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MECHANISMS OF MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS BY METHYLNITROSOUREA

By

Scott Everette Boley

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ABSTRACT

MECHANISM OF MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS BY METHYLNITROSOUREA

by

Scott Everette Boley

The transformation of human cells from a normal to a tumorigenic state requires multiple stages, with each stage associated with a more transformed phenotype. The purpose of my research was to determine whether the methylating agent methylnitrosourea (MNU) induces transformation of human cells and whether the O^6-methylguanine adduct (O^6MeG) is causally involved in this transformation. Two populations of the infinite life span, karyotypically stable, human fibroblast cell strain MSU-1.1 differing in their ability to repair O^6MeG were exposed to MNU and assayed for the ability to proliferate under low serum conditions, as evidenced by the ability to form foci on a lawn of normal cells. The results showed that O^6MeG plays a causal role in the MNU-induced transformation of MSU-1.1 cells to focus formation. Use of a transgenic yeast assay to determine the transactivating ability of the p53 gene in 40 independent cell strains derived from representative foci revealed that 37.5% lacked functional p53 and one cell strain was heterozygous for *p53*. Assays of the ability of 35 strains to form tumors in athymic mice showed that no cell strain containing wild type p53 formed tumors. In contrast, the p53 heterozygous strain and 10 of 15 strains with mutant p53 formed malignant tumors. The data indicate that loss of p53 transactivating ability is not sufficient for malignant transformation of MSU-

1.1 cells, but greatly facilitates it. Sequence analysis of several strains revealed each contained an identical mutation, a A-to-G transition at codon 215, and this same mutation was found in the mutant allele of the heterozygous strain, strongly suggesting that a subpopulation of cells altered at codon 215 pre-existed within the MSU-1.1 population used for this study. The data indicate that such cells do not form foci, but that loss of the wild type *p53* allele facilitates focus formation. Analysis of the p arm of chromosome 17 of a series of strains with mutant p53 for evidence of mitotic homologous recombination, i.e., loss of informative restriction fragment length polymorphisms and microsatellite markers, revealed seven patterns of homologous. Only two of these seven patterns were found in p53 mutant cell strains derived from ionizing radiation-induced foci.

This dissertation is dedicated to my family for their never-ending support and patience. To my mother, who always told me that I could be whatever I wanted to be, and my father, who showed me that through hard work I could achieve anything. To my brothers and sisters, who always remind me who I am and where I came from. To my son Jordan, who is a constant source of pride, and my daughter Kierstin, who brings a ray of sunshine into each day. In conclusion I want to dedicate this work to my wife, Alana, for without her there would be no me.

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ABBREVIATIONS

AGT	O^6-alkylguanine-DNA alkyltransferase
AI	anchorage independence
APC	adenopolyposis coli
CHO	Chinese hamster ovary cells
DNA-PK	DNA-dependent protein kinase
dsDNA	double-stranded DNA
HPRT	hypoxanthine phosphoribosyltransferase
LFS	Li-Fraumeni Syndrome
MAPK	mitogen-activated kinase
MMR	mismatch repair
NER	nucleotide excision repair
O^6BzG	O^6-benzylguanine
O^6MeG	O^6-methylguanine
O^2MeT	O^2-methylthymine
O^4MeT	O^4-methylthymine
PCNA	proliferating cell nuclear antigen
Rb	retinoblastoma
RFLP	restriction fragment length polymorphisms
SCE	sister chromatid exchange
ssDNA	single-stranded DNA
TCR	transcriptional coupled repair

XP	xeroderma pigmentosum
XRCC	x-ray cross complementing genes

INTRODUCTION

It is estimated that one out of every three people in the United States will be diagnosed with cancer at some point in their lives. Therefore the study of the process by which cancer develops, carcinogenesis, has significant public health importance. In the carcinogenesis process, a cell accumulates individual genetic alterations associated with a transformed phenotype. These alterations are the result of random mutations in a subset of critical genes. The majority of these genes can be categorized as either tumor suppressor genes or oncogenes. These genes play opposing roles in regulating cell growth, with oncogenes promoting growth and tumor suppressor genes involved in the inhibition of cell growth. It is the alteration of both types of genes that relieves the growth constraints associated with a normal phenotype. Therefore the determination of how these genes are involved in cellular transformation is central to cancer research.

The research for my dissertation involved studying the carcinogen-induced transformation of a human fibroblast cell strain, MSU-1.1 (Morgan et al., 1991), from a nontumorigenic to a tumorigenic state. Previous studies from this laboratory had shown that MSU-1.1 cells could be malignantly transformed by a single carcinogen treatment (Yang et al., 1992; Reinhold et al., 1996; O'Reilly et al., 1998). This indicates that MSU-1.1 cells represent a late stage in the multistep carcinogenesis model needing only one or two additional changes to become transformed. To detect transformed cells, I utilized a focus formation

assay designed to select for the clonal growth of cells that are able to proliferate under conditions in which the majority of cells stop proliferating. Previous results from this laboratory had shown that this assay was useful for detecting the ability of benzo(a)pyrene diol epoxide (Yang et al., 1992) or ionizing radiation (Reinhold et al., 1996; O'Reilly et al., 1998) to induce malignant transformation of MSU-1.1 cells. I undertook a study designed to determine if the simple methylating carcinogen methylnitrosourea (MNU) could induce transformation of MSU-1.1 cells, and if so, whether the principal lesion responsible for cell killing and mutagenesis i.e., O^6-methylguanine (O^6MeG) was directly involved. By manipulation of the ability of MSU-1.1 cells to repair that specific MNU-induced DNA damage, I could determine its role in the MNU-induced transformation of MSU-1.1 cells. Since earlier results from this laboratory had suggested loss of the *p53* gene on chromosome 17 might be involved in the malignant transformation of MSU-1.1 cells (Reinhold et al., 1996), I analyzed the transactivating ability of the p53 gene in the cell strains derived from independent foci, using a transgenic yeast assay (Scharer and Iggo, 1992; Ishioka et al., 1993). Focus-derived cell strains that were found to differ in their p53 transactivating capacity were tested for their ability to form tumors in athymic mice.

Chapter I consists of a review of evidence from the literature that carcinogenesis is a multistep process, involving the progression of a cell from a normal to a malignant phenotype. The critical importance of oncogenes and tumor suppressor genes is reviewed with particular emphasis placed on the *p53*

tumor suppressor gene, since it is centrally involved in my research. Since the alterations important to carcinogenesis are mainly mutations resulting from the replication of a damaged DNA template, the components and mechanisms of two critical DNA repair mechanisms are examined, with an emphasis on DNA damage resulting from methylating agents. Because methylating agents can induce homologous recombination in human fibroblasts (Zhang et al., 1996) I also reviewed the factors involved in homologous recombination and discussed the most probable mechanisms involved.

Chapter II consists of a manuscript prepared for submission to the journal **Cancer Research**. In that study, I manipulated the ability of human MSU-1.1 fibroblasts to repair a specific DNA adduct, i.e., O^6MeG, in order to determine if this adduct plays a causal role in the MNU-induced transformation of MSU-1.1 cells to focus formation. My results indicate that it does. Analysis of focus-derived cell strains revealed inactivation of p53 transactivating function might play a role in focus formation. Through analysis of polymorphic genetic loci in the focus-derived cell strains, I obtained data that strongly suggest the existence of a subpopulation of cells containing a specific *p53* mutation within the bulk MSU-1.1 population I used for the study. The data further support the hypothesis that this subpopulation is heterozygous and that the loss of the wild type p53 allele results from homologous recombination. The recent demonstration by Zhang et al. (1998, 1999) in this laboratory that O^6MeG is capable of inducing homologous recombination in human fibroblast cell in culture, strongly supports this hypothesis.

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CHAPTER I

LITERATURE REVIEW

I. Multistep Theory of Carcinogenesis

It is widely accepted that carcinogenesis, the process by which a normal call becomes tumorigenic, is a process that involves multiple stages. Each stage is the consequence of a genetic or epigenetic alteration that results in a phenotypic change in the cell. As a cell progresses from one stage to another, it accumulates the alterations characteristic of a malignant cell.

A. Clonal expansion

Clonal expansion is based on the premise that when genetic alterations relevant to carcinogenesis occur in a cell the result is an increased population of altered cells. These alterations are typically the result of mutations although they could also be the result of epigenetic events. This increase in population size could occur in one of two ways: 1) the alteration results in a growth advantage being conferred to the affected cell, allowing the altered cell to clonally expand and 2) this alteration results in the affected cell being resistant to cell death, while the surrounding unaltered cells perish. Either of these routes results in the accumulation of a population of cells that contain the same alteration, thereby increasing the likelihood of a second mutational event occurring within a cell that already has the first mutation, yielding a doubly mutated cell. As before, this

additional alteration could result in a new growth advantage, culminating in a population of cells containing two mutations. It is by the repetition of these events, mutation followed by clonal expansion and subsequent mutation, that a normal cell may gain all of the mutations associated with a tumorigenic cell.

