series of rabbit polyclonal antibodies against hRev1-specific peptides. Western blotting with these antibodies indicated that the level of expression of hRev1 in the parental cell strain used in the above mutation studies, i.e., cells not transfected with antisense, was very low, too low to quantify a decrease in protein expression caused by *hREV1* antisense. As an alternative approach to see if the level of hRev1 protein determines the frequency of UV-induced mutants, we transfected an *hREV1* gene coding for a Flag-tagged protein into the antisense-expressing cell strain that exhibited a UV-induced mutant frequency only ~ 5% of its normal control parental cell strain. Two transfectant strains expressing the exogenous Flag-hRev1 protein at a detectable level were assayed for the frequency of UV-induced mutants. For each dose of UV in both recipients, the frequency of mutants increased ~ 8-fold over that of the recipient cell strain, i.e., from 5% of that induced in the normal parent to 40%. This series of cell strains was also used for a similar investigation of the role of hRev1 in mutagenesis caused by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE). The frequency of mutants

induced by BPDE in the recipient cell strain expressing antisense RNA was ~ 4% of that induced in the normal parental strain. The frequency of mutants induced by BPDE in one of the two transfectant cell strains expressing Flag-tagged hRev1 protein increased ~ 7.3-fold, i.e., to 29% of that found in the normal parental strain; in the other strain, it increased ~3.3-fold, to 13% of the parent. Sequencing of the *HPRT* gene of independent mutants induced by BPDE in these cell strains showed that the majority were base substitutions, and that the kinds of base substitutions did not differ from those induced by BPDE in the parental control cell strains or those found previously in this laboratory to be induced by BPDE in foreskin-derived finite life span human fibroblasts.

Introduction

In *Saccharomyces cerevisiae* yeast exposed to a variety of DNA damaging agents, the products of the *REV3*, *REV7* and *REV1* genes contribute only minimally to cell survival, but are absolutely required for damage-induced mutagenesis (Lawrence and Hinkle, 1996). The Rev3 and Rev7 proteins constitute DNA polymerase zeta, with Rev3 being the catalytic subunit, and Rev7 the non-catalytic regulatory subunit (Nelson et al., 1996a). Pol zeta is capable of replicating past UV-induced thymine-thymine cyclobutane dimers, the major photoproduct of UV radiation (Nelson et al., 1996a). In cell-free assays, Rev1 protein has been shown to exhibit a DNA template-dependent deoxycytidyl transferase activity, by which it transfers a dCMP opposite an undamaged G template, but it does not insert other deoxynucleotides opposite their respective template deoxynucleotides. *S. cerevisiae* Rev1 can also efficiently incorporate a

dCMP opposite an abasic site in vitro, and this can subsequently be extended efficiently by DNA polymerase zeta (Nelson et al., 1996b). Lawrence and colleagues (Nelson et al., 2000) also showed that in *S. cerevisiae, REV1* is required for replicating past (6-4) T-T UV photoproducts, a process that usually does not require dCMP insertions, suggesting that Rev1 has a second function in participating in translesion synthesis.

The potential relevance of the error-prone translesion synthesis mechanism involving *REV3*, *REV7* and *REV1* to human carcinogenesis stimulated a successful search for human homologs of these yeast genes (Xiao et al., 1998a; Gibbs et al., 1998, 2000; Lin et al., 1999b; Murakumo et al., 2000). Studies designed to eliminate, or greatly decrease, the level of hRev3 and hRev1 protein in human cells by use of antisense showed that the frequency of 6-thioguanine resistant mutants induced by UV in human fibroblasts expressing high levels of *hREV3* or *hREV1* antisense RNA was reduced very significantly (Gibbs et al., 1998; 2000). It has not been possible to demonstrate directly the relationship between reduced UV-mutagenicity and reduced hRev3 or hRev1 proteins in cell strains expressing antisense RNA using Western blotting. As an alternative approach, we transfected a cell strain that expressed *hREV1* antisense RNA at a high level and exhibited 95% reduction in the frequency of UV-induced mutants (Gibbs et al., 2000) with an exogenous *hREV1* gene coding for hRev1 protein Flag-tagged at the amino-terminus (Flag-hRev1). Transfectants expressing the transgene were identified by Western blotting using an antibody against the tag. Two of these latter cell strains, along with several control strains

transfected with an empty vector, as well as the original parental human fibroblast strain and its *hREV1* antisense-expressing derivative strain, i.e., the one used as the recipient for the Flag-tagged hRev1, were compared for the frequency of mutants induced by UV radiation and also BPDE, a reactive metabolite of benzopyrene. The results show that expression of hRev1 protein in the transfectant strains significantly increased the frequency of UV or BPDE-induced mutants. DNA sequencing of the cDNA of the *HPRT* gene in the BPDE-induced mutants revealed that the kinds of mutations induced were virtually identical to those found previously in this laboratory (Yang et al., 1989).

Materials and methods

Cell strains and cell culture

The cells strains were derived from MSU-1.2, a derivative of MSU-1.1 cell strain, an infinite life span, near-diploid, karyotypically-stable cell strain established in this laboratory from the foreskin of a normal neonate (Morgan et al., 1991). Unless otherwise noted, the cell strains were cultured in Eagle's minimal medium (Gibco, Gaithersburg, MD), pH 7.2, supplemented with 0.2 mM L-aspartic acid, 0.2 mM L-serine, 1.0 mM sodium pyruvate, and 10% (v/v) supplemented calf serum (HyClone, Logan UT). The culture medium also contained penicillin (100 U/ml), streptomycin (100 μg/ml), and hydrocortisone (1 μg/ml). Cells were cultured in 5% CO_2 humidified incubators at 37^0C.

Plasmids and DNA transfection

The plasmid used for expression of the Flag-hRev1 protein, pcDNA3.1(+)/FLAG-hREV1 was described previously (Murakumo et al., 2000). The recipient cells were plated at 10^5 per 60 mm-diameter culture dish and transfected ~18 h later. Because pcDNA3.1(+)/FLAG-hREV1 contains the gene for neomycin resistance as its selectable marker, and the recipient MSU-1.2 cells already express this marker (Morgan et al., 1991), a co-transfection with a plasmid pcDNA6/V5-HisA which carries the gene for resistance to blasticidin was performed, using 5 μg pcDNA3.1(+)/FLAG-hREV1 and 0.5 μg pcDNA6/V5-HisA per 60 mm-diameter culture dish. The plasmid DNA was introduced in lipofectamine following the manufacturer's instructions (Gibco, Bethesda). After 48 h, the medium was changed to medium containing blasticidin (5 μg/ml) to select for transfectants. The cells were given fresh culture medium containing blasticidin (5 μg/ml) every 5 days.

Analysis for the expression of hRev1 protein

Cells growing exponentially were washed with cold phosphate-buffered saline (PBS) and then lysed on ice with lysis buffer composed of 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM sodium vanadate, 1 mM DTT, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 μg/ml aprotinin and 25 μg/ml leupeptin. The cell lysates were collected with a rubber scraper and centrifuged at 14,000 rpm on a microcentrifuge (Eppendorf, Westbury, NY) at 4^0C. The supernatant was collected, an aliquot was taken for protein quantification using the Coomassie protein assay reagent (Pierce, Rockford, IL), and the rest was stored at -80^0C. Unless otherwise noted, lysates

(100 μg of protein) were loaded on a 6% SDS-PAGE gel, and the gel was run at constant 35 mA. The proteins were electrotransfered onto an Immobilon-P membrane (Millipore, Bedford, MA) for ~18 h at constant 35 mA. The membranes were blocked at room temperature for 2-3 h in 5% (w/v) non-fat dry milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.2, 137 mM NaCl) containing 0.1% (v/v) Tween-20 (TBST), and incubated with the mouse monoclonal anti-Flag antibody (Clone M1, Sigma, St. Louis, MO) at the recommended concentration of 10 μg/ml or the rabbit anti-hRev1 polyclonal antibody (antibody 468) at 1:600 dilution for 2 h at room temperature. The membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Sigma, St. Louis, MO) that had been diluted 1:20,000 in TBST solution supplemented with 5% milk. Enhanced chemiluminescence substrate (SuperSignal Pico, Pierce, Rockford, IL) was used for blots using anti-Flag antibody, and Maximum Sensitivity Substrate (Supersignal Femto, Pierce, Rockford, IL) was used for blots using anti-hRev1 antibody. The amount of Ku-80 protein, assessed with rabbit polyclonal anti-Ku80 antibody (Serotec, Raleigh, NC) and same chemiluminescent substrates as those for detection of hRev1 protein on the same blot, was used as the loading control. For the Western blotting experiments designed to explore the detection limit of protein using a Femto sensitivity substrate, a Dot-Blot anaysis was first performed to optimize the concentrations of primary and secondary antibodies, and SuperBlock blocking reagent (Pierce, Rockford, IL) and nitrocellulose membrane (Amersham Pharmacia, Piscataway,

NJ) were used as recommended by the manufacturer of the chemiluminescent substrate.

Determination of the cytotoxic and mutagenic effects of UV radiation

The cytotoxicity of UV was determined from the number of colonies formed at each dose, and the mutagenic effect of UV was determined from the frequency of UV-induced *HPRT* mutants, as selected by resistance to 40 μM 6-thioguanine (TG). The procedures used have been described previously (Gibbs et al., 2000; Li et al., 2002). Briefly, to determine the cytotoxic effect of UV, sufficient cells were plated per 100-mm-diameter dish to obtain ~50 colonies per dish. The number plated depended on the expected survival and the cloning efficiency of the strain. To determine the frequency of induced mutants, cells were plated into a series of 150-mm-diameter dishes (P150's) at a density of 0.5×10^6 to 2×10^6 cells per dish, depending on the expected survival at each dose and allowing cells enough room to grow for 4 days. For each dose, sufficient dishes were used to ensure at least 1×10^6 surviving target cells. Approximately 12-16 h after plating, the medium was removed, and the cells were rinsed with PBS and irradiated, with the dishes revolving on a turntable, at an incidence dose of ~0.3 $J/m^2/sec$. Cells were given fresh medium immediately after irradiation and refed again after 24 h. For determining cytotoxicity, cells were allowed 14 days to form colonies, with one additional feeding. To determine the frequency of mutants, cells were allowed 8-10 days of an expression period to allow depletion of preexisting Hprt protein, with one harvesting, pooling of cells of the same dose, and plating of $1\text{-}2 \times 10^6$ cells at the 4^{th}-5^{th} day post irradiation. A total of $1 \times$

10^6 to 2× 10^6 cells from each population were selected for resistance to 40 μM 6-TG at a density of ~500 cells/cm^2. To assay their colony-forming ability at the time of selection, cells from each population were plated in nonselective medium at cloning density. This value was used to correct the observed frequency of TG-resistant (TG^r) mutants. Parental MSU-1.2 cells have a cloning efficiency of ~50%. To increase the cloning efficiency of any derivative strain that cloned at a lower frequency, and to increase the number of cells per clone, feeder cells composed of TG^r clonal populations derived from the same cell strain were γ-irradiated with 30Gy to prevent them from replicating and were plated at a density of 4× 10^3 cells/cm^2 at the end of the expression period, i.e., when the target cells were selected for frequency of mutants and cloning efficiency. The cells used for cloning efficiency were stained with crystal violet and the colonies were counted. After ~14 days of selection, with one feeding of the cells after 7 days, the dishes containing cells assayed for mutant frequency were searched for colonies using a focused beam of light from below. The TG^r colonies were isolated and stored at -80^{0}C for later sequencing of their *HPRT* gene. The dishes were then stained and any remaining colonies observed were added to the total number.