A few important points about the concept of clonal expansion need to be clarified. First, the mutations must affect a gene capable of conferring a growth advantage to the target cell. For example, the mutation of a hemoglobin gene would not be expected to have an effect on the growth properties of a fibroblastic cell. Second, the order in which the mutations occur is not defined, but rather the overall accumulation of mutations is the crucial factor (Fearon and Vogelstein, 1990). Unless the first change results in a greatly increased mutation rate, the cells will not be able to acquire a sufficient number of changes before they reach the end of their lifespan. However, the acquisition of an infinite life span is generally accepted as an early change in tumorigenesis in cell culture because it is needed if later changes are to occur. McCormick and Maher (1989) showed that several changes are required for the tumorigenic transformation of cells in culture, and phenotypically normal human cells exhibit a life span in culture of approximately 60 population doublings. Based on a mutation frequency of 1 x 10^{-6}, a cell that has incurred the first mutation in carcinogenesis requires an additional 20 population doublings to reach 10^6 cells and thereby have an appreciable chance of undergoing a second mutation. If a cell requires only three mutations to become tumorigenically transformed, the cell would require an infinite or greatly extended life span in order to accumulate these changes.

Third, the fact that a cell incurs an initial mutation does not mean that it is destined to become malignant. A cell may accumulate only one or two mutations, and not progress past that point. Fourth, the mutation conferring a growth advantage is central to the concept of clonal expansion, since without an expansion of mutated cells, the chances of a second mutation occurring within a cell that contains the first mutation would be greatly reduced. However, it is important to note that the growth advantage may be a direct consequence of the mutation, as in the overexpression of a growth factor, or it may be indirectly connected, as in the mutation of a gene critical to DNA repair. Altered DNA repair would not directly result in a growth advantage, but it decreases the number of population doublings required because it would increase the chance of subsequent mutations occurring due to faulty repair of exogenous and endogenous DNA damage, and in that way it would fit with the clonal expansion model.

One of the most widely used techniques to determine the clonality of tumors in females was developed by Vogelstein et al. (1987) and involves restriction endonuclease digestion of genomic DNA. The DNA is first digested to reveal restriction fragment length polymorphisms (RFLP) between paternal and maternal X chromosomes. The second digest utilizes a methylation-sensitive endonuclease to reveal the methylation status, and therefore the transcriptional status (for a review see Jost and Bruhat, 1997) of a gene on the X chromosome, usually hypoxanthine phosphoribosyltransferase (*HPRT*). Using both types of enzymes, investigators can determine if the tumor is comprised of cells that had

the same X chromosome activated, which would indicate the tumor was derived from a single cell.

Herman et al. (1990) used RFLP analysis to determine the clonality of benign pituitary tumors from 16 female patients, and found 12 to exhibit results indicating a monoclonal origin. Namba et al. (1990), investigated the clonality of thyroid tumors in females using the combination of the RFLP and methylation-sensitive restriction endonucleases discussed above and found six adenomas and three carcinomas exhibiting a monoclonal origin. In both the Namba and Herman studies, a few samples gave results indicating a polyclonal origin but this may well be the result of contaminating normal tissue present in the tumor sample. Fearon et al. (1987) analyzed 50 human colorectal tumors and found all to show monoclonal patterns of X chromosome inactivation. Of these tumors, 30 were adenomas and the remaining 20 were carcinomas. The fact that the adenomas showed monoclonal origins is significant since various reports suggest that adenomas are precursors of carcinomas (Muto et al., 1975; Shinya and Wolff, 1979).

Although the above reports show a monoclonal origin for human tumors, results from the study by Hsu et al. (1983) indicated a polyclonal origin for polyps in patients diagnosed with Gardners Syndrome. The method they used was based on isozyme analysis of polymorphisms in the X-linked gene glucose-6-phosphate dehydrogenase. As described above, this technique relies on the fact that one X chromosome is inactivated, and therefore only one isozyme is expressed per cell. The drawback to this method is that it relies on protein

expression and is therefore susceptible to alterations in transcription and translation, common features of transformed cells. Again, the presence of normal cells within the tumor sample would make it appear to represent a polyclonal population and this could alter the results. Furthermore, the results from Hsu et al. (1983) may be specific to a tumor type or cancer syndrome. Since the majority of tumors studied reveal a monoclonal origin it is generally accepted that the majority of human tumors originate from a single cell.

B. Multistep model of colorectal carcinogenesis

The reports described above are evidence that the majority of human tumors are monoclonal, which is consistent with the multistep model and clonal expansion. However, those reports dealt with the end product of tumorigenesis, the tumor sample, while what was lacking was an illustration of the multistep model itself. A detailed description of how a tumor evolves from a normal cell was required that included examples of the distinct stages that occur during the transformation of a normal cell to a tumorigenic state.

Fearon and Vogelstein (1990) depict an excellent *in vivo* illustration of a multistep model for colorectal carcinogenesis. Patients afflicted with familial adenomatous polyposis, an autosomal dominant cancer syndrome, are predisposed to colorectal tumor formation and develop hundreds of colorectal adenomas, providing a large sample of neoplastic tissue. Multiple tissue samples were isolated and separated into distinct phenotypic stages depicting the progression of tumorigenesis, from the normal epithelium of the colon to adenomas of varying size, and ultimately carcinomas. Fearon and Vogelstein

found that each stage could be characterized by a common genetic change, suggesting the mutation was associated with the progression to that particular stage, although it was possible the mutation responsible may have been in some unknown gene. Since the same genetic change, i.e. inactivation of *p53* or activation of *ras*, was found in different samples representing a distinct phenotype, the data strongly suggested a connection between the observed mutation and the phenotype.

The first discernible phenotypic change noted was that normal colonic cells had converted to a hyperproliferative state. Through molecular analysis of hyperproliferative samples, the authors found that loss or inactivation of the adenopolyposis coli (*APC*) gene located on the q arm of chromosome 5 (Bodmer et al., 1987; Leppert et al., 1987) correlated with the increased proliferation. The APC protein has been shown to interact with β-catenin (Rubinfeld et al., 1993) which plays a role in intercellular interactions and cytoskeleton arrangement (Su et al., 1993). Therefore, alterations in *APC* could lead to a disruption in cell to cell signaling responsible for inhibiting unregulated growth. The next phenotypically identifiable stage was early adenoma (less than 1 cm in size), and analysis revealed that most early adenomas exhibited hypomethylation of their genomic DNA, i.e., there was an overall decrease in the level of 5-methylcytosine in the DNA. Alterations in the level of 5-methylcytosine affects the transcriptional activity of genes (for a review see Jost and Bruhat, 1997). For example, Feinberg and Vogelstein (1983a,b) have shown that hypomethylation could result in the increased expression of genes, including oncogenes, and contribute to the

progression of the carcinogenesis process. Other studies have shown that decreased methylation of cytosine increases genomic instability by affecting chromosomal condensation and therefore affecting proper segregation of the chromosomes during mitosis (Schmid et al., 1984). A recent report by Chen et al. (1998) showed that hypomethylation of DNA in murine embryonic stem cells results in a significant increase in the mutation rate.

It is important to note that in their analyses Fearon and Vogelstein found that not all samples from a given phenotypic stage contained the same mutation, but rather that the mutation was seen in the majority of samples. An important point made by the authors based on this evidence was that the accumulation of the changes was the critical factor to tumorigenesis, not the order in which they occurred. For example, mutation of *p53* and *ras* were found to occur at various stages of the process, indicating the timing of these particular changes was not crucial.

C. The MSU-1.1 cell strain

The model described above is an excellent description of carcinogenesis *in vivo*, but to further understand the mechanisms involved in the carcinogenic process a model that can be manipulated in the laboratory under controlled conditions is required. This would allow researchers to study the characteristics of the various stages involved in the conversion of a normal cell to a tumorigenic state as well as to design early detection techniques and aid in the development of effective cancer therapies.

McCormick and colleagues (Morgan et al., 1991) developed an infinite life span, karyotypically stable, nontumorigenic, human fibroblast cell strain designated MSU-1.1. They transfected a phenotypically normal, finite life span, human fibroblast cell line derived from neonatal foreskin, designated LG1, with a viral form of the *myc* oncogene. Several drug resistant clones transfected with that *v-myc* vector and two from a control vector were isolated and serially cultured until the cells entered crisis and then senesced, i.e., they did not exhibit further cellular replication. Among the senescent progeny of one of the clones, the only one that later studies showed was expressing *v-myc*, it was noted that a few areas of cells exhibiting growth appeared. Cells from these areas were isolated, propagated, and characterized. These cells exhibited an infinite life span, contained 45 chromosomes, including two marker chromosomes, were karyotypically stable, nontumorigenic, and partially growth factor independent. When earlier frozen stocks of the progeny of that *v-myc* expressing clone were examined, another infinite life span cell strain was isolated. Characterization of these cells revealed they were diploid, karyotypically stable, nontumorigenic, and had normal growth factor dependence. Since the frequency of generating an infinite life span cell strain in culture is extremely rare, it is assumed that the strain with the marker chromosomes, designated MSU-1.1, arose from the diploid strain, designated MSU-1.0. MSU-1.1 cells are unique in that they, unlike MSU-1.0 cells, are able to be transformed to a tumorigenic phenotype by overexpression of transfected oncogenes or following a single carcinogen

treatment (Hurlin et al., 1989; Wilson et al., 1990; Yang et al., 1992; Reinhold et al., 1996; O'Reilly et al., 1998)

Generation of the infinite life span cell strains MSU-1.0 and MSU-1.1 allowed McCormick and colleagues to study pure populations of cells that represented the progression of a normal fibroblast cell line, LG1, through to the tumorigenic derivatives of MSU-1.1. This allowed them to study of specific differences in isogenic cell strains at each stage of tumorigenesis. In addition, it presented the unique opportunity to identify any genetic alteration associated with a particular stage of the model.

D. Detection of mutated cells

Both models discussed above begin with normal cells that proceed through various stages as the result of mutations or epigenetic events, culminating in tumor formation. The MSU-1.1 model provides a method to detect progression from nontumorigenic state to tumorigenic state in a controlled laboratory setting. As detailed above, MSU-1.1 cells can be malignantly transformed by a single carcinogen treatment, however a method was required to detect those cells that had been altered in some way that was relevant to carcinogenesis.

1. *HPRT* mutation assay

As stated above, tumorigenic transformation of human cells in culture requires the mutation of critical genes. The most common method of studying mutation frequency in cultured human cells is measuring the frequency of cells that acquire resistance to 6-thioguanine (DeMars, 1974) following treatment. This resistance results from functional inactivation of the *HPRT* gene, located on

the X chromosome. Since MSU-1.1 cells are derived from a male cell, *HPRT* represents a single copy gene. Therefore, the frequency of *HPRT* mutation obtained following treatment of MSU-1.1 cells with a mutagen is a measure of the mutation of a single copy gene. The HPRT assay can also be used with female cells since only one X chromosome is active. The frequency of induced *HPRT* mutations can be taken to represent the likelihood of mutating any one gene within the MSU-1.1 genome by treatment with a carcinogen. Human fibroblasts treated with a dose of a methylating agent that results in a survival of 20% typically yields $100x10^{-6}$ *HPRT* mutants (Domoradzki et al., 1984).

Although this assay is an efficient and effective method for measuring mutation frequencies, the *HPRT* gene is not a critical gene involved in carcinogenesis. The majority of genes that have been found to be important in cancer are involved in the regulation of cell growth. Assays are needed that detect a mutation in a gene(s) involved in tumorigenic transformation.

2. Focus formation assay

When MSU-1.1 cells are seeded into a culture dish they proliferate until they come into contact with other cells, at which time they stop growing and form a confluent monolayer. A common characteristic of transformed cells is that they have lost this contact inhibition property (Abercrombie, 1979). Therefore an assay designed to detect loss of contact inhibition might be useful to detect carcinogen transformed MSU-1.1 cells. Reznikoff et al. (1973) developed an assay to detect C3H10T1/2 mouse fibroblasts that had overcome contact inhibition following treatment with various chemicals. Cells were treated with

doses designed to result in 200 surviving cells per dish, and these cells were allowed to replicate in the dish for six weeks, at which time the cells were stained and foci, or clones of cells that had overcome contact inhibition and formed piled colonies, were visible.

McCormick and colleagues also used the focus formation as an assay identifying transformed human fibroblasts (Yang et al., 1992, Reinhold et al., 1996, O'Reilly et al., 1998). The focus assay described in these reports differs from the mouse assay used by Reznikoff and colleagues in that following treatment, the MSU-1.1 fibroblasts are kept in exponential growth for a short period, then plated at a density of 1×10^3 or 4×10^3 cells/cm^2 under low serum conditions and allowed to grow to confluence. The 7 to 8 day expression period prior to replating is intended to allow expression of newly mutated genes and the degradation of wild type gene products. Lowering of the serum level of the culture medium was from 10% to 2% or 0.5% for the cells plated for foci allowed the assay to screen for cells that have a greatly reduced requirement for growth factors, another characteristic of transformed cells (Hurlin et al., 1989; Wilson et al., 1992; Reinhold et al., 1996; O'Reilly et al., 1998). The usefulness of the focus assay as a means of identifying transformed cells is evidenced by the fact that a high percentage of focus-derived cell strains isolated following carcinogen treatment of MSU-1.1 cells and tested for tumorigenicity were found to form tumors in athymic mice (Yang et al., 1992; O'Reilly et al., 1998).

3. Anchorage independence assay

The ability to form colonies in soft agarose, commonly called anchorage independence (AI), is another common characteristic of transformed cells (McCormick and Maher, 1988). Normal fibroblast cells are anchorage dependent, i.e., they require a substrate to attach to in order to proliferate. To accommodate the need for attachment, the plastic vessels used in cell culture research are treated by the manufacturer to generate a suitable charge density so that they can serve as a substitute for the ECM proteins. Contact between the cell and the plastic culture vessel initiates a signaling cascade that is mediated by integrins (Schwartz 1997). The activated integrins transmit the signals to mediators that are also involved in growth factor-induced pathways, and so there is crosstalk between the two pathways. As in many signaling pathways, it is possible to mutate one of the components of the integrin pathway resulting in the constitutive activation of the pathway. This removes the requirement for an attachment factor and the cell grows as if it is on a solid support. Schwartz et al. (1996) showed that activation of the small G protein Rho resulted in the ability of rodent cells to become anchorage independent. A recent study by Qui et al. (1997) indicated that Rat1 fibroblasts could be induced to become anchorage independent by transfection with an activated form of *cdc42*, which is another member of the Rho family of genes.

Various papers from our laboratory and others have shown a correlation between AI and tumorigenicity of human cells (Freedman and Shin, 1974; Yang et al., 1992; Reinhold et al., 1996; O'Reilly et al., 1998). This same correlation

holds for NIH3T3 cells (Wang et al., 1998) Syrian hamster embryo cells (Suzuki 1997) and rat liver epithelial cells (Presnell et al., 1995). Therefore, the combination of the focus assay as an initial screen followed by the AI assay provides a powerful selection tool to isolate tumorigenic cell strains resulting from carcinogen treatment of MSU-1.1 cells.

4. Tumorigenicity assay

The ultimate measure of the tumorigenic potential of a cell strain is its ability to form tumors in an animal model. The predominant model used is the athymic mouse, which lacks a thymus gland and is therefore unable to mount a immunological response against foreign cells. To test the tumorigenic potential of transformed human fibroblasts in athymic mice, gelatin sponges placed under the skin are used to provide an attachment matrix for the cells. This keeps the cells in one location and prevents them from diffusing under the skin. In addition to determine the tumorigenic potential of a particular cell strain, athymic mice can also serve as a screening tool. McCormick and colleagues have data showing injection of a total of 10^7 human fibroblasts that contain 10^5 or more sarcomagenic cells into an athymic mouse results in the development of a tumor with a latency period of 32 weeks compared to a 11 week latency period for 10^7 sarcomagenic cells. Injection of 10^7 normal fibroblasts has never formed tumors in this assay (McCormick, unpublished data). Therefore the athymic mouse can also serve as a screen to detect cell strains that contain a small tumorigenic subpopulation.

II. Critical Genes in Carcinogenesis

As noted above, the mutations involved in the multistep process of tumorigenesis must occur in a critical subset of genes. The protein products of these genes are often involved in the control of cellular proliferation, and these genes can be grouped into two broad categories oncogenes and tumor suppressor genes.

A. Oncogenes

Oncogenes were first discovered through the study of retroviruses that produced tumors in birds and rodents (for review see Bishop 1987). Analysis of the genes encoded in the retroviruses revealed the virus contained genes that had normal cellular counterparts termed proto-oncogenes, with oncogenes being the activated form of a proto-oncogene. The viral genes differ from the cellular genes by being in an activated form, e.g., the viral genes often lack the regulatory functions found in the cellular genes. This is the result of mutation or deletion of critical residues in the proto-oncogene. When activation of proto-oncogenes induces transformation, it does so through a direct approach, i.e., the oncogene constitutively activates the growth stimulatory pathway. The consequence of this activation is that the requirement for exogenous factors to stimulate the pathway is removed. This activation is a dominant event since mutation of one allele exerts a dominant effect over the wild type allele.

The most intensely studied family of proto-oncogenes is the *ras* family (for review, see Gomez et al., 1998). The family consists of three genes, H-*ras*, K-*ras* and N-*ras* that are activated by a wide variety of extracellular stimuli that bind

to membrane receptors (Khosravi and Der, 1994). The activity of ras proteins is defined by the phosphorylation status of the bound guanine nucleotide accessory factor (Bourne et al., 1990). When bound to GTP, ras is in its active form. The conversion of GTP to GDP results in the inactivation of ras, and the GDP is subsequently released. This regulation of ras activity is accomplished through the action of two classes of regulatory proteins (for review see Boguski and McCormick, 1993). First a GTPase activating protein stimulates the inherent GTPase activity of ras, resulting in the conversion of the active ras-GTP complex to the inactive ras-GDP complex (Bourne et al., 1990). Second, guanine nucleotide exchange factors function to promote the dissociation of GDP from the inactive complex, thereby enabling ras to be reactivated by binding to GTP (Feig 1993). The most frequent types of *ras* mutations seen in human tumors are alterations in codons 12,13 and 61 to disrupt this regulatory cycle of *ras*. Typical of an oncogene, *ras* mutations act dominantly meaning only one allele needs to be mutated to achieve constitutive activation of the pathway.