Determination of the cytotoxic and mutagenic effects of BPDE

The procedures for determining the cytotoxic and mutageneic effects BPDE have been described previously (Yang et al., 1991). Briefly, cells in exponential growth were plated into a series of p100's at a density of 5.5× 10^5cells/dish. When cells had attached and flattened out, at ~12-16h post plating,

the medium was removed, the cells were rinsed with PBS, and fresh serum-free medium was added. The BPDE, dissolved in anhydrous dimethylsulfoxide, was delivered into each culture dish with micropipette and mixed immediately. After 1h, the medium was removed and replaced with fresh culture medium. The cells in two dishes for each dose, and for the mock-treated control, were immediately harvested, pooled and plated at cloning density to determine the cytotoxicity of BPDE. The rest of cells, were allowed an expression period of 8-10 days before being selected with 40 μM 6-TG. The procedure for expression and selection was the same as that used for determining the mutagenic effects of UV.

Sequencing of mutant *HPRT* cDNA

Cell pellets of TG^r colonies (~500-2,000 cells/pellet) derived from the mutagenesis assays were lysed and the *HPRT* cDNA was amplified using procedures and reagents essentially described by Yang et al. (1989). The amplified *HPRT* cDNA was purified with QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced using Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Pharmacia, Piscataway, NJ) with primer C (AAACTCAACTTGAACTCTCA) labeled with Cy5.5 and primer 7 (GACATTCTTTCCAGTTAAAGT) labeled with Cy5.0. The sequencing was analyzed using Microgene Clipper System (Visible Genetics, Toronto, Ontario, Canada).

Results

Efforts to determine the cellular level of hRev1 protein

In the previous study to determine if *hREV1* plays a role in UV-induced mutagenesis (Gibbs et al., 2000), we showed that two human fibroblast strains expressing antisense *hREV1* RNA at high levels, i.e., 7AGM-17C-R1 and 7AGM-12B-R1, exhibited a significant reduction in the frequency of UV-induced mutants, compared to that of their parental strain 7AGM. For each dose, the mutant frequencies observed were 40% and 5% that of the parental cell strain, respectively. Subsequently, a third cell strain expressing a high level of antisense RNA was tested. It gave a frequency ~18% that of the parent (data not shown). Additional studies showed that the low mutant frequency in these three strains was not the result of such artifacts as loss of *HPRT* mutant cells by metabolic cooperation from neighboring *HPRT* wild-type cells; a slower rate of growth of the cells, allowing longer time for excision repair prior to DNA replication past the photoproducts; or an increase in the number of X chromosomes, on which the *HPRT* gene is located (data not shown). Taken together, these data strongly suggest that the function of *hREV1* is required for mutagenesis induced by UV light in human cells.

The more direct way to test the hypothesis that the reduction of UV mutability of these cells strains resulted from impaired function of *hREV1* was to determine that antisense reduced or eliminated expression of the target protein, as was seen for hMms2 in studies from this laboratory (Li et al., 2002). We independently prepared rabbit polyclonal antisera against three discrete hRev1

peptides of 18, 19 and 21 amino acid residues, each of which was predicted to have high antigenicity. To facilitate the identification of hRev1 protein on Western blots, we prepared a positive control hRev1 protein by overexpressing a Flag-tagged hRev1 protein in kidney 293 cells, using a transient expression protocol. The overexpressed Flag-hRev1 protein could be easily detected by Western blotting using anti-Flag antibody (data not shown). Using this positive control lysate, we tested the potential of the rabbit anti-hRev1 peptide antisera for use in Western blotting. Our pilot studies indicated that two of the polyclonal antisera failed to recognize even the hRev1 protein overexpressed in 293 cells, and the third, designated 468, recognized the overexpressed Flag-hRev1 protein, as shown in Figure 1 (Lane 1). However, this best antibody, i.e., antibody 468, could not detect an endogenous hRev1 band at that position in the control 293 cells or in the parental cell strain 7AGM used in our study. We determined that the 293 cells transiently overexpressing Flag-hRev1 protein had at least 20-fold more hRev1 protein than the control non-transfected 293 cells or 7AGM parental cell strain. Western blot analyses using carefully optimized blotting conditions, including optimum primary antibody concentration, secondary antibody concentrations, blocking reagent, and maximum sensitivity fluorescent substrate, revealed that the lowest amount of protein that could be detected was about 6.3 μg (Lane 7), 16-fold less the amount of protein displayed in Lane 1, and linear range fell between 50 μg and 12.5 μg of protein (Lane 4, 5 and 6). Under the

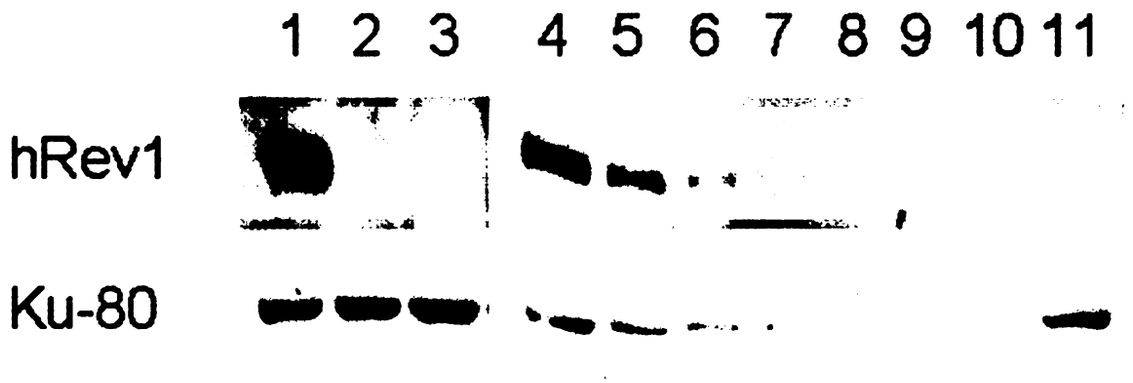


Figure 1. Evidence that endogenous hRev1 protein in the parental strain 7AGM was below the detection limit of Western blotting. Lane 1, positive control human kidney 293 cells, transiently expressing Flag-hRev1; lane 2, control kidney 293 cells; lanes 3 and 11, original parental cell strain 7AGM. 100 μg of protein was loaded in these lanes. Lanes 4 through 10, kidney 293 cells transiently expressing Flag-hRev1 protein, but with only 50, 25, 12.5, 6.3, 3.1, 1.5, 0.8 μg of protein loaded, respectively. The hRev1 protein was probed with rabbit polyclonal antibody 468. Ku-80 protein was used as the loading control. These blots were developed using maximum sensitivity chemiluminescence substrate.

same conditions, 100 μg of protein prepared from the parental 7AGM cells yielded no obvious band (Lane 11). Tests showed that use of lysates prepared from nuclei rather than from whole cells did not produce any advantage (data not shown).

Cell strains expressing exogenous Flag-hRev1 protein

To circumvent the difficulty of not being able to detect endogenous hRev1 protein on Western blots as a means of determining that the decreased frequency of mutants induced by UV resulted from a decrease in expression of hRev1 as a result of antisense RNA-expression, we employed an alternative strategy. We introduced an exogenous *hREV1* gene into the 7AGM-12B-R1 antisense RNA-expressing cell strain that showed the greatest reduction in UV mutagenesis, i.e., 20-fold (Gibbs et al., 2000). If the reduction in mutagenesis in this antisense RNA-expressing cell strain was caused by lower level of hRev1 protein, expression of exogenous hRev1 protein should increase the UV-mutability of this cell strain. To facilitate the detection of exogenous protein expressed in the transfectant clones, we transfected this recipient strain with a plasmid expressing a hRev1 protein with a Flag-tag fused in-frame at the amino-terminus. The expression of Flag-hRev1 could be detected by Western blot analysis using an anti-Flag mouse monoclonal antibody. Following transfection, ~90 blasticidin-resistant transfectant clones were expanded and screened for expression of Flag-hRev1 protein. A total of 14 clones showed expression of the exogenous Flag-hRev1 protein. As shown in Figure 2, two cell strains, designated 7AGM-12B-R1-FR1C1 and 7AGM-12B-R1-FR1C2, expressed Flag-