In addition to requiring GTP for activation, the cellular localization of ras is also important for its function. Since the common activating signal for ras is stimulation of a membrane receptor, ras needs to be located at the plasma membrane to be activated. The carboxy-terminus of ras is crucial to its targeting to the membrane (Willumsen et al., 1984) and post-translational modifications at the C-terminus are responsible for positioning ras at the plasma membrane (Casey et al., 1989).

To determine the downstream targets of ras, Moodie et al. (1993) anchored an activated ras to an affinity column and showed the activated ras specifically bound the serine/threonine kinase raf-1. Their report further showed that the ras-raf-1 complex is capable of activating mitogen-activated kinase (MAPK). Among the targets of the MAPKs are transcription factors that activate genes involved in differentiation, apoptosis and proliferation. Therefore, constitutive activation of ras leads to uncontrolled transcription of a variety of genes.

To define specific physiological consequences of activated ras, Mulcahy et al. (1985) showed that inactivation of ras by microinjection of an antibody prevented the cells from entering S phase in response to serum stimulation. In addition, they showed this abrogation of ras function results in a dramatic reduction of DNA synthesis in the human cell line MRC-5, suggesting that ras activity is required for the proliferation of both human and rodent fibroblasts. Furthermore, their results indicate that ras is a required mediator for the multiple growth factors present in serum. In a related study, Dobrowolski et al. (1994) demonstrated that ras activity is required at multiple points in the G0/G1 phase of the cell cycle for BALB/c 3T3 cells following their release from quiescence. Feig and Cooper (1988) showed that H-ras, mutated at position 17, functions as a dominant negative mutant and inhibits the growth of NIH 3T3 cells, supporting the studies described above.

The report by Lovec et al. (1994) indicates that overexpression of cyclin D1 cooperates with an activated H-*ras* to transform primary rat embryo fibroblasts. Cyclin D1 is important in the regulation of the G1/S transition of the cell cycle (for

a review, see Sherr, 1996). This cooperation is significant since Albanese et al. (1995) showed the level of cyclin D1 is dependent on the status of *ras*. They showed transforming mutants of ras induced the cyclin D1 promoter in a variety of cell lines, including human cells. These reports establish a link between ras and cell cycle control.

In addition to examining the role of ras in cellular proliferation detailed above, use of dominant negative ras mutants has revealed a role for ras in neuronal differentiation. Szeberenyi et al. (1990) showed the dominant negative version of ras blocks the nerve growth factor induced differentiation of PC12 cells. In a related study, Ferrari et al. (1994) showed that inhibition of ras activity by a dominant negative mutant would not only inhibit proliferation of PC12 cells, but also block apoptosis caused by withdrawal of nerve growth factor.

The above illustrates the many aspects of the cellular machinery that the ras family of proteins touches upon and serves as a typical example of a proto-oncogene. From all the data that has been accumulated on *ras* it is obvious how unregulated activity of the *ras* family, and proto-oncogenes in general, could lead to cellular transformation.

B. Tumor suppressor genes

The other major category of genes critical to carcinogenesis is the tumor suppressor genes. The presence of tumor suppressor genes was first suspected after fusion of malignant cells with normal cells suppressed the tumorigenic phenotype, suggesting that loss of gene function is involved in the formation of a tumor. The first tumor suppressor gene isolated was the retinoblastoma gene

(*Rb*) (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). Tumor suppressor genes are often involved in negative regulation of cellular proliferation. In contrast to proto-oncogene activation, both copies of a tumor suppressor must be mutated to inactivate the gene, since the remaining wild type allele may compensate for the mutation of one allele. The mutation of both alleles of a tumor suppressor gene results in a loss of function of the gene product for the mutated cell, compared to a gain of function seen upon activation of a proto-oncogene. Another characteristic of this class of genes is that germline mutations of tumor suppressor genes are often associated with a familial cancer syndrome, e.g., the *p53* gene and Li-Fraumeni Syndrome, *Rb* and familial retinoblastoma. Tumor suppressor genes can be further characterized based on their interactions with the regulatory mechanisms of proliferation (Kinzler and Vogelstein, 1998)

1. Caretaker genes

Genes that fit into this category play an indirect role in tumor suppression, i.e., the gene product does not actively participate in the regulation of cell growth. These include genes involved in DNA repair such as those involved in mismatch repair and nucleotide excision repair. As mentioned above, mutation of a DNA repair gene does not directly result in a growth advantage, however, a loss in DNA repair ability affects a cells ability to maintain a normal phenotype and greatly increases the chances of mutation. The hallmark for a caretaker gene is that it is not absolutely required for neoplasia, and restoration of a defective caretaker gene does not affect growth properties.

2. Gatekeeper genes

The products of gatekeeper genes are directly involved in tumor suppression through control of cell growth. Unlike the caretaker genes described above, restoration of gatekeeper function reverses the neoplastic phenotype. Examples of gatekeeper genes are the *p53* gene, which will be discussed in greater detail below, and the *Rb* gene. In its hypophosphorylated state, the *Rb* gene product plays a direct role in cell growth by binding the E2F transcription factor (Dyson 1998), preventing it from transcribing genes necessary to promote the cell cycle. Once phosphorylated, the Rb protein releases E2F, which activates the transcription of various genes initiating proliferation (for a review, see Johnson and Schneider-Broussard, 1998). Loss of functional Rb leads to unregulated E2F-mediated transcription and loss of cell cycle control.

3. Landscaper genes

This is the most recently defined of the tumor suppressor categories. Genes in this category are involved in regulating the microenvironment surrounding neoplastic growth. In studying patients with juvenile polyposis syndromes, Howe et al. (1998) found that mutation of *SMAD4/DPC4* is involved in the formation of hamartomatous polyps in the colon that arise from stromal cells. These polyps are at a low risk for tumorigenic transformation. However, due to the altered stromal environment, the epithelial cells in the vicinity of the polyps are at a elevated risk for tumorigenic transformation.

III. p53

A. Background

The p53 gene is the currently most intensely studied gene in cancer research. A recent search of the database in the National Library of Congress revealed over 12,000 citations for a gene that was discovered less than 25 years ago (Lane and Crawford, 1979; Linzer and Levine, 1979). Early characterization of *p53* by Sarnow et al. (1982) indicated the p53 protein can associate with viral proteins in murine cells, and Crawford et al. (1981) showed that *p53* was overexpressed in 12 out of 13 human tumor lines tested. Furthermore, the study by Eliyahu et al. (1984) demonstrated *p53* cooperated with an activated Ha-*ras* in the immortalization and transformation of rat embryo fibroblasts. Based on these characteristics, *p53* was originally categorized as an oncogene.

This classification of *p53* began to be questioned when Mowat et al. (1985) found *p53* to be inactivated in several viral-induced tumor lines, and Masuda et al. (1987) demonstrated *p53* was inactivated in primary osteogenic sarcomas as the result of genomic rearrangement. In addition, Finlay et al. (1989) showed that wild type *p53* suppressed transformation of rat embryo fibroblasts, and Baker et al. (1990) showed the wild type function of *p53* was required to suppress growth of colorectal carcinoma cells in culture. These results were not typical of an oncogene. The paradox was solved when Hinds et al. (1989) demonstrated that in the initial studies that classified *p53* as an oncogene, the cDNA employed encoded a mutated version of *p53*. This led to *p53* being reclassified as a tumor suppressor gene. Further analysis of the properties of the

p53 gene, some of which will be discussed below, revealed that it belongs to the gatekeeper category of tumor suppressor genes referred to earlier.

The *p53* gene is located on the p arm of chromosome 17, at 17p31.1 (McBride et al., 1986) and is roughly 20 kb in size (Lamb and Crawford, 1986). The p53 transcript is approximately 1.3 kb in size and contains 11 exons, with the first exon being untranslated. The p53 gene product has been found to have a variety of functions. I will limit my discussion to what are considered major functions of *p53* with regard to carcinogenesis.

B. Domains of p53

The p53 protein can be divided into four domains: 1) the transcriptional activation domain (residues 1-42) 2) the sequence-specific DNA binding domain (102-292) 3) the oligomerization domain (324-355) and 4) regulatory domain (367-393).

1. Transcriptional activation domain (residues 1-43)

The first 43 residues at the amino terminus comprise the transcriptional activation domain. It contains a stretch of ten amino acids (13-23) that are highly conserved among various species (Levine 1997). Residues 19, 22 and 23 are essential for the transactivating ability of *p53* (Lin et al., 1994). These same residues are now known to be involved in the binding of the *p53* activation domain to the TATA-associated factors $TAF_{II}40$ and $TAF_{II}60$ of *Drosophila* (Thut et al., 1995). In addition, these residues are also involved in the binding of p53 to either MDM2 or to the E1B-55 kDa proteins that negatively regulate the transcriptional activity of *p53* (Lin et al., 1994). The amino terminus has also

been shown to interact with the TATA box-binding protein (Horikoshi et al., 1995). Furthermore, this domain has been shown to interact with the single stranded DNA-binding protein RPA, which functions in DNA replication and repair (Dutta et al., 1993; He et al., 1993).

2. DNA binding domain (residues 102-292)

The central core of p53 contains four of the five highly conserved regions of p53 (Soussi et al., 1990), in addition to the sequence-specific binding domain of p53 (Bargonetti et al., 1993; Wang et al., 1993a, 1995b). Wang et al. (1995b) showed that purified core domains of p53 bind their recognition sequences as four monomers. Furthermore, Balagurumoorthy et al. (1995) showed the purified core domains of p53 can cooperate with each other in binding DNA and that the bound p53 is capable of bending the DNA. This ability to alter the conformation of DNA would be essential in the transcription of p53-responsive genes that contain p53 binding sequences separated in the genome (Zauberman et al., 1995).

Cho et al. (1994) were the first to obtain information on the structure of the core domain. From their crystallization data, it was shown that the four conserved regions contained within the central domain are involved in contacting the DNA. These domains are arranged in two α-helical loops that interact with the DNA. The remaining portion of the core domain is arranged mainly in β-sheets that serve in a structural capacity to maintain the proper conformation for binding. The DNA binding domain is where the vast majority of p53 missense mutations are found (Hainaut et al., 1997) and the most frequently mutated

residues in this domain are those involved in DNA binding. In an interesting study, Halazonetis et al. (1993) showed that upon binding DNA, not only does p53 alter the conformation of the DNA, but the protein itself undergoes a conformational change, enabling antibodies raised against mutant p53 to recognize wild type p53 bound to DNA.

3. Oligomerization domain (residues 324-355)

This domain is involved in forming p53 tetramers and is connected to the central domain by a flexible linker (Jeffrey et al., 1995). Shaulian et al. (1992) showed fusion proteins that contain this domain are able to bind and inactivate endogenous p53. This reflects a dominant negative mechanism for p53-dependent transformation, i.e., the protein transcribed from a single mutant p53 allele can exert a dominant effect over protein transcribed from a wild type allele. Pietenpol et al. (1994) showed oligomerization of p53 to be essential for transcriptional activation, which is required for p53-mediated tumor suppression. In contrast, the report by Shaulian et al. (1993) showed deletion of the oligomerization domain does not eliminate the ability of p53 to suppress transformation. Suprisingly they showed truncated p53 is able to activate expression of a p53-responsive gene, indicating that oligomerzation is not necessary for p53 transactivation.

4. Regulatory domain (residues 367-393)

The 26 residues at the C-terminus are capable of binding single stranded regions of DNA created by DNA mismatches, implying a role for p53 in DNA mismatch repair (Lee et al., 1995). This domain also serves to catalyze the

reannealing of single-stranded DNA or RNA to double-stranded DNA (Bakalkin et al., 1994). Various alterations of this domain such as phosphorylation, deletion or antibody binding result in the activation of the DNA binding properties of the central domain (Hupp and Lane, 1994). Jayarman and Prives (1995) have shown that this domain is capable of interacting with short stretches of DNA (20-39 nucleotides) which results in activation of the core domain, whereas longer pieces of double stranded DNA inhibit DNA binding.

C. Transcriptional targets

As stated above the most studied function of p53 is its ability to activate transcription of various genes. The ability of p53 to suppress tumor formation has been found to be dependent on its transactivating ability (Crook et al., 1994; Pietenpol et al., 1994). To function as a transcription factor, p53 binds DNA as a tetramer in a sequence specific manner. The conserved recognition sequence of p53 is two copies of the 10-mer (5' – RRRCA/TT/AGYYY – 3') arranged in an inverted repeat. Although there are many genes that are transcribed in a p53-dependent manner I will only focus on two of the most relevant target genes.

1. *p21/WAF1/Cip1*

Perhaps the most important of the p53 target genes, at least in relationship to cell cycle control, is the p21 gene (el-Diery et al., 1993; Harper et al., 1993). *p21* is located on chromosome 6p21.2 and has been shown to negatively affect the growth of human tumor cells in culture (el-Diery et al., 1993). The p21 protein binds to cyclin dependent kinases, particularly those acting in the G1 phase of the cell cycle (Harper et al., 1993; Xiong et al., 1993), inhibiting their ability to

phosphorylate the Rb protein (Harper et al., 1993). As stated earlier, Rb in a hypophosphorylated state binds and inhibits the E2F transcription factor that is required for cell cycle progression, thereby arresting the cells in the G1 phase of the cell cycle. p21 has also been shown to bind proliferating cell nuclear antigen (PCNA) (Waga et al.,1994), and the data indicate that this binding interferes with the role of PCNA in proliferation. In addition, Polyak et al. (1996) showed that in colorectal cell lines, *p21* might play a role in protecting cells from apoptosis.

Despite the strong association between *p53* and *p21*, there are *p53*-independent mechanisms of *p21* expression. For example, Micheli et al. (1994) showed that various growth factors could stimulate expression of *p21* in *p53* null mouse fibroblasts. The study by Macleod et al. (1995) indicated that *p21* is expressed during the differentiation of mouse erythroleukemia cells in *p53* null mice. However, their results also showed that p53 is required for induction of *p21* following DNA damage.

2. *MDM2*

Another transcriptional target of *p53* is the *MDM2* gene (Barak et al., 1993; Perry et al., 1993). The MDM2 protein binds the N-terminal activation domain of p53 and inhibits the ability of p53 to activate transcription (Momand et al., 1992; Oliner et al., 1993). This interaction involves residues 13-29 of p53 being bound in a hydrophobic pocket of mdm2 (Kussie et al., 1996). The interaction of MDM2 and p53 represents an autoregulatory feedback loop. The significance of this interaction and its consequences becomes apparent when one learns that *MDM2*

is overexpressed in 30-40% of human sarcomas (Oliner et al., 1992). Such sarcomas would be functionally lacking p53.

In addition to its ability to inhibit the transcriptional activation of genes by p53, MDM2 can also interfere with the ability of p53 to suppress activation of promoters containing TATA boxes (Chen et al., 1995). Two groups have recently shown that p53 is rapidly degraded in a proteosome-dependent manner after being bound by MDM2 (Haupt et al., 1997; Kubbutat et al., 1997). Lundgren et al. (1997) recently showed that overexpression of MDM2 induced tumors in both p53 wild type and p53 null mice.

D. Activation of p53

In most cells, the level of p53 is extremely low, but it can be induced to a high level under certain conditions. Interestingly, this induction is not the result of increased transcription but reflects post-translational stabilization (Kastan et al., 1991). Midgley et al. (1995) exposed mice to ionizing radiation and found the level of *p53* induction varied in a tissue-dependent manner. The study by Nelson and Kastan (1994) demonstrated that DNA strand breaks resulting from ionizing radiation or chemical treatment induces accumulation of p53 in human cells, but that DNA lesions themselves do not induce *p53*. In addition, Zhan et al. (1993) found that serum deprivation also induces *p53* activity in human cells. Radiolabeling of human or murine cells also results in increased *p53* activity (Yeargin and Haas, 1995). In addition, Renzing and Lane (1995) showed that calcium phosphate-mediated transfection of mammalian cells elevates *p53* activity. Therefore it appears that induction of p53 occurs under a wide variety

conditions of cellular stress. In an intriguing study, Jayaraman et al. (1995) found that short pieces of single-stranded DNA can stimulate the DNA-binding properties of p53, an indication of a possible connection between p53 and DNA repair.

E. p53 and apoptosis

Lotem and Sachs (1993) showed bone marrow myeloid progenitor cells from *p53* null mice fail to undergo apoptosis in response to radiation or heat shock. The study by Caelles et al. (1994) used cells that contained temperature-sensitive version of *p53* and found apoptosis to occur in a p53-dependent manner in the presence of transcriptional and translational inhibitors, indicating that p53-dependent apoptosis is not the result of p53-mediated transcription of genes involved in apoptosis. In addition, Merritt et al. (1994) found no apoptosis to occur in the stem cells of the small intestine of *p53* null mice following full body irradiation. Their study further showed that the level of spontaneous apoptosis is not affected by *p53* status. Zhu et al. (1994) used antisense techniques to show that loss of *p53* function results in a loss of apoptosis in acute myeloblastic leukemia blasts. Furthermore two genes involved in apoptosis, *bax* and *bcl-2* (White 1996) are controlled by p53 transactivating ability (Miyashita and Reed, 1995; Miyashita et al., 1994). Sabbatini et al. (1995) used a temperature-sensitive p53 mutant to show that transcriptionally functional p53 is required to induce apoptosis in baby rat kidney cells. However, Haupt et al. (1995a) showed transcriptionally inactive p53 still induced apoptosis in HeLa cells. In an interesting study, Haupt et al. (1995b) showed that *p53*-mediated apoptosis in

HeLa cells is overcome by the overexpression of Rb protein, implying that Rb-related proteins may be involved in deciding whether a cell undergoes apoptosis once *p53* halts the cell cycle. The study by Yonish-Rouach et al. (1993) used myeloid leukemia cells transfected with a temperature-sensitive p53 mutant to show cells in the G1 phase of the cell cycle are more susceptible to apoptosis, indicating a relationship between cell cycle and apoptosis. Based on these reports, it is likely that *p53* has both transcription-dependent and transcription-independent roles in apoptosis.