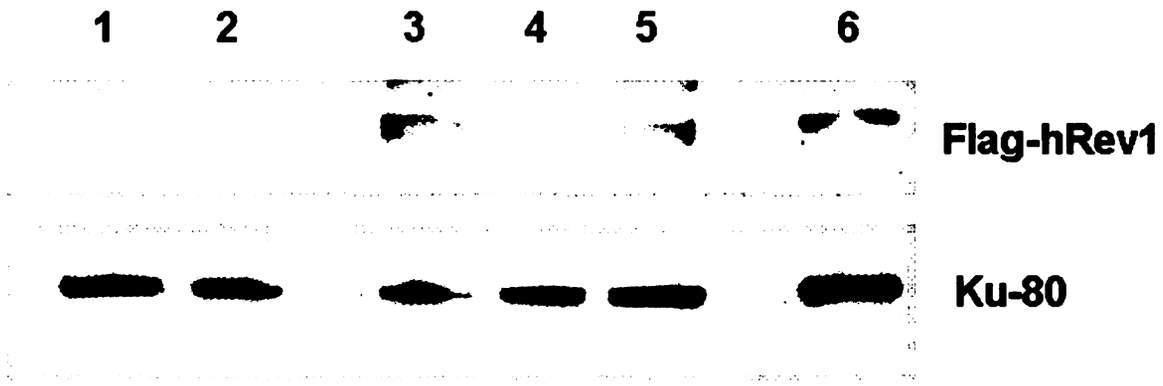


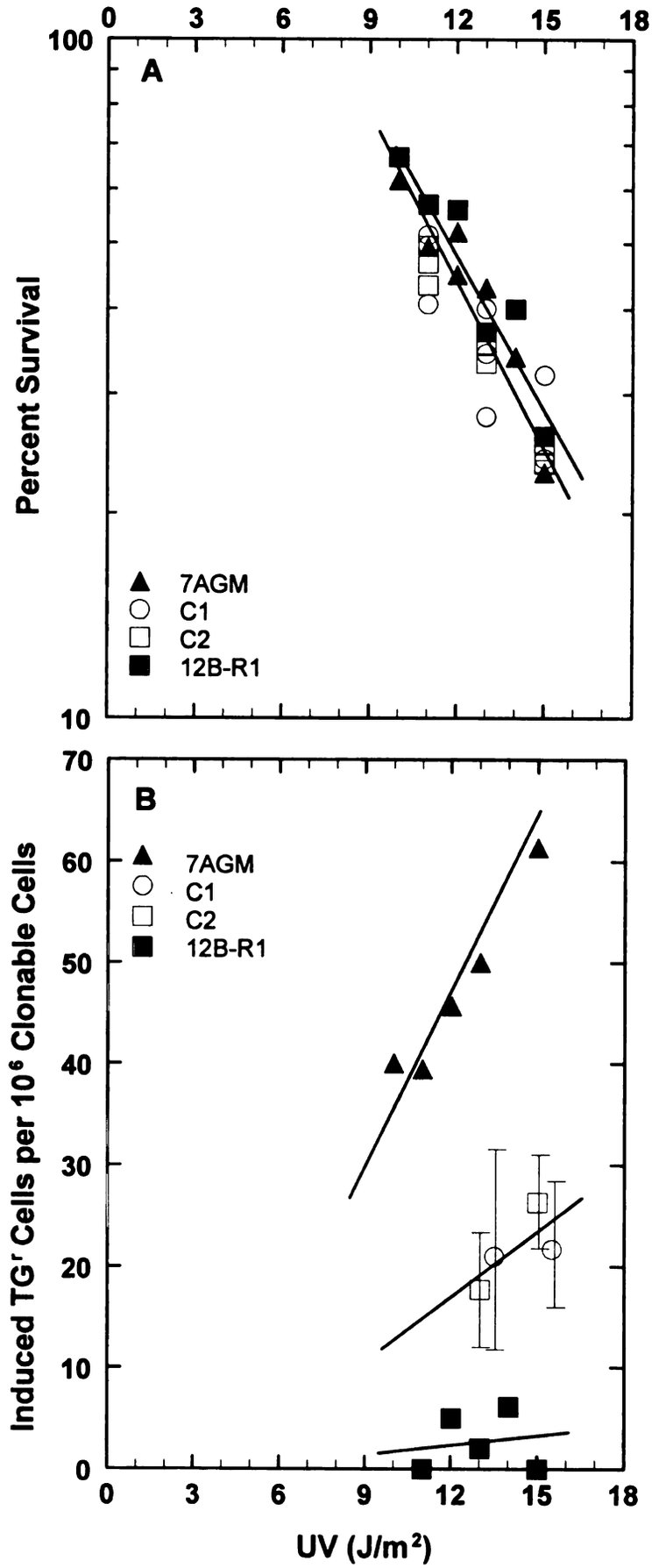
Figure 2. Western blot analysis of transfected cell strains for the expression of exogenous Flag-hRev1 protein. Lanes 1 and 2, cell strains transfected with the vector alone, as a control; lanes 3 and 5, transfectant strains, C1 and C2, expressing Flag-hRev1 protein; lane 4, a transfectant that failed to express Flag-hRev1 protein; lane 6, the positive control, i.e., 293 cells transiently transfected with FLAG-hREV1 plasmid. The Flag-hRev1 protein was detected using mouse monoclonal anti-Flag antibody. Ku-80 was used as a loading control.

hRev1 at a higher level than other transfectants. These were chosen for further studies.

Effects of Flag-hRev1 expression on UV-induced mutagenesis

These two transfectant cell strains expressing exogenous Flag-hRev1 protein, hereafter designated C1 and C2 for brevity, were used as target cell strains in UV-induced mutagenesis assays to determine whether UV-mutability of the antisense-expressing recipient cell strain could be restored as a result of the exogenous hRev1 expression. That latter strain, 7AGM-12B-R1, hereafter referred to as 12B-R1 for brevity, which expressed high level of antisense *hREV1* RNA, had shown an approximately 95% reduction in frequency of UV-induced mutants compared to the normal parental strain (Gibbs et al., 2000), a level of reduction comparable to that seen in a yeast *rev1* mutant strain (Lawrence and Hinkle, 1996). Three independent experiments were carried out to determine the cytotoxic and mutagenic effects of UV (254nm) in the two transfectant cell strains. As shown in Figure 3A, the cell strains did not exhibit a significant change in sensitivity to the cytotoxic effect of UV. This result was not unexpected because even the strains expressing high levels of antisense *hREV1* RNA showed a survival comparable to the parental 7AGM cell strain (Gibbs et al., 2000). However, as shown in Figure 3B, the two transfectan strains, C1 and C2, showed a significantly increased frequency of UV-induced TG^r mutants, compared to their recipient strain, 12B-R1. Both of them exhibited approximately 8-fold higher frequency than the parental strain 7AGM-12B-R1, to a level of ~40% of the frequency of the original normal fibroblasts 7AGM. In contrast, cell

Figure 3. Evidence that expression of Flag-hRev1 protein does not greatly affect cell survival, but significantly increases the frequency of UV-induced mutants. A) UV cytotoxicity. B) Frequencies of UV-induced TG^r mutants. The data for the original parental strain 7AGM (▲) and the antisense RNA-expressing strain 12B-R1 (■) from Gibbs et al. (2000) are shown for comparison. (○), cell strain C1; (□), cell strain C2. The data shown by open symbols represent three independent experiments. For the mutagenicity data, the latter are shown as the mean ± standard deviation. The data shown by (○) have been offset slightly from the dose used, i.e., 13 and 15 J/m^2 for clarity. To obtain UV-induced frequencies, the background frequencies of mutants, which ranged from 0 to 9 per 10^6 clonable cells, have been subtracted. The mutant frequencies were corrected for the cloning efficiency of the cells at the time they were plated into selective medium. These ranged from ~30% to 90%. The survival curves for strains C1 and C2 are the same. They are virtually superimposed on the lower curve and have been omitted for clarity.

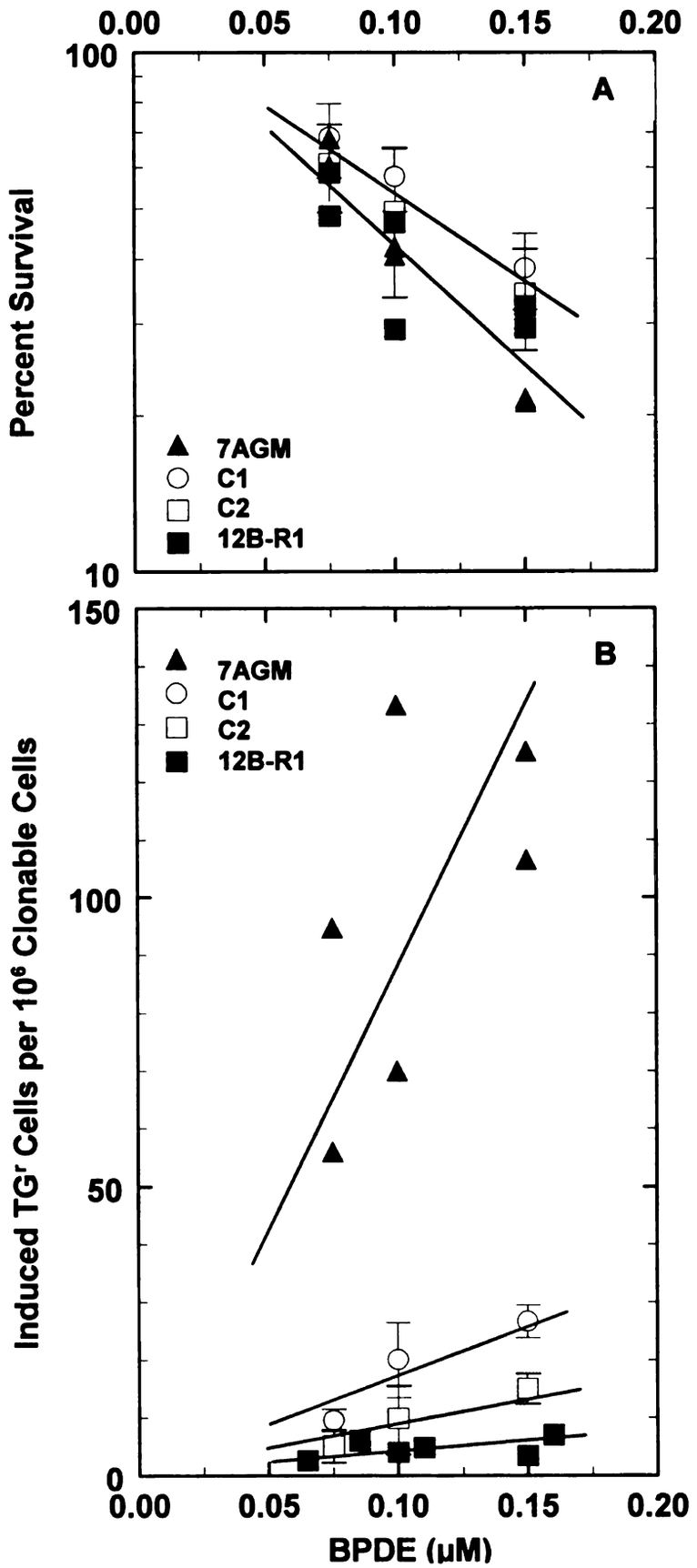

A
Percent Survival
100
10
0
3
6
9
12
15
18
7AGM
C1
C2
12B-R1
B
Induced TG^r Cells per 10^6 Clonable Cells
70
60
50
40
30
20
10
0
UV (J/m^2)

strains transfected with an empty vector, or that were transfected with the *FLAG-hREV1* plasmid, but did not express the exogenous protein, remained virtually immutable (data not shown). These data indicate that the reduced capability of the antisense RNA-expressing strain in UV-induced mutation can be restored by exogenous expression of hRev1, strongly suggesting that the function of *hREV1* is required for UV-induced mutagenesis.

Effects of Flag-hRev1 expression on BPDE-induced mutagenesis

Both the photoproducts induced by UV light and the bulky adducts formed by benzo[a]pyrene, including BPDE, cause DNA helix distortion, and these DNA lesions are repaired by the same repair mechanism, i.e., nucleotide excision repair (Friedberg et al., 1995). To explore the possibility that the mutagenic effect of these two kinds of lesions requires the function of the same pathway, i.e., the pathway involving *hREV1*, we determined the frequency of BPDE-induced mutants in the original parental strain 7AGM, strain 12B-R1 that expresses a high level of antisense RNA, and the two transfectant strains that express exogenous hRev1 protein. As shown in Figure 4A, the cytotoxic effect of BPDE as a function of dose in the parental strain 7AGM and the antisense RNA-expressing strain, 12B-R1, were very similar; that in the two exogenous hRev1 expressing strains, C1 and C2, was slightly less. However, as shown in Figure 4B, in contrast to the high frequency of mutants induced by BPDE in the parental fibroblasts, cell strain 7AGM, values similar to those found previously in this laboratory for finite life span human fibroblasts (Yang, et al., 1982; Aust et al., 1984), the frequency of induced mutants in 12B-R1 was very low, suggesting that the disruption of

Figure 4. Evidence that expression of Flag-hRev1 protein does not greatly affect cell survival but significantly increases the frequency of BPDE-induced mutants. A) BPDE cytotoxicity. B) Frequencies of BPDE-induced TG^r mutants. (▲), the original parental strain 7AGM; (■), the antisense RNA-expressing strain 12B-R1; (○), C1; (□), C2. The data shown by open symbols represent three independent experiments. For the mutagenicity data, the latter are shown as the mean ± standard deviation. Four data points shown by (■) have been offset slightly from the dose used, i.e., 0.075, 0.10, and o.15 μM for clarity. To obtain BPDE-induced frequencies, the background frequencies of mutants, which ranged from 0 to 4 per 10^6 clonable cells, have been subtracted. The mutant frequencies were corrected for the cloning efficiency of the cells at the time they were plated into selective medium. These ranged from ~30% to 90%. The survival curves for strains C1 and C2, and the survival curves for 7AGM and 12B-R1 are virtually the same. Only two lines are drawn for clarity.