F. p53 and DNA repair

It is well established that p53-dependent transcription of *p21* is required in preventing cells containing damaged DNA from replicating, but whether *p53* has any direct role in repair is an area of some debate. p53 binds and inhibits the single-stranded DNA binding protein RP-A (Dutta et al., 1993) which has well-defined roles in replication and repair. This provides another route by which *p53* can stop cells with damaged DNA from cycling. However, other transcription factors such as *VP16* and *GAL4* can also bind RPA (He et al., 1993), and so this interaction may be a general characteristic of transcription factors, and not *p53* in particular. p53 can also bind various components of the TFIIH protein complex, as well as the strand-specific repair protein CSB (Wang et al., 1995a). In addition, p53 inhibits the helicase activity of the XPB and XPD components of TFIIH, indicating that p53 can have a negative effect on DNA repair (Wang et al., 1995a). In addition, the C-terminal domain of p53 binds DNA containing small insertion/deletion mismatches (Lee et al., 1995). Furthermore, Reed et al. (1995)

showed the C-terminal domain binds DNA that contains strand breaks which may provide a signal for repair machinery. Lack of *p53* affects global DNA repair in human cells. However, the repair of active genes is normal for these cells (Ford and Hanawalt, 1995). In addition to a direct role for *p53* in DNA repair, Waga et al. (1994) showed that the p53-dependent gene *p21* (see above) inhibits the role of PCNA in DNA replication *in vitro*. However, Li et al. (1994b) showed p21 does not affect the role of PCNA in DNA repair.

G. Relevance of p53 to carcinogenesis

As stated above, *p53* is the most commonly mutated gene in human cancer, with over 50% of all human tumors studied to date exhibiting *p53* mutations. From the functional aspects of *p53* illustrated above, it is obvious that loss of *p53* could easily lend itself to carcinogenesis.

1. *p53* knockout mice

Donehower et al. (1992) used homologous recombination to engineer mice that lacked *p53*. The mice develop normally, indicating that *p53* is dispensable for development. Mice that were null for *p53* develop tumors, mainly lymphomas and sarcomas, by 6 months of age. Some of the null mice exhibit multiple primary tumors of different origins, indicating *p53* plays a general role in tumorigenesis in these animals. The parental heterozygous mice develop tumors after a longer latency. However, the fact that the mice develop normally called into question the fact that cells with mutant *p53* are genomically unstable (Smith and Fornace, 1995). One possible explanation is that p53 is not required during development, which is supported by the fact that the null mice develop normally.

Another possibility is the fact that mouse cells do not express any protein represents a situation different from cells that express a mutant form of p53 since mutant forms of p53 exhibit properties distinct from the wild type form. Bouffler et al. (1995) showed that cells derived from the bone marrow of these mice exhibit elevated spontaneous chromosomal abnormalities. However, these cells showed that loss of *p53* did not have an effect on ionizing radiation-induced chromosomal abnormalities, again raising questions about the role of *p53* in genomic stability. Harvey et al. (1993) characterized cells from these mice and found the *p53*-null cells grow faster, require less growth factors, and achieve a higher density than either the heterozygous or wild type cells. They further showed that the p53-null cells are genetically unstable during culturing. An unexpected result was that although the heterozygous cells lost their wild type allele early in passaging, they failed to achieve an infinite life span common for the *p53*-null cells. Donehower et al. (1995) showed that when transgenic mice predisposed for mammary tumors were crossed with *p53*-null mice, the *p53*-null progeny exhibit mammary tumors at an earlier age. In addition, it was shown that approximately half of the tumors from the *p53*-heterozygous progeny exhibit loss of the wild type *p53* allele, indicating a selection against wild type *p53* in mouse mammary tumors. Kemp (1995) showed that loss of *p53* in these mice does not affect the incidence of diethylnitrosamine-induced hepotocellular carcinomas or adenomas. These mice are an invaluable tool to examine the effects of mutagens in relationship to the p53 status.

2. Li-Fraumeni syndrome (LFS)

As stated above, germline mutations of p53 are common among families diagnosed with LFS. Li and Fraumeni (1969a,b) studied numerous medical records and death certificates and found five families that exhibited a higher than normal incidence of cancer. Each family showed siblings or cousins that had childhood sarcomas, and the ancestry of one parent showed a high incidence of diverse types of cancer. In a similar situation, Lavigueur et al. (1989) showed that mice that overexpress a mutant *p53* exhibit many of the same tumors seen in LFS patients. Analysis by Malkin et al. (1990) of DNA from these LFS families revealed that all the families show the presence of a germline heterozygous mutation in *p53*. When tumors from these individuals were examined, they were found to have lost the wild type allele (Malkin et al., 1990). A recent study (Santibanez-Koref et al., 1992) showed that not all LFS families carry germline mutations. The report by Barnes et al. (1992) documented a family whose cells, instead of carrying a heterozygous mutation in *p53*, overexpress wild type *p53*. It is possible that families that exhibit wild type *p53* could have their p53 function abrogated by some other mechanism such as *MDM2* overexpression or promoter silencing. It is also possible there is some other unknown gene that is critical in LFS.

In an effort to study LFS in a laboratory setting Srivastava et al. (1992) determined expression levels of the mutant and wild type p53 alleles in LFS skin fibroblast and showed that both alleles are equally expressed. In an *in vitro* translation assay, some LFS mutations exhibit a dominant negative effect over

wild type *p53* (Srivastava et al., 1993) although this was not always the case (Felix et al., 1993; Horio et al., 1994).

IV. DNA Repair Systems

As stated above, mutations in critical genes are essential in the tumorigenic transformation of human cells and mutations are the result of the replication of a damaged DNA template. However, cells contain various DNA repair mechanisms to remove damage prior to replication. I will review two of the major repair pathways in this section, and a third in the next section.

A. Nucleotide excision repair (NER)

Human NER recognizes many different types of damage, from UV-inducted photoproducts to chemical adducts (Huang et al., 1994). NER also recognizes mismatched base pairs, although NER does not have the capability of distinguishing the newly replicated strand as does the mismatch repair system (Modrich 1994)(see below). Mu et al. (1994) showed that NER of a bulky adduct in HeLa cell extracts is affected by sequence context. In an *in vitro* study of human NER, Huang and Sancar (1994) showed that NER components are capable of repairing damage on a 100 bp template, indicating that the proteins involved in NER form a tight complex.

1. Components of NER.

Most of what is known about the components of human NER comes from the study of the familial cancer-prone syndrome xeroderma pigmentosum (XP). These individuals exhibit a significantly enhanced susceptibility to sunlight-

induced skin cancers, and cells derived from these individuals are defective in NER. Complementation assays with rodent cells revealed seven complementation groups designated XPA through XPG. There is also a variant group of XP and cells from patients in this group exhibit normal NER (Tung et al., 1996) but are error-prone in the replication of unrepaired UV lesions (Wang et al., 1991; Wang et al., 1993b; McGregor et al., 1998). Cells from individuals within a specific XP group were found to be defective in a particular aspect of NER. The genes found to be involved with these defects are named for the particular XP group (XPA-XPG) from which the cells were derived. In the discussion below, I will review the major components of human NER and also discuss the cellular consequences of defects in these components.

a. *XPA – RPA*

The XPA protein was shown by Asahina et al. (1994) to exhibit strong binding properties towards DNA damaged by either radiation or chemical treatment. He et al. (1995) showed it associates with human replication protein, RPA, and this association increased the specificity of XPA for damaged DNA. RPA is a trimer of proteins that exhibit single stranded DNA binding properties and play a central role in DNA replication (Henricksen et al., 1994) as well as in the damage recognition step of NER (Coverley et al., 1991; He et al., 1995). Using highly purified proteins to reconstitute NER *in vitro*, Mu et al. (1995) showed RPA is required for the incision stage of NER. In addition, Li et al. (1994a) used a yeast two-hybrid system to show that XPA is capable of interacting with ERCC1. Park and Sancar (1994) presented data that indicated XPA forms a complex with both

ERCC1 and XPF. In a separate study, Park et al. (1995) showed XPA specifically binds the general transcription factor TFIIH, which is also a component of NER (see below). Based on these interactions with other components of the NER pathway, it appears that XPA is the central protein for NER. Defects in *XPA* would render a cell unable to recognize damaged DNA and unable to initiate NER.

b. TFIIH

TFIIH is a basal transcription factor for RNA polymerase II (Drapkin and Reinberg 1994) and is composed of multiple subunits. Further characterization of TFIIH has reveals that it has functions other than its role in transcription (for review, see Svejstrup et al., 1996). The TFIIH complex exhibits helicase activity, suggesting TFIIH plays a role in NER by opening the DNA (Schaeffer et al., 1993). This helicase activity is attributed to two subunits of TFIIH, the XPD and XPB proteins, which exhibit helicase activities (Wang et al., 1996). In an *in vitro* assay, Drapkin et al. (1994) showed that TFIIH is essential for NER. Cells from XPB patients exhibit reduced basal transcription levels in addition to defective NER (Hwang et al., 1996) and cells from XPD patients were found to exhibit a similar phenotype (Boulikas, 1996).

c. *XPC*

van der Spek et al. (1994) showed the XPC protein copurifies with the HHR23B protein and Sugasawa et al. (1996) showed HHR23B enhances the ability of XPC to function *in vitro*. In addition, the study by Reardon et al. (1996) revealed that XPC tightly binds to DNA and further showed that XPC does not

require HHR23B for activity *in vitro*. Furthermore, the study by Drapkin et al. (1994) showed an association between XPC and TFIIH. Despite being associated with other NER factors XPC is not required for NER of a thymine dimer *in vitro* (Mu and Sancar, 1997). In a separate study Mu et al. (1996) showed NER of a cholesterol adduct *in vitro* is also independent of the presence of XPC. While its presence may be dispensable for *in vitro* NER, Bohr (1987) showed XPC cells are deficient in repairing transcriptionally silent regions of the genome. In a recent study, Baxter and Smerdon (1998) showed XPC cells are unable to unfold the chromatin surrounding DNA damage, indicating this protein functions as an accessibility factor *in vivo*.

d. *ERCC1-XPF*

XPF, a 120kDa protein, forms a stable heterodimer with the 33 kDa protein ERCCI, which as stated earlier interacts with the XPA protein (Li et al., 1994a). This heterodimer exhibits endonuclease activities with specificity for single-stranded DNA near the border with duplex DNA. Sijbers et al. (1996) showed this complex is involved in the making the incision 5' to the distortion caused by the DNA damage, and the complex does not require ATP for activity. In a related study, Bessho et al. (1997) showed that the presence of RPA stimulates the endonuclease activity of the XPF-ERCC1 complex. Matsumara et al. (1998) showed XPF cells show an increased sensitivity to UV damage, but are not completely deficient in NER, since they show a slow rate of NER most likely the result of residual endonuclease activity.

e. *XPG*

The XPG protein is a 133 kDa protein that has a loose association with TFIIH and RPA (He et al., 1995). In addition, XPG exhibits single-stranded DNA endonuclease activity (Habraken et al., 1994; O'Donovan et al., 1994a). As was seen with the XPF-ERCC1 complex, XPG was shown by Sijbers et al. (1996) not to require ATP for activity, and O'Donovan et al. (1994b) further showed XPG is responsible for making the incision 3' to the distortion caused by the DNA damage. Cells lacking *XPG* would be expected to exhibit a phenotype similar to XPF cells, however the XPG cells are more sensitive to DNA damage. A possible explanation for the enhanced sensitivity comes from the recent study by Klungland and Lindahl (1997) which showed XPG plays a role in base excision repair as well as NER. Therefore, elimination of *XPG* affects two DNA repair pathways.

2. Mechanism of NER

a. Recognition of damage

The damage is initially recognized by the XPA-RPA complex that binds to the DNA. The bound complex recruits TFIIH and the ATP-dependent helicase activity of the XPB and XPD components of TFIIH unwinds the DNA, forming the preincision complex. Evans et al. (1997) showed that the formation of the preincision complex requires both XPA and ATP. In addition, they showed the preincision complex comprises approximately 25 nucleotides being unwound around the DNA damage.

b. Removal of damage

TFIIH recruits the XPG protein that then makes the incision 3' to the damage while the ERCC1-XPF complex is recruited by the XPA protein to make the incision 5' to the damage (Matsunaga et al., 1995). Moggs et al. (1996) inhibited DNA synthesis in an *in vitro* NER system and showed that the incisions occur primarily at the 9^{th} phosphodiester bond 3' to the damage and to the 16^{th} phosphodiester bond 5' to the damage. This pattern of removal is the same in yeast (Budd and Campbell 1995) and *Xenopus laevis* (Svoboda et al., 1993), implying that the pattern is universal for eukaryotes.

c. Repair synthesis

After removing the damage, a DNA polymerase uses the remaining strand as a template to fill in the gap. This is considered to be error-free since DNA polymerases exhibit a high fidelity rate and there are less than 30 nucleotides to replace. Two separate groups have shown that PCNA is required for the synthesis stage of NER (Nichols and Sancar, 1992; Shivji et al., 1992). This would indicate a role for polymerase δ or ε in a repair synthesis since both associate with PCNA (Shivji et al., 1992). Budd and Campbell (1995) used mutagenesis in yeast to show that either polymerase δ or ε could function as the polymerase in the DNA synthesis stage. The study of Zeng et al. (1994) showed that the addition of antibodies specific to polymerase δ inhibits NER in HeLa extracts, indicating polymerase δ is involved in human NER.

3. Transcription-coupled repair

A unique feature of NER is what is termed transcription-coupled repair (TCR). It was found that those genes expressed at high levels are preferentially repaired compared to areas of DNA not transcribed, indicating a correlation between transcription and repair (for review, see Hanawalt 1994). The exact mechanism for TCR in humans is not understood, however Selby and Sancar (1993) showed that the *mfd* gene is required for TCR in bacteria. Characterization of the mfd protein revealed that it is capable of dislodging RNA polymerase stalled at a DNA lesion, and subsequently stimulate repair of the lesion. Cells from patients suffering from Cockaynes Syndrome are defective in transcription coupled repair (Venema et al., 1990), and therefore investigators are studying the Cockaynes Syndrome genes *CSA* (Henning et al., 1995) and *CSB* (Troelstra et al., 1992) to see if they play a role in human TCR similar to the bacterial *mfd* gene.

B. Mismatch repair

Mismatch repair (MMR), as the name implies, is involved in the repair of mispaired bases and also insertion/deletion mismatches. These mispairs may be the result of polymerase errors, or they may be found in the heteroduplex intermediate formed during recombination. Most of what is known about MMR comes from bacterial studies, but the information about human MMR is accumulating rapidly.

1. Bacterial MMR

Since the errors generated during replication involve the incorporation of incorrect nucleotides, effective MMR must be able to distinguish newly replicated

DNA. In bacteria, DNA is specifically methylated at d(GATC) sequences. Since newly replicated DNA has not had time to be methylated at these sequences, and this provides bacterial MMR with the ability to distinguish newly synthesis DNA (Modrich and Lahue 1996). Repair is initiated by the binding of a MutS homodimer to the mispaired DNA in an ATP-dependent reaction, forming a heteroduplex loop in the DNA (Allen et al., 1997). The MutL protein is then recruited to the opened DNA containing MutS (Grilley et al., 1989). Drotschmann et al. (1998) showed that MutL enhances the binding of MutS to the mismatch and the study by Yamaguchi et al. (1998) demonstrated that MutL enhances the activity of MutU, a DNA helicase. The MutH protein then binds and incises the unmethylated strand 5' to the d(GATC) sequence (Au et al., 1992). Using electron microscopy, Grilley et al. (1993) showed that the incision occurs on the portion of unmethylated strand at a d(GATC) site that is closest to the mismatch. The excision of the newly synthesized strand proceeds in a bidirectional manner (Grilley et al., 1993) and is terminated within 100 bp on either side of the mismatch. It had been suggested that exonuclease I and exonuclease VII are involved in the excision step (Grilley et al., 1993). However, the recent findings of Viswanathan and Lovett (1998) showed that mutation of these enzymes had no effect on bacterial MMR. Lahue et al. (1989) showed DNA polymerase III functions to fill in the excised DNA.

2. Human MMR

a. Background

Human MMR recognizes all eight base pair mismatches as well as small insertions and deletions (Thomas et al., 1991). The repair of these defects is affected by sequence context, with purine: purine and purine: pyrimidine mispairs being the best substrates and C:C being the worst substrate (Holmes et al., 1990). In addition, human MMR effectively repairs insertions/deletions up to 5 nucleotides in size (Umar et al., 1994a)

b. Components

One of the human homologs of the bacterial Mut S protein is the hMSH2 protein. This protein forms a heterodimer with hMSH6, also known as the G/T binding protein (Palombo et al., 1995), to form the Mut Sα complex (Drummond et al., 1995). This complex recognizes mismatches as well as one base insertions (Drummond et al., 1995). The study by Drummond et al. (1995) also showed that cells deficient for MutSα are resistant to the cytotoxic effects of methylating agents. These results indicate that MMR is involved in the cytotoxic effect of methylating agents. HMSH2 also dimerizes with the hMSH3 protein to form the MutSβ complex (Acharya et al., 1996; Palombo et al., 1996). This complex binds to insertions/deletions, but shows little affinity for mismatches (Palombo et al., 1996). Therefore, hMSH2 is the general damage recognition protein and hMSH3 and hMSH6 provide the specificity.