0.00
0.05
0.10
0.15
0.20
100
10
A
Percent Survival
7AGM
C1
C2
12B-R1
150
100
50
0
B
Induced TG^r Cells per 10^6 Clonable Cells
BPDE (μM)

hREV1 function by expression of antisense RNA greatly abolished the mutations induced by BPDE. Conversely, as also shown in Figure 4B, with expression of the exogenous Flag-hRev1 protein, both cell strains C1 and C2 showed a restoration of BPDE-induced mutagenesis, to a level significantly higher than their parental strain 12B-R1, 5-fold and 3-fold respectively. All the data were results from at least two independent mutagenesis assays, with selection of 1×10^6 cells for each dose. Figure 5 showes the relationship between frequency of induced mutants and cytotoxicity of BPDE, using the same set of data as used for Figure 4. The dependence of mutagenesis induced by BPDE on the cells' functional status of *hREV1* strongly supports the indispensable role of *hREV1* in mutagenesis induced by BPDE.

hREV1 is required for the majority of UV- and BPDE-induced base substitutions

The restoration of UV and BPDE mutability of the antisense RNA-expressing strain by exogenous expression of Flag-hRev1 protein strongly indicated that the function of *hREV1* had been markedly impaired in this cell strain, and that the greatly reduced mutation was caused by decreased function of *hREV1*. The fact that cell strain 12B-R1 exhibited an ~ 94% reduction in frequency of UV-induced mutants indicated that the function of *hREV1* is required for the vast majority of mutations induced by UV. In finite life span foreskin-derived human fibroblast cells, the vast majority of mutations induced by UV are base substitutions, with about 50%-60% being C→T transitions (McGregor et al., 1991; Wang et al., 1993). These data, along with the data in

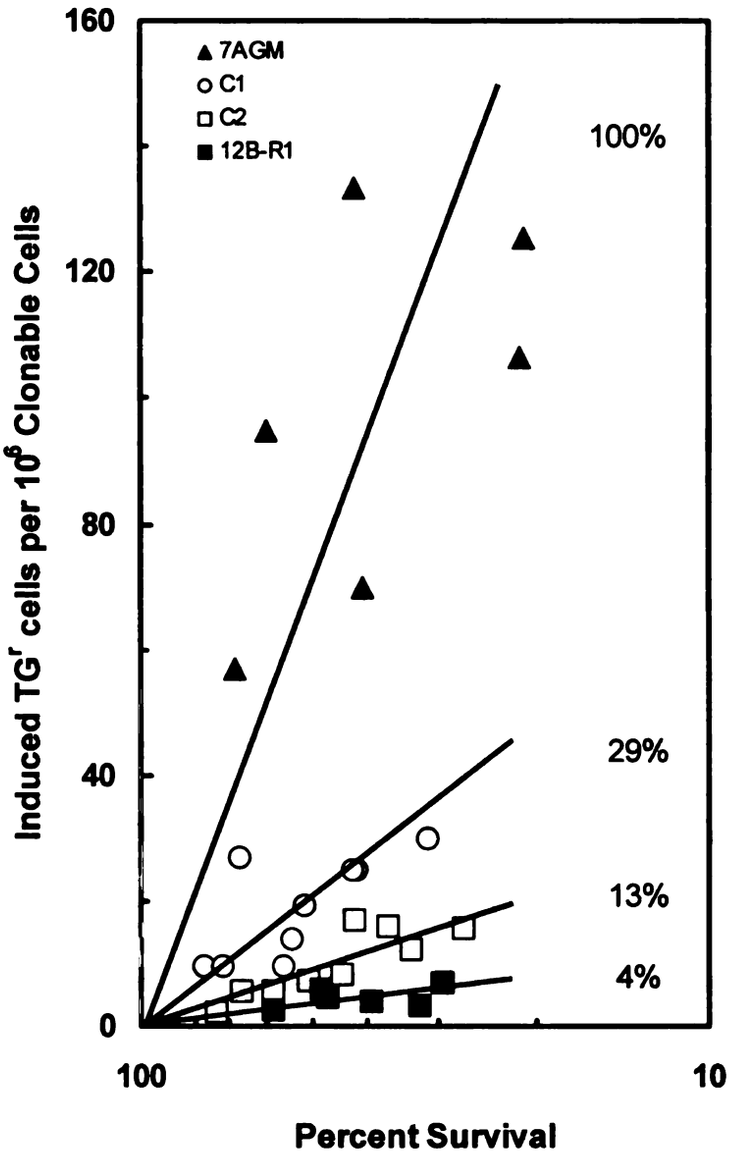


Figure 5. Frequency of mutants induced by BPDE expressed as a function of percent survival of the target cells. This figure shows all the data points used for preparing Figure 4. They demonstrate the degree of increase in mutagenesis observed when exogenous hRev1 protein was expressed in the *hREV1* antisense-expressing recipient cell strain, 12B-R1. (▲), 7AGM; (■), 12B-R1; (○), C1; (□), C2.

Figure 3B, predict that hRev1 is involved in generation of C→T transitions. Similarly, the fact that the cell strain 12B-R1 also showed a ~96% reduction in the frequency of BPDE-induced mutants predicts that *hREV1* is involved in the vast majority of mutations induced by BPDE.

To test this prediction for BPDE, we sequenced the *HPRT* cDNA of BPDE-induced *HPRT* mutant clones derived from the cell strain C1, which expressed exogenous Flag-hRev1 protein. If hRev1 is required for nearly all BPDE-induced mutagenesis, the kinds of mutations generated in cell strains expressing exogenous Flag-hRev1 should resemble those of the original parental strain 7AGM. We first obtained the spectrum of BPDE-induced *HPRT* mutants derived from asynchronously growing cells of the original parental strain 7AGM, an immortal, near-diploid fibroblast strain, and compared with that of a normal human fibroblast strain previously obtained in this laboratory (Yang et al., 1991). In asynchronously growing normal diploid human fibroblasts, the majority of mutations induced by BPDE are base substitutions, with approximately 94% of them involving guanine, primarily G→T transvesions and ~5% involving adenine (Yang et al., 1991). As summarized in Table 1, the mutation spectrum induced by BPDE in 7AGM resembled that of the normal fibroblast strain (Yang et al., 1991). Approximately 80% of these base substitutions are transversions and the most predominant transversion is G→T transversion. As expected, sequencing of the BPDE-induced *HPRT* mutants derived from the Flag-hRev1 expressing strain C1 revealed a very similar spectrum. The similarity of mutation spectrum from C1 and that of its parental strain 7AGM, along with the data shown in Figure 4B and

5, clearly demonstrates that the function of *hREV1* is required for virtually all BPDE-induced mutageneis.

Discussion

The difficulty of detecting hRev1 protein might reflect the low expression level of hRev1 protein in human cells. Indeed, the 5' untranslated region of *hREV1* mRNA contains an out-of-frame start codon, which would initiate the translation of an unrelated small peptide and would severely reduce the translation efficiency of full-length hRev1 protein (Gibbs et al., 2000; Lin et al., 1999b). *hREV3*, believed to function in the same pathway as *hREV1*, also has properties in its mRNA that would greatly reduce the translation efficiency of the hRev3 protein. Such properties are the presence of an out-of-frame start codon (Gibbs et al., 1998), and the propensity of forming a stem-loop structure at the 5' untranslated region (Lin et al., 1999a). As a consequence, hRev3 protein might also be very low. The low expression level of Rev1 and Rev3 proteins appears to be evolutionarily conserved, because the yeast *REV3* gene also has an out-of-frame ATG start codon (Morrison et al., 1989), and efforts to detect endogenous yeast Rev3 or Rev1 proteins using antibody-based techniques were not successful either (Lawrence, unpublished studies).

The biochemical studies of many proteins involved in mutagenesis have been greatly facilitated by the use of protein tags and their corresponding commercially available ligands that the tags bind to and/or high-quality monoclonal antibodies. Fusion of a glutathione S-transferase (GST) tag to the N-terminus of yeast Rev3 protein allowed affinity purification of sufficient amount of

Table 1. Comparison of the kinds of base substitutions induced by BPDE in the *HPRT* gene of cells that express *hREV1* antisense RNA, but also exogenous Flag-tagged hRev1 protein with the kinds induced in the MSU-1.2 parental strain and finite life span diploid human fibroblasts.

Base substitutions	Number of base substitutions (% in all substitutions) observed in:		
	7AGM-12B-R1-FR1C1	Parental strain 7AGM	Normal finite cell line[a]
Transversions	19 (83%)	14 (78%)	25 (78%)
G → T	14 (61%)	9 (50%)	19 (59%)
G → C	2 (9%)	2 (11%)	5 (16%)
A → C	2 (9%)	2 (11%)	0
A → T	1 (4%)	1 (6%)	1 (3%)
Transitions	4 (17%)	4 (22%)	7 (22%)
G → A	2 (9%)	2 (11%)	6 (19%)
A → G	2 (9%)	2 (11%)	1 (3%)
Total	23	18	32

[a] These data, for a foreskin-derived finite life span normal human fibroblast cell line, were derived in this laboratory by Yang et al. (1991). They are included here for comparison.

Rev3 protein, and helped elucidate the interaction of Rev7 to Rev3 and the formation of the Pol zeta entity by this interaction (Nelson et al., 1996a). The deoxycytidyl transferase activity of yeast Rev1 protein was also originally identified using recombinant Rev1 protein fused in-frame with GST tag at the N-terminus and purified with glutathione-sepharose columns (Nelson et al., 1996b). In the characterization of human Rev1 protein (hRev1), Lin et al. (1999b) expressed and purified a recombinant hRev1 protein fused with six histidine residues at its N-terminus as well. The data indicate that these proteins, tagged at the N-terminus, retained their functions. The present study was designed to determine whether the reduced mutagenesis observed with cell strains expressing antisense *hREV1* RNA was a result of a loss of *hREV1* function. We introduced expression of an exogenous hRev1 protein in one of these strains. To facilitate the detection of the exogenous protein, we expressed hRev1 protein tagged with the Flag tag at the N-terminus. Evidence that this fusion protein retains its wild-type conformation comes from its ability to bind hRev7 protein shown by co-immunoprecipitation using anti-Flag monoclonal antibody (Murakumo et al., 2001).

The present studies with two derivative cell strains, showing that expression of this exogenous tagged hRev1 protein partially restored the mutability of the recipient cell strain expressing antisense RNA, strongly support a role for *hREV1* in mutagenesis induced by UV and BPDE. The fact that expression of hRev1 did not increase the frequency of UV-induced mutants to the level seen in the parental cell strain 7AGM, but only to 40% of the value,

probably reflects the continued expression of *hREV1* antisense in these two cell strains. Alternatively, the transfectants might simply not have produced as much protein, or activity of the recombinant fusion protein in participating in translesion synthesis is less than that of endogenous hRev1 protein. The observation that the increase in the frequency of mutants induced by BPDE in the same exogenous hRev1-expressing strains is not quite as high as that induced by UV might indicate that the tagged hRev1 protein participates better in bypassing UV photoproducts than BPDE adducts. Nevertheless, the reduction of mutation frequency in cell strains expressing antisense RNA and the reversal of this reduction by exogenous hRev1 protein clearly indicate the requirement for *hREV1* in damage-induced mutagenesis.

The human homolog of yeast *REV1* (*hREV1*) appeared to have functions similar to the yeast gene. The human Rev1 protein (hRev1) has a specific G template-dependent transferase activity (Lin et al., 1999b), and the ability to incorporate dCMP opposite abasic sites (Lin et al., 1999b). Our previous studies showed that human fibroblasts expressing high level of antisense RNA of *hREV1* (Gibbs et al., 2000) and *hREV3* (Gibbs et al., 1998), a gene believed to function in the same pathway as *hREV1*, exhibited greatly reduced mutation frequency induced by UV. Very recently, hRev1 protein has been shown to incorporate dCMP opposite damaged guanines, including 8-oxoguanine and the bulky guanine adduct formed by benzo[a]pyrene in in vitro primer extension assays (Zhang et al., 2002). If, in human cells, hRev1 protein inserts dCMP opposite template G damaged by benzo[a]pyrene, and DNA polymerase Pol kappa

extends from the 3' -OH, as demonstrated by in vitro primer extension assay (Zhang et al., 2002), this bypassing will be error-free, as C is the correct base pair for G. Then, reduction in the function of *hREV1* will cause an increase in BPDE-induced mutagenesis. However, we did not obtain such a result. Our data showed that expression of antisense RNA greatly reduced the frequency of BPDE-induced mutants, and expression of exogenous hRev1 protein in this antisense-expressing strain restored the frequency of mutants, strongly suggesting that hRev1 is involved in an error-prone mechanism. Sequencing of BPDE-induced mutants also showed that hRev1 is required for the vast majority of base substitutions induced by BPDE. The error-free bypassing of BPDE adduct by hRev1 protein might play a minor role in human cells.

In summary, by introducing expression of exogenous hRev1 protein in the cell strain expressing antisense *hREV1* RNA and seeing that this increased the frequency of mutants 8-fold, we confirmed our previous conclusion that the function of *hREV1* is required for UV-induced mutagenesis. In the present study, our data with the cell strain expressing high level of antisense (12B-R1) exhibiting a 96% reduction in the frequency of mutants induced by BPDE and the reversal of this reduction by exogenous hRev1 protein also indicated that *hREV1* is involved in BPDE-induced mutagenesis. Furthermore, we provided evidence that *hREV1* is involved in generating nearly all kinds of base substitutions induced by BPDE.

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APPENDIX

Roles of DNA Polymerase zeta and Rev1 Protein in Eukaryotic Mutagenesis and Translesion Replication

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Running Head: DNA polymerase zeta and Rev1 protein

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In addition to mechanisms that specifically repair DNA, such as those that excise damaged nucleotides or bases, eukaryotes possess several processes that promote the tolerance of unrepaired DNA damage. These processes are concerned with overcoming a potentially lethal property of unrepaired template damage, namely its propensity to block or severely impede DNA replication. One of the strategies used to overcome this inhibition is translesion replication, in which extension of the nascent strand at blocked forks is promoted by means of specialized DNA polymerases that are presumably more tolerant of alterations in primer/ template structure than the normal replicases. In budding yeast, the eukaryote in which tolerance mechanisms have been most intensively studied, the major process for translesion replication employs products encoded by the *REV1*, *REV3*, and *REV7* genes (Lemontt 1971; Lawrence et al. 1985a,b). Rev3p is the catalytic subunit of DNA polymerase zeta (Morrison et al. 1989; Nelson et al. 1996a), a distant relative of DNA polymerase delta (Braithwaite and Ito 1993), and Rev7p is another subunit of pol zeta (Torpey et al. 1994; Nelson et al. 1996a). Rev1p contains domains found in a superfamily that includes the *E. coli* UmuC and DinB gene products, the DinB homolog encoded by yeast *RAD30*, and the products of several mammalian *RAD30* homologs (reviewed in Woodgate 1999; Gerlach et al 1999). Although the process employing the pol zeta and Rev1p enzymes provides only a modest fraction of the capability of yeast to tolerate unrepaired damage, the activities of these enzymes are a major source of both spontaneous and induced mutations. For the most part, pol zeta and Rev1p are responsible for the production of basepair substitutions and single

nucleotide frameshifts, rather than larger additions or deletions, and their activities are usually the principal source of single nucleotide alterations. Greater than 98% of the basepair substitutions induced by UV irradiation, and > 90% of the UV-induced frameshift mutations depend on the function of the pol zeta, for example (Lawrence and Christensen 1979; Lawrence et al. 1984). Rev1p function is responsible for > 94% of UV-induced basepair substitutions, but appears to have less involvement in the induction of frameshift mutations, at least at some genetic sites (Lawrence and Christensen 1978; Lawrence et al. 1984). Although less well investigated, the mutagenic effectiveness of other mutagens such as ionizing radiation (McKee and Lawrence 1979) and chemical agents (Lawrence et al 1985b) also depend substantially on pol zeta and Rev1p function. In addition, pol zeta is responsible for between 50% and 75% of spontaneous mutations (Quah et al. 1980), for the increased spontaneous mutation resulting from transcription (Datta and Jinks-Robertson 1995), recombination (Holbeck and Strathern 1995), and the overexpression of 3-methyl adenine glycosylase (Glassner et al. 1998), and for a substantial fraction of the enhanced spontaneous mutagenesis in an excision-defective strain (Roche et al 1994). Genetic evidence suggests that, aside from translesion replication, pol zeta and Rev1p play no part in any other replication, recombination, or repair activity. Recent evidence suggests that yeast possesses a second translesion pathway, which employs DNA polymerase eta (Johnson et al. 1999), encoded by *RAD30* (McDonald et al. 1997). A comparison between the phenotypes of

RAD30 and *REV* mutants indicates that pol eta has a relatively restricted range of substrates.

Translesion replication is one of several DNA damage tolerance activities carried out by the yeast *RAD6* pathway. The activities of this pathway are often referred to as post replication repair, a double misnomer because they do not repair DNA and they do not necessarily occur after replication in excision repair proficient cells; they are more accurately called mechanisms for the restoration of replication competence (Lawrence 1994). The mutant phenotypes of the *RAD6* and *RAD18* genes, which encompass those of all other genes in the *RAD6* epistasis group and are the most extreme (Lawrence et al. 1974, Cassier-Chauvat and Fabre 1991), suggesting that they regulate the pathway (McKee and Lawrence 1979). Rad6p, which is an E2 ubiquitin conjugase (Jentsch et al. 1987) and Rad18p, which binds single-stranded DNA (Jones et al 1988), form a heterodimer that perhaps targets the conjugase activity to sites of unrepaired damage (Bailly et al. 1994). The DNA polymerase zeta / Rev1p translesion replication subpathway also appears to be regulated by the checkpoint genes *RAD9*, *RAD17*, *RAD24*, and *MEC3* because, like the *REV* loci, deletion mutants of these genes are deficient in UV-induced mutagenesis (Paulovich et al. 1998). A plausible, but as yet untested, hypothesis is that the checkpoint gene products are part of a system that monitors the status of replication forks, and activates the pol zeta / Rad1p subpathway when forks are blocked. If this is correct, ubiquitination of some target protein is presumably part of the monitoring process. Unlike the bacterial SOS system, there is little evidence for

transcriptional control of this subpathway in yeast (Larimer et al. 1989; Singhal et al. 1992).

PROPERTIES OF YEAST POL zeta AND Rev1p

The properties of pol zeta have been investigated by co-expressing a glutathione-*S*-transferase-Rev3 fusion protein (Gst-Rev3p) and normal Rev7p in yeast, and purifying the complex by affinity chromatography on glutathione-Sepharose (Nelson et al. 1996a). Although Gst-Rev3p alone shows a low level of DNA polymerase activity, the Gst-Rev3p/Rev7p complex is 20- to 30-fold more active. A plasmid expressing Gst-Rev3p fully complements the mutant phenotype of a *rev3* deletion strain, indicating that the fusion protein retains a substantial level of activity *in vivo*, and cleavage of the Gst moiety from the fusion protein only increased its activity by about two-fold. Since pol zeta has not yet been purified from cells without overexpression of Rev3p and Rev7p, we cannot rule out the possibility that the native enzyme contains additional subunits.

Pol zeta is a poorly processive enzyme, dissociating from half of the template molecules after adding only ≤ 3 nucleotides under conditions in which extension mostly resulted from a single polymerase binding event, though in some instances up to ~ 200 nucleotides were added. Pol zeta does not possess a 3′ to 5′ exonuclease proofreading activity, and is relatively insensitive to aphidicolin (> 90% activity at 200 mM) and dideoxynucleotide triphosphates (> 90% activity at 100 μM), and only moderately sensitive to butylphenyl-guanosine triphosphate (70% activity at 10 μM). Of particular relevance to its role in translesion replication, pol zeta appeares to be much more tolerant of abnormal

structure in the template and at the primer terminus than the major replicases. For example, under conditions apparently involving only a single binding event, pol zeta extended the primer past a thymine-thymine *cis-syn* cyclobutane dimer with ~ 10% efficiency but with yeast DNA polymerase alpha the efficiency was < 1% (Nelson et al. 1996a). Although, as described below, it has subsequently been discovered that pol zeta is unlikely to be primarily responsible for dimer bypass *in vivo*, such a result nevertheless suggest that pol zeta possesses a superior capability for tolerating template distortion. In addition, pol zeta extends a terminally mismatched primer more efficiently than pol alpha on dimer-free templates, again indicating a reduced stringency for normal duplex structure at the primer terminus (Table1). Apparent extension efficiencies were determined for all combinations of terminal nucleotide in the primer paired or mispaired with each nucleotide in the template, using templates in which the next nucleotide beyond the primer terminus was either dA or dG, and extension driven by increasing concentrations of dTTP or dCTP respectively. Extension efficiencies with pol zeta were from two-fold to more than 1000-fold higher than for pol alpha for any given mismatch. For pol zeta the actual efficiencies ranged from 0.3% to 54%, whereas for pol alpha the range was 0.002% to 3%. It appears that pol zeta is less accurate than the major replicases not only by virtue of a lack of proofreading, but also because it lacks some of the stringent requirements for correct primer/template structure.

As shown by the *REV1* mutant phenotype, lesion bypass by pol zeta also requires the function of Rev1p. To date, the clearest enzymatic function

Table 1. Apparent efficiencies for extension by pol zeta or pol alpha from single terminal base-pair mismatches

Terminal base pair (P·T)*	Apparent extension efficiency: $\frac{V_{max} / K_m \text{ (mismatch)}}{V_{max} / K_m \text{ (match)}}$					
	DNA polymerase zeta		DNA polymerase alpha		Efficiency ratio pol zeta/pol alpha	
	apparent extension efficiency		apparent extension efficiency			
	dTTP	dCTP	dTTP	dCTP	dTTP	dCTP
T·A	1	1	1	1		
A·A	1.6×10^{-1}	1.1×10^{-1}	1.7×10^{-3}	2.9×10^{-4}	96	379
C·A	2.7×10^{-2}	4.4×10^{-2}	5.3×10^{-3}	2.5×10^{-3}	5	17
G·A	4.1×10^{-2}	1.9×10^{-2}	4.6×10^{-5}	2.1×10^{-5}	893	912
G·C	1	1	1	1		
A·C	6.7×10^{-2}	2.7×10^{-1}	1.0×10^{-2}	8.3×10^{-3}	6	33
C·C	3.8×10^{-3}	3.1×10^{-3}	2.2×10^{-3}	1.6×10^{-5}	2	196
T·C	6.3×10^{-2}	1.5×10^{-1}	9.4×10^{-4}	2.0×10^{-3}	68	75
C·G	1	1	1	1		
A·G	1.3×10^{-1}	2.0×10^{-1}	4.3×10^{-4}	2.8×10^{-3}	299	71
G·G	9.5×10^{-2}	2.0×10^{-1}	7.5×10^{-5}	4.3×10^{-4}	1,265	478
T·G	9.1×10^{-2}	7.0×10^{-2}	1.5×10^{-2}	3.3×10^{-3}	6	21
A·T	1	1	1	1		
C·T	4.2×10^{-2}	2.9×10^{-2}	2.0×10^{-2}	5.9×10^{-3}	2	5
G·T	5.4×10^{-1}	3.5×10^{-1}	1.7×10^{-2}	3.3×10^{-3}	31	108
T·T	6.7×10^{-2}	8.3×10^{-2}	3.0×10^{-2}	6.8×10^{-5}	2	1,226

* P·T, primer·template.

associated with this protein is a deoxycytidyl transferase activity which inserts dCMP opposite an abasic site in the template and, with a much reduced efficiency, opposite template guanine and adenine nucleotides (Nelson et al. 1996b). No evidence was found to indicate utilization of any nucleotide triphosphate other than dCTP. As shown below, this activity is responsible for the preferential insertion of dCMP opposite abasic sites *in vivo* that had been observed previously (Gibbs and Lawrence 1995); unlike *E. coli*, in which an "A-rule" for such insertion is observed (Lawrence et al. 1990), yeast exhibits a "C-rule". Replication past an abasic site *in vitro* by pol zeta is strongly stimulated by Rev1p (Nelson et al. 1996b), and the same is probably true *in vivo*. The Rev1p dC transferase activity has received the greatest attention, in large part perhaps because other members of the Rev1/UmuC/DinD/Rad30 superfamily have subsequently been found to be DNA polymerases (reviewed in Woodgate 1999; Gerlach et al. 1999). Nevertheless, the transferase activity is unlikely to constitute the Rev1p function required for the bypass of most lesions other than abasic sites, and the protein appears to possess a second activity whose enzymatic basis is at present poorly understood.