Li and Modrich (1995) used a complementation assay to isolate the human homolog of *MutL* from HeLa cells. There are three human MutL homologs,

MLH1, *PMS1*, and *PMS2* (Bronner et al., 1994; Nicolaides et al., 1994). Human MLH1 forms a heterodimer with PMS2 to generate the MutLα complex. Cells unable to form the MutLα complex are deficient in the repair of mismatched DNA, in addition to exhibiting instability of nucleotide repeats (Parsons et al., 1993; Risinger et al., 1995). Further analysis of these cells revealed that the defect blocked MMR prior to the incision step, indicating the MutLα complex plays an early role in MMR, most likely in a role similar to the MutL protein in bacteria, i.e., to recruit additional components of the MMR machinery (see above).

c. Mechanism

The entire mechanism of human MMR is not known, however certain aspects of the process have been elucidated. As stated earlier, the MutSα and MutSβ complexes are involved in the recognition of mismatched DNA. In addition, the study by Fang and Modrich (1993) showed that the excision of mismatched DNA in human MMR proceeds by a bidirectional manner, just as in bacteria. Human MMR also repairs mismatches in a strand specific manner, much the same as bacterial MMR. The exact mechanism is not yet understood. However, the presence of a strand break targets human MMR to remove the strand containing the break *in vitro* (Thomas et al., 1991; Holmes et al., 1990). However, the components involved in the excision stage of human MMR have not yet been determined. Working with yeast, Johnson et al. (1995) showed mutation of *RTH1*, a 5' to 3' exonuclease, results in the instability of simple DNA repeats, a hallmark of defective MMR (Umar et al., 1994b). In addition, Szankasi and Smith (1995) showed mutation of exonuclease 1, a 5' to 3' DNA exonuclease, also

results in yeast that displayed altered MMR. Currently investigators are looking for human homologs for these genes.

The synthesis step in human MMR was shown to be blocked by the addition of aphidicolin to HeLa extracts (Holmes et al., 1990; Thomas et al., 1991). This implied that the synthesis is performed by either polymerase α, δ and/or ε. In a pivotal study, Longley et al. (1997) showed HeLa extract depleted of polymerase δ are unable to perform the synthesis stage, indicating that polymerase δ is responsible for the synthesis stage of MMR.

V. Methylation Damage and Repair

A. Sources of DNA methylation

1. Dietary exposure

Huber and Lutz (1984) showed dietary nitrites and secondary amines can react under acidic conditions similar to those found in the stomach and form reactive nitroso compounds, including methylating agents. The main route by which humans are exposed to nitrite is through the processing of ingested nitrates, from either food or water supplies. In addition, Walters (1980) reported low levels of nitrite are found in potatoes, tomatoes and beets. Secondary amines are found in foods such as fish and shrimp (Lin and Lai, 1980) as well as tobacco smoke and flavorings (Lijinsky & Epstein, 1970). In addition, thiocyanates, found in foods such as cauliflower, can catalyze the reaction between nitrites and secondary amines (Boyland and Walker, 1974). However, dietary factors such as ascorbic acid, which is used to preserve meat, can inhibit

nitrosation in the stomach. Lin et al. (1983) showed that squid and octopus contain high levels of the secondary amines dimethylamine and methylamine. They further demonstrated that these compounds can react with nitrite to form the methylating compound N-nitrosodimethylamine and proposed this may be a factor in the increased incidence of stomach cancer seen in Japan and China where these foods are popular. Shih-Hsin et al. (1986) showed that the gastric juices of residents of Lin-Xian in China contained several N-nitrosamines, including methylating agents, and found there was a correlation between the frequency of epithelial lesions of the esophagus and the level of the nitrosamines in the diet.

2. Chemotherapeutic exposure

An additional route by which humans are exposed to methylating agents is through treatment with chemotherapeutic drugs that form the O^6-methylguanine (O^6MeG) adduct. These drugs are effective cytotoxic agents, and the O^6MeG lesion is suggested as the lesion that causes the cytotoxic effect seen. Souliotis et al. (1991) showed increased levels of O^6MeG in leukocytes taken from patients with Hodgkin's disease that were being treated with the chemotherapeutic drug dacarbazine. In a separate study Kyrtopouloss et al. (1993) showed similar results for patients treated with either dacarbazine, a drug forming O^6MeG, or the related drug procarbazine.

B. Sites of DNA methylation and their consequences

As a model for methylating agents, I will discuss methylating nitroso compounds, since these are of environmental importance (for a review see

Montesano and Bartsch 1976). There are other compounds that also induce formation of O^6MeG such as 4-(methylnitosamino)-1-(3-pyridyl)-1-butanone found in tobacco smoke (Morse et al., 1989) and chemotherapeutic drugs such as those listed above. Methylating nitroso compounds can be divided into two groups based on their mode of activation. Nitrosamides produce electrophiles during spontaneous decomposition. Nitrosamines require metabolic activation to generate the reactive species (Montesano and Bartsch, 1976). Despite the differences in their mechanism of activation, methylating nitroso compounds react with cellular macromolecules through the formation of the methyl carbonium ion. It is important to note that this ion is capable of interacting with proteins and RNA (Singer and Fraenkel-Conrat, 1969) as well as targeting nucleophilic sites of DNA. I will limit my discussion to the methylation of DNA.

The reaction of the methylcarbonium ion with nucleic acids proceeds by unimolecular nucleophilic substitution and can result in the formation of at least 12 methylated bases (Beranek 1990). The N7 position of guanine is the most nucleophilic site of DNA and is therefore the most reactive site for methylating agents comprising 65-83% of all methyl adducts (Beranek, 1990). The next most prevalent adducts are those generated by reaction at the O atoms of DNA and these account for 25% of the DNA adducts formed by methylating agents. It is interesting to note that all methylating nitroso compounds studied react at the same sites on the DNA, although differences exist in the extent of reaction at a particular site (Beranek, 1990). The biological importance of these differences was shown in the study by Loveless (1969) where the reaction of a methylating

agent at a particular site correlated closely with the compound's mutagenic or carcinogenic effects. These results implied that only certain adducts formed by methylating agents are responsible for the cellular effects seen. One possible explanation for the cellular effects resulting from methylation damage is that methylation of DNA increases the rate of depurination and depyrimidination, as well as strand scission (Margison and O'Connor, 1973; Margison et al., 1973). However, these types of damage are readily repaired, and therefore pose little threat to the cell.

Abbott and Saffhill (1977) used RNA polymerase to replicate a methylated poly (dA-dT) template. Their result showed O^4-methylthymine (O^4MeT) is miscoding with the polymerase incorporating guanine opposite each O^4MeT. Their results further showed that other methylation products such as 1, 3, and 7-methyladenine as well as phosphotriester adducts, are not miscoding. Since RNA polymerase exhibits a lower fidelity rate compared to DNA polymerases (Drake and Baltz 1976; Topal and Fresco 1976) it is possible these results reflect the lower fidelity of the RNA polymerase. However, Saffhill (1985) showed similar results using rat spleen DNA polymerase α, thereby extending the results to mammalian polymerases and establishing O^4MeT as a miscoding lesion. In addition, his results showed that O^2-methylthymine (O^2MeT) is also miscoding, but at a lower efficiency than O^4MeT. Although both of these lesions are miscoding they represent only a few percent of the total methylation products formed *in vivo* (Beranek 1990), and therefore cannot explain the mutation frequencies achieved with methylating agents.

The most widely studied adduct formed by methylating agents is the O^6MeG adduct. Gercham and Ludlum (1973) showed that RNA polymerase incorporates primarily UMP and to a lessor extent AMP across from O^6MeG when they used a copolymer of dC and O^6MeG as a template. A study by Abbott and Saffhill (1979) used *E. coli* DNA polymerase I to replicate a methylated poly (dC-dG) template and showed that O^6MeG is miscoding with thymine being incorporated across from O^6MeG. Furthermore, their results showed that the miscoding frequency of O^6MeG is dependent on nucleotide availability. In their assay, O^6MeG is miscoding 40% of the time if the dNTP pool is not limiting, but if dCTP levels are reduced, the frequency of miscoding increased. Singer et al. (1989) replicated an oligonucleotide containing O^6MeG with DNA polymerase I and showed the miscoding properties of O^6MeG are dramatically influenced by the sequence 3' to the O^6MeG. One explanation for the miscoding properties was shown in the work by Swann (1990) who used ^{31}P-NMR to show that O^6MeG closely resembles adenine in bond angles and bond lengths. He further showed that the O^6MeG:T base pair maintains a more normal Watson Crick alignment of the phosphate backbone than O^6MeG:C and therefore O^6MeG:T facilitates phosphodiester bond formation. The above studies showed O^6MeG is a miscoding lesion *in vitro*, but whether it is miscoding in cells was not addressed nor were the potential biological effects.

One of the first indications that O^6MeG may have biological significance was a report from Kleihues and Margison (1974) that showed O^6MeG is a persistent lesion in rat brain for animals treated with MNU. Domoradzki et al. (1984, 1985)

showed the ability of human cells to repair O^6MeG correlated with the cytotoxic and mutagenic effects of MNNG treatment, indicating that O^6MeG is both cytotoxic and mutagenic in human cells. These results were confirmed by Lukash et al. (1991) who showed that the complete depletion of the ability of human fibroblasts to repair O^6MeG results in an increased induction of *HPRT* mutations