Evidence pointing to the existence of this second function includes the observations that Rev1p-dependent bypass of a thymine-thymine pyrimidine (6-4) pyrimidinone UV photoproduct *in vivo* does not entail the incorporation of dC, and that bypass of an abasic site in a *rev1-1* mutant is much reduced, even though Rev1-1p retains a substantial fraction of the normal level of dC transferase activity, which bypass of an abasic site is expected to require (Nelson

et al. 2000). In these experiments, the frequencies of lesion bypass were estimated by transforming *REV*$^+$ or *rev* mutant yeast strains with duplex DNA plasmids that carried either a T-T (6-4) photoadduct, a T-T cyclobutane dimer, or abasic site centrally located in a 28 nucleotide single-stranded region. The number of these transformant colonies, normalized to the number obtained with a lesion-free but otherwise identical sample of control plasmid, provided an estimate of bypass frequency, since replication past the lesion and gap-filling is a necessary precursor to the creation of transformants. Replicated plasmid products were then analyzed to identify the nucleotides inserted opposite the lesion.

As shown in Table 2, the presence of a T-T (6-4) photoproduct in the plasmid strongly inhibits gap filling, and only 17% of the plasmids were completely replicated in a *REV*$^+$ strain. Lesion bypass in the successfully replicated plasmids was largely dependent on Rev1p, since the fraction of fully replicated plasmids fell to 1% in the *rev1* deletion strain. Incorporation of dCMP opposite one or the other of the thymine nucleotides occurred in only 1 of 72 *REV*$^+$ transformants analyzed, however, suggesting that the transferase activity was at best rarely employed during bypass of the T-T (6-4) lesion. A low frequency of dCMP insertion was also seen in a previous study (Gibbs et al. 1995). Bypass of the T-T (6-4) photoproduct was also much reduced in the *rev1-1* (G193R) and *rev3* deletion strains. Interestingly, neither Rev1p nor Rev3p function appeared to be required for the bypass of a T-T cyclobutane dimer,

Table 2. Bypass frequencies of an abasic site, T-T *cis-syn* cyclobutane dimer, and T-T pyrimidine (6-4) pyrimidinone adduct in *REV*$^{+}$, *rev1* deletion, *rev1-1*, and *rev3* deletion strains of yeast

Genotype of strain	T-T (6-4) (% bypass)	T-T dimer (% bypass)	Abasic site				
			nucleotide inserted during bypass				Number analyzed
			% bypass	dCMP	dAMP	other	
REV^{+}	17	82	23	89	11	0	18
rev1 deletion	1.0	76	0.4	0	100	0	18
rev1-1	0.5	66	2.3	75	25	0	4
rev3 deletion	0.4	72	0.9	0	100	0	8

indicating that bypass of this lesion is carried out by another DNA polymerase, most probably by pol eta (Johnson et al. 1999).

As expected, since dCMP is incorporated preferentially *in vivo* (Gibbs and Lawrence 1995), bypass of an abasic site during gap-filling was also found to be Rev1p-dependent. Twenty three percent of plasmid molecules were fully replicated in the *REV*$^+$ strain, but only 0.4% in a *rev1* deletion strain. In keeping with the earlier study, dCMP was incorporated opposite the abasic site in 89% of the bypass events in the *REV*$^+$ strain (Table 2), but such insertion was not observed in the few bypass events occurring in the *rev1* deletion strain, indicating that this event is Rev1p dependent. Significantly, however, reduced bypass and gap filling (2.3%) also occurs in the *rev1-1* mutant strain. The G193R *rev1-1* mutant protein retains about 60% of the wildtype Rev1p dC transferase activity (Figure 1), and bypass events in the *rev1-1* mutant entailed the incorporporation of dCMP in roughly the same proportion as seen in the *REV*$^+$ strain (Table 2). It therefore appears that bypass of an abasic site requires both the transferase and the second Rev1p function.

The enzymatic basis of this second Rev1p function is not known. Simultaneous overproduction of Rev1p, Gst-Rev3p, and Rev7p in yeast, followed by glutathione sepharose chromatography of cleared cell extracts, provides no evidence for the association of Rev1p with pol zeta. However, as noted above, the native enzyme may embody other subunits in addition to Rev3p and Rev7p, and one of these may mediate a Rev1p association. Alternatively, pol zeta and Rev1p may be independently recruited to a stalled replication fork, resulting in a

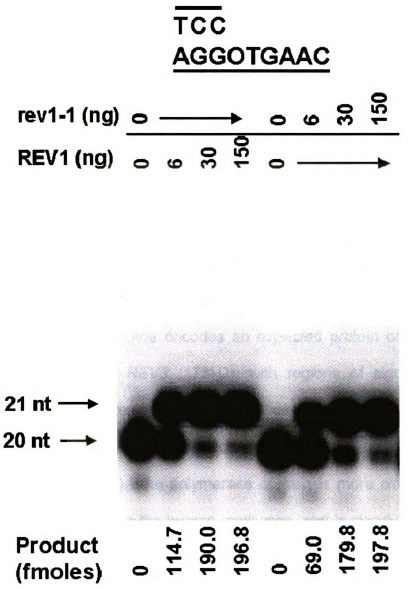


Figure 1. Assay of dCMP transferase activity for Gst-Rev1p and Gst-Rev1-1p fusion proteins. These activities insert dCMP opposite the abasic site in the template, designated O, extending the 20 nucleotide ^{32}P end labelled primer by one nucleotide. Amounts of the 21 nucleotide product were estimated by phosphorimaging.

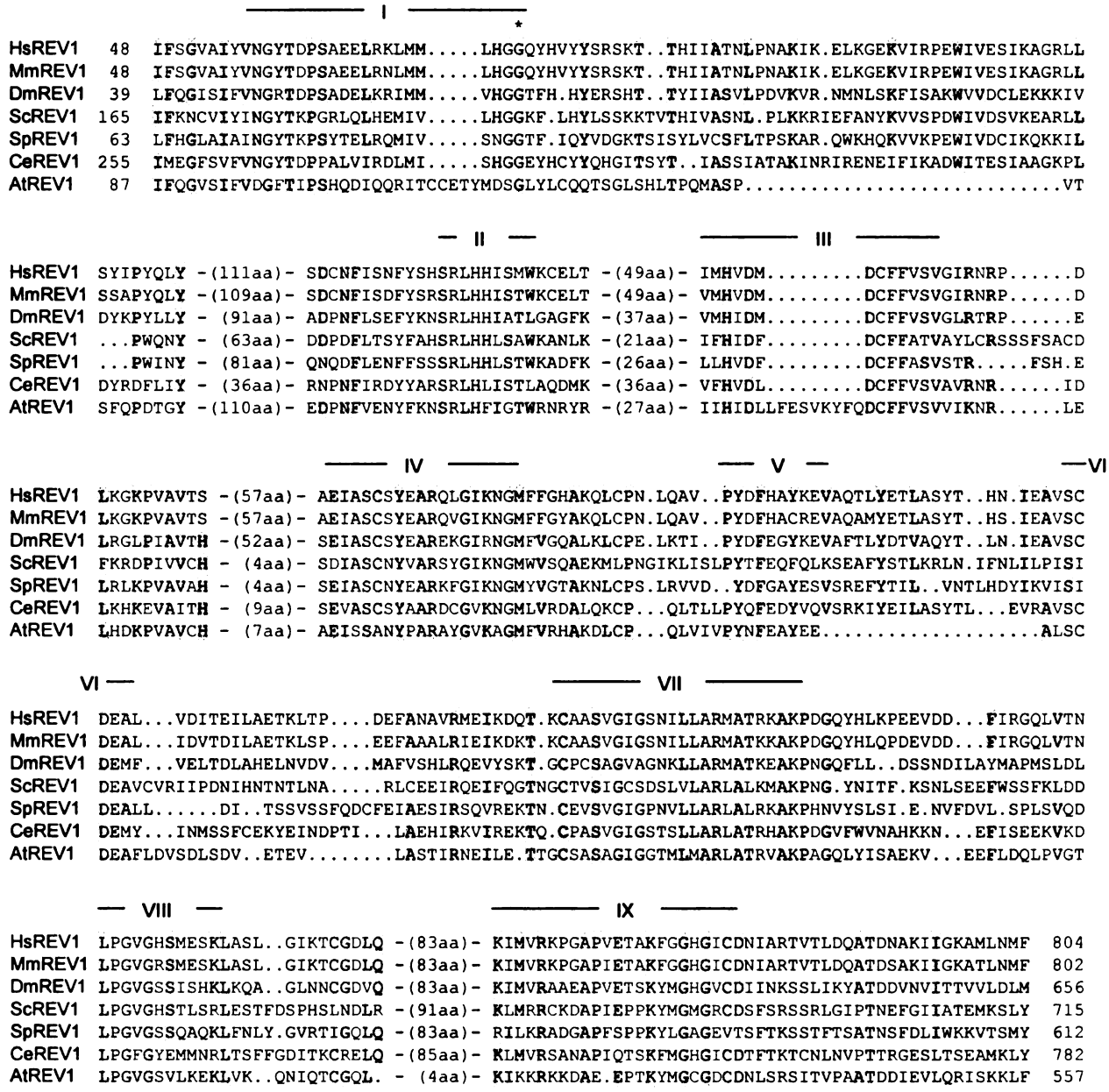
transient association. Whatever the nature of the association, Rev1p is likely to facilitate nucleotide insertion and primer extension by pol zeta, since genetic evidence suggests that the second function is more concerned with base substitution mutagenesis than with misalignment and frameshift mutagenesis.

FUNCTIONS OF POL zeta AND REV1p IN HUMANS

Saccharomyces cerevisiae has proved to be a good model for mammalian repair functions, and repair proteins are often conserved from budding yeast to humans. We have therefore used the yeast Rev1 and Rev3 protein sequences to isolate cDNA clones that encode their human counterparts (Gibbs et al. 1998; 2000). The human *REV3* gene encodes an expected protein of 353kD, about twice the mass of yeast *REV3* (173kD), with regions of significant identity principally at the amino terminus and at the carboxy terminus, which is the polymerase domain. Similar regions of homology are found in *REV3* genes from other organisms (Figure 2). The polymerase domain is more closely related to that of yeast than to those in the human replicases, and is clearly of the pol zeta type. About 40% of human *REV3* mRNA contains a 128 nucleotide insertion, probably the result of alternate splicing, at position +139/140 of the open reading frame which introduces a stop codon, and upstream out-of-frame ATG codons, features indicating that, as with yeast, cellular levels of Rev3p are probably quite low. The human *REV1* gene encodes a 138kD protein with significant identity to the 112kD yeast protein over the amino two-thirds of the protein (Gibbs et al. 2000). Alignments with the Rev1p from other species (Figure 3) identify 9 sequence motifs, the first of which lies in a region with weak homology to the

Figure 2. Sequence comparisons of conserved regions in the amino- and carboxy-terminus of Rev3p from various species. Sc, *Saccharomyces cerevisiae* (Morrison et al. 1989); Sp, *Schizosaccharomyces pombe* (accession No.AL355632); Hs, *Homo sapiens* (accession No. AF058701); Mm, *Mus musculus* (accession No. AF083465); Dm, *Drosophila melanogaster* (J. Eeken, P. van Sloun, and R. Romeijn, unpubl.); At, *Arabidopsis thaliana* (accession No. AC011020); Lm, *Leishmania major* (accession No. AL122012). Residues conserved in at least 5 of the sequences are highlighted.

Figure 3. Sequence comparisons of conserved regions of Rev1p from various species. Hs, *Homo sapiens* (accession No. AF206019); Mm, *Mus musculus* (P. Gibbs and C. Lawrence, unpubl.); Dm, *Drosophila melanogaster* (CG12189, accession No. AE003469); Sc, *Saccharomyces cerevisiae* (accession No. M22222); Sp, *Schizosaccharomyces pombe* (accession No. AL035548); Ce, *Caenorhabditis elegans* (accession No. Z46812); At, *Arabidopsis* thaliana (accession No. AC002342). Residues conserved in at least 5 of the sequences are highlighted. Conserved sequence motifs (Gibbs *et al.*, 2000) are overlined and numbered. The conserved glycine mutated in *rev1-1* is marked with an asterisk.


I
II
III
IV
V
VI
VII
VIII
IX
HsREV1 48 IFSGVAIYVNGYTDPSAEELRKLMM.....LHGGQYHVYYSRSKT..THIIATNLPNAKIK.ELKGEKVIRPEWIVESIKAGRLL
MmREV1 48 IFSGVAIYVNGYTDPSAEELRNLMM.....LHGGQYHVYYSRSKT..THIIATNLPNAKIK.ELKGEKVIRPEWIVESIKAGRLL
DmREV1 39 LFQGISIFVNGRTDPSADELKRIMM.....VHGGTFH.HYERSHT..TYIIASVLPDVKVR.NMNLSKFISAKWVVDCLEKKKIV
ScREV1 165 IFKNCVIYINGYTKPGRLQLHEMIV.....LHGGKF.LHYLSSKKTVTHIVASNL.PLKKRIEFANYKVVSPDWIVDSVKEARLL
SpREV1 63 LFHGLAIAINGYTKPSYTELRQMIV.....SNGGTF.IQYVDGKTSISYLVCSFLTPSKAR.QWKHQKVVKPEWIVDCIKQKKIL
CeREV1 255 IMEGFSVFVNGYTDPPALVIRDLMI.....SHGGEYHCYYQHGITSYT..IASSIATAKINRIRENEIFIKADWITESIAAGKPL
AtREV1 87 IFQGVSIFVDGFTIPSHQDIQQRITCCETYMDSGLYLCQQTSGLSHLTPQMASP.....VT
HsREV1 SYIPYQLY -(111aa)- SDCNFISNFYSHSRLHHISMWKCELT -(49aa)- IMHVDM.....DCFFVSVGIRNRP.....D
MmREV1 SSAPYQLY -(109aa)- SDCNFISDFYSRSRLHHISTWKCELT -(49aa)- VMHVDM.....DCFFVSVGIRNRP.....D
DmREV1 DYKPYLLY - (91aa)- ADPNFLSEFYKNSRLHHIATLGAGFK -(37aa)- VMHIDM.....DCFFVSVGLRTRP.....E
ScREV1 ...PWQNY - (63aa)- DDPDFLTSYFAHSRLHHLSAWKANLK -(21aa)- IFHIDF.....DCFFATVAYLCRSSSFSACD
SpREV1 ...PWINY - (81aa)- QNQDFLENFFSSSRLHHLSTWKADFK -(26aa)- LLHVDF.....DCFFASVSTR.....FSH.E
CeREV1 DYRDFLIY - (36aa)- RNPNFIRDYYARSRLHLISTLAQDMK -(36aa)- VFHVDL.....DCFFVSVAVRNR.....ID
AtREV1 SFQPDTGY -(110aa)- EDPNFVENYFKNSRLHFIGTWRNRYR -(27aa)- IIHIDLLFESVKYFQDCFFVSVVIKNR.....LE
HsREV1 LKGKPVAVTS -(57aa)- AEIASCSYEARQLGIKNGMFFGHAKQLCPN.LQAV..PYDFHAYKEVAQTLYETLASYT..HN.IEAVSC
MmREV1 LKGKPVAVTS -(57aa)- AEIASCSYEARQVGIKNGMFFGYAKQLCPN.LQAV..PYDFHACREVAQAMYETLASYT..HS.IEAVSC
DmREV1 LRGLPIAVTH -(52aa)- SEIASCSYEAREKGIRNGMFVGQALKLCPE.LKTI..PYDFEGYKEVAFTLYDTVAQYT..LN.IEAVSC
ScREV1 FKRDPIVVCH - (4aa)- SDIASCNYVARSYGIKNGMWVSQAEKMLPNGIKLISLPYTFEQFQLKSEAFYSTLKRLN.IFNLILPISI
SpREV1 LRLKPVAVAH - (4aa)- SEIASCNYEARKFGIKNGMYVGTAKNLCPS.LRVVD..YDFGAYESVSREFYTIL..VNTLHDYIKVISI
CeREV1 LKHKEVAITH - (9aa)- SEVASCSYAARDCGVKNGMLVRDALQKCP...QLTLLPYQFEDYVQVSRKIYEILASYTL...EVRAVSC
AtREV1 LHDKPVAVCH - (7aa)- AEISSANYPARAYGVKAGMFVRHAKDLCP...QLVIVPYNFEAYEE.....ALSC
HsREV1 DEAL...VDITEILAETKLTP....DEFANAVRMEIKDQT.KCAASVGIGSNILLARMATRKAKPDGQYHLKPEEVDD...FIRGQLVTN
MmREV1 DEAL...IDVTDILAETKLSP....EEFAAALRIEIKDKT.KCAASVGIGSNILLARMATKKAKPDGQYHLQPDEVDD...FIRGQLVTN
DmREV1 DEMF...VELTDLAHELNVDV....MAFVSHLRQEVYSKT.GCPCSAGVAGNKLLARMATKEAKPNGQFLL..DSSNDILAYMAPMSLDL
ScREV1 DEAVCVRIIPDNIHNTNTLNA.....RLCEEIRQEIFQGTNGCTVSIGCSDSLVLARLALKMAKPNG.YNITF.KSNLSEEFWSSFKLDD
SpREV1 DEALL......DI..TSSVSSFQDCFEIAESIRSQVREKTN.CEVSVGIGPNVLLARLALRKAKPHNVYSLSI.E.NVFDVL.SPLSVQD
CeREV1 DEMY...INMSSFCEKYEINDPTI...LAEHIRKVIREKTQ.CPASVGIGSTSLLARLATRHAKPDGVFWVNAHKKN...EFISEEKVKD
AtREV1 DEAFLDVSDLSDV..ETEV.....LASTIRNEILE.TTGCSASAGIGGTMLMARLATRVAKPAGQLYISAEKV...EEFLDQLPVGT
HsREV1 LPGVGHSMESKLASL..GIKTCGDLQ -(83aa)- KIMVRKPGAPVETAKFGGHGICDNIARTVTLDQATDNAKIIGKAMLNMF 804
MmREV1 LPGVGRSMESKLASL..GIKTCGDLQ -(83aa)- KIMVRKPGAPIETAKFGGHGICDNIARTVTLDQATDSAKIIGKATLNMF 802
DmREV1 LPGVGSSISHKLKQA..GLNNCGDVQ -(83aa)- KIMVRAAEAPVETSKYMGHGVCDIINKSSLIKYATDDVNVITTVVLDLM 656
ScREV1 LPGVGHSTLSRLESTFDSPHSLNDLR -(91aa)- KLMRRCKDAPIEPPKYMGMGRCDSFSRSSRLGIPTNEFGIIATEMKSLY 715
SpREV1 LPGVGSSQAQKLFNLY.GVRTIGQLQ -(83aa)- RILKRADGAPFSPPKYLGAGEVTSFTKSSTFTSATNSFDLIWKKVTSMY 612
CeREV1 LPGFGYEMMNRLTSFFGDITKCRELQ -(85aa)- KLMVRSANAPIQTSKFMGHGICDTFTKTCNLNVPTTRGESLTSEAMKLY 782
AtREV1 LPGVGSVLKEKLVK..QNIQTCGQL. - (4aa)- KIKKRKKDAE.EPTKYMGCGDCDNLSRSITVPAATDDIEVLQRISKKLF 557

BRCT domain of the BRCA1 gene, which may be concerned with protein-protein interactions. This region contains G76, the residue homologous to G193 in yeast and the site of the G193R mutation found in the Rev1-1 mutant protein, which substantially lacks the second function of yeast Rev1p, but retains much of the dC transferase activity. The highly conserved DXD and DE sequences found in motifs III and VI respectively appear to be concerned with polymerase function (Johnson et al. 1999; Gerlach et al. 1999). As with the *REV3* locus, the human *REV1* gene contains an upstream out-of frame ATG in a good context, again suggesting that cellular levels of Rev1p may be low. Essentially identical *REV3* and *REV1* clones have also been isolated by other groups (Morelli et al. 1998; Lin et al. 1999a,b).

The human Rev1p not only exhibit significant homology to the yeast proteins, but also appear to perform similar functions to their fungal counterparts. To investigate this issue, cells derived from MSU-1.2, an infinite life-span, non-tumorigenic, near diploid, human fibroblast cell line, were transformed with plasmids expressing high levels of either a *REV1* or *REV3* antisense RNA fragment, and the cells assayed for UV-induced mutagenicity and cytotoxicity (Gibbs et al. 1998, 2000). In cells expressing the antisense RNA, the frequency of 6-thioguanine resistant mutants induced by UV was much reduced compared to those seen in control cells in which antisense RNA was absent (Figure 4), a phenotype similar to that of yeast *rev* mutants. Unlike yeast *rev* mutants, however, the lower frequencies of UV-induced mutations were not accompanied by an increased sensitivity to the cytotoxic effects of the UV radiation, perhaps

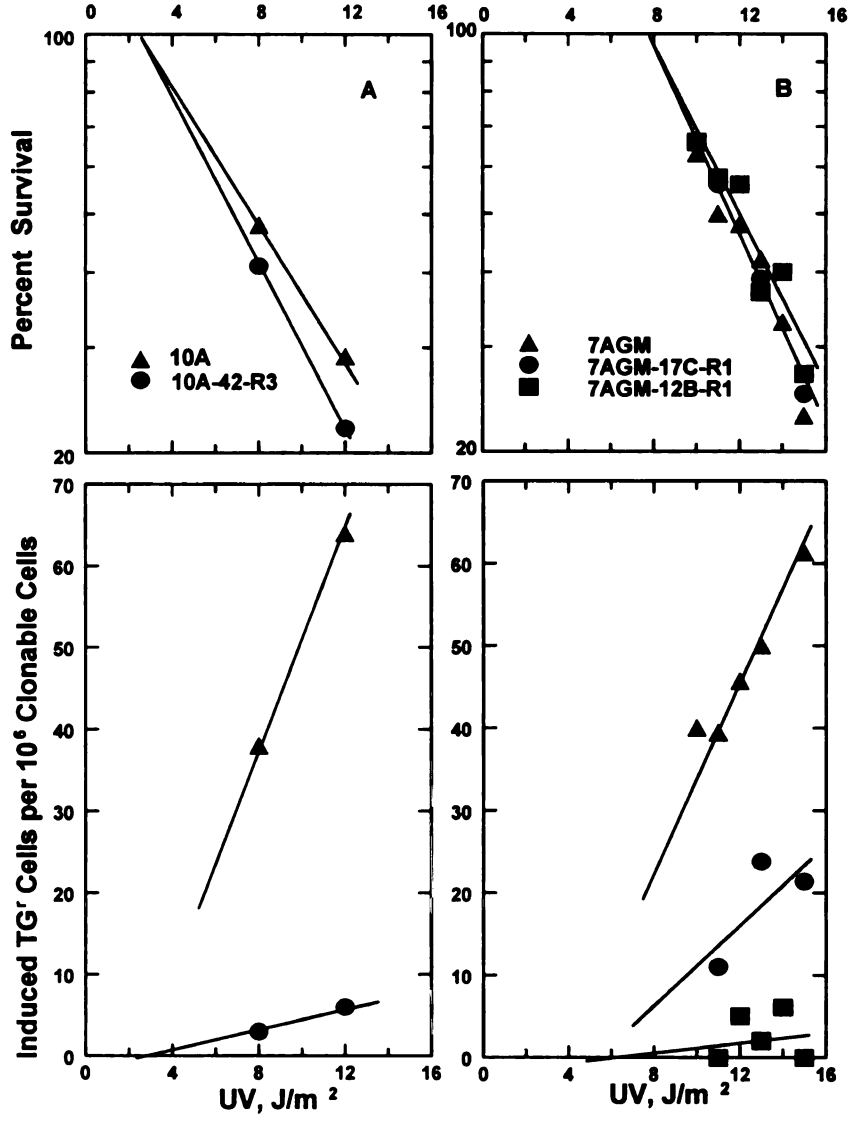


Figure 4. Percent survival and frequency of 6-thioguanine mutants in UV-irradiated human cells expressing *REV3* antisense RNA (A) or *REV1* antisense RNA (B), together with their antisense-free controls.

because the unrepaired damage was channeled into another repair or tolerance pathway. In addition to the reduction in UV-induced mutagenesis, expression of *REV3* antisense RNA also reduces spontaneous mutation frequencies in a *msh6* mutant strain (X. Li and V.M. Maher, unpublished data), suggesting that human pol zeta also resembles its yeast counterpart with respect to involvement in spontaneous mutagenesis. Reinforcing the similarity between the yeast and human proteins, Zigang Wang and co-workers have additionally shown that human Rev1p possesses a dC transferase activity (Lin et al. 1999b). Curiously, however, although dCMP is predominantly inserted opposite an abasic site *in vivo* in yeast (Gibbs and Lawrence 1995), incorporation of dAMP is the predominant event in monkey cells (Takeshita and Eisenberg, 1994). Finally, a putative human *REV7* cDNA clone has been isolated (Murakumo et al. 2000); whether the latter plays a role in mutagenesis remains to be determined.

DISCUSSION AND CONCLUSIONS

DNA polymerase zeta and Rev1 protein are employed in a translesion replication pathway in yeast that has a wide range of substrates and is a major source of DNA-damage induced mutations. Pol zeta is also responsible for the majority of spontaneous mutations in yeast. Judging from the results with human cells and from the widespread occurrence of homologs of the yeast *REV1* and *REV3* genes in other organisms, pol zeta and Rev1p are probably found in most eukaryotes. In addition to their presence in humans, *REV1* and *REV3* homologs have been jointly found in the mouse (accession No. AL355632; P. Gibbs, unpubl.), *Arabidopsis thaliana* (accession Nos. AC011020, AC002342),

Drosophila melanogaster (J. Eeken, P. van Sloun, and R Romeijn, pers. comm., accession No. AE003469) and *Schizosaccharomyces pombe* (accession Nos. AL355632, AL035548). In addition, *Leishmania major* has also been found to possess a *REV3* gene (accession No. AL122012), (see Figures 2 and 3). Interestingly, *Caenorhabditis elegans* possesses a *REV1* gene (accession No. Z46812) but not, apparently, a *REV3* gene and since the entire genome of this organism has been sequenced, it appears to lack an enzyme of the pol zeta type. Translesion replication in the worm may therefore be accomplished by Rev1p in combination with a different DNA polymerase such as pol delta, the enzyme to which pol zeta is most closely related. If so, the eukaryotic ancestral pol delta may perhaps have originally performed multiple functions in normal replication, repair, and translesion replication with some, though not necessarily all, lineages acquiring pol zeta by duplication of the gene encoding the catalytic subunit of pol delta and functional specialization of the resulting Rev3p.

At the present, putative Rev7p homologs have been discovered only in *Drosophila* (CG2948, accession No. AE003602) and humans (accession No. AF157482), perhaps indicating poor conservation of sequence. Although future work may uncover an enzymatic activity for this pol zeta subunit, its ability to promote the activity of the Rev3p catalytic subunit may in fact represent its chief function. Enhancing the activity of Rev3p may represent a mechanism for the activation of pol zeta when unrepaired damage in the genome prevents the completion of replication. The observation that the mutant phenotype of the yeast checkpoint genes *RAD9*, *RAD17*, *RAD24*, and *MEC3* includes not only a failure

to observe checkpoints but also a lack of UV-induced mutability characteristic of *rev* mutants (Paulovich et al 1998), suggests that a signalling pathway extends from the detection of blocked replication forks to the mobilization of the enzymes needed to remedy this situation. For pol zeta, such a mobilization might entail the release of sequestered Rev7p and the formation of a more active enzyme. Sequestering of Rev7p might involve association with the checkpoint gene products themselves, or by gene products phosphorylated or otherwise altered by these proteins. A variety of genetic evidence, including the introduction of in-frame stop codons by alternate splicing in human *REV3* and the existence of out-of-frame upstream ATG codons in several *REV* genes, suggests that cells maintain only very low levels of pol zeta and Rev1p, which may be necessary to prevent competition with replicases and high frequencies of spontaneous mutations.

Since pol zeta and Rev1p appear to be concerned with translesion replication in both yeast and humans, they presumably perform the same function in most eukaryotes. In the single-celled eukaryotes, such a process is clearly useful; the advantage of completing replication is likely to outweigh the disadvantage of possibly generating mutations. In mammalian cells, the balance in favor of translesion replication is at first sight less clear, since such cells have the additional option of apoptosis. Why, then, do mammals possess a translesion replication pathway? Part of the reason may be that the adverse consequences of lesion bypass are not necessarily high. The error-rate of translesion replication is usually very much less than 100%, and may be quite low. Even if mutations

are produced, their occurrence in non-informational DNA, or the formation of synonomous changes in coding sequences and the tolerance of many proteins towards residue substitutions are all factors that diminish the adverse effects of translesion replication. At the same time, the occurrence of unscheduled apoptosis may also itself carry risks, particularly in early development. A *REV3* knockout in the mouse is lethal (P. van Sloun, R. Romeijn, N. de Wind, and J. Eeken, pers. comm.), though the reasons for this are not yet known. By contrast, cultured human cells that apparently contain very little Rev3p or Rev1p are fully viable and grow at a normal rate (Gibbs et al. 1998, 2000). Significantly, such cells appear to be relatively resistant to the induction of apoptosis. Interrelationships of the regulation of pol zeta, apoptosis, tumor suppression, and cell cycle control is perhaps suggested by the observation that $p33^{ING1}$, the product of the human *ING1* gene, strongly associates with Rev3p in a two-hybrid assay (R. Murante, unpublished data). The candidate tumor suppressor $p33^{ING1}$ has been reported to negatively regulate cell proliferation, and apoptosis, to facilitate transcription from the p21/WAF1 promoter, and to physically associate with p53, indicating that it is part of the p53 signalling pathway (Garkavtsev et al. 1998). Lethality of the mouse *REV3* knockout may also arise because the mammalian enzyme provides another essential function, independent of its role in translesion replication. The much greater size of the Rev3p in mammals suggests that it may interact with a wider range of proteins or possess some additional activity; it has been suggested, for example, that the mouse gene is involved in a stress response (Kajiwara et al. 1996).

In addition to its role in translesion replication and the tolerance of unrepaired DNA damage originating from exogenous mutagens, pol zeta is also concerned with the production of spontaneous mutations; yeast *rev3* mutants are strong antimutator strains (Quah et al. 1980; Roche et al 1994; Datta and Jinks-Robertson 1995; Holbeck and Strathern 1995), and spontaneous mutation frequencies are reduced in *msh6* mutant human cells producing high levels of *REV3* antisense RNA (Z. Li and V. M. Maher, unpub.). Although replication past unrepaired spontaneously arising lesions may generate some of these mutations, it is unlikely that sufficient damage occurs to explain more than a small fraction of them. Instead, the majority may be produced by pol zeta when this enzyme is called upon to restart replication at forks that are blocked for reasons other than template damage. Roel Schaaper and co-workers (Fijalkowska et al. 1997) have suggested that the enhanced spontaneous mutagenesis resulting from derepression of the *E. coli* SOS regulon is caused by increased extension from terminally mismatches that temporarily stall DNA polymerase III. Such extension is presumably carried out by the UmuD′$_2$C complex, now known to constitute DNA polymerase V (Tang et al. 1999). This observation raises the possibility that *E. coli* pol V and its eukaryotic counterpart, pol zeta, may best be viewed as enzymes engaged principally in normal replication. From this perspective, these DNA polymerases are concerned with restarting replication at forks stalled for any reason, whether it is by virtue of a terminal mismatch, hairpin structure, or refractory DNA sequence, as well as by virtue of unrepaired DNA damage. Of these, the terminal mismatch and template damage are likely to have the

greatest propensity to generate mutations, but the other causes of stalling may also result in replication errors because pol zeta lacks proofreading.

Because it appears to be the major source of basepair substitution and frameshift mutations, investigation of the pol zeta/Rev1p translesion replication pathway is likely to have implications for human genotoxicology and for genetic diseases such as cancer. Reduction in the activity of this pathway may reduce spontaneous and induced mutation frequencies and therefore the risk of developing cancer. It has been calculated that merely halving the spontaneous mutation frequency might sufficiently delay the onset of cancer as to reduce its incidence substantially (Loeb & Christians, 1996). Since the majority of spontaneous mutations are likely to depend on pol zeta/Rev1p function, such a reduction should be easily achievable. Although the pathway is essential for viability during early development, it remains to be established whether it is also essential in mature mammals, and whether ways of dissociating mutability and viability can be found.

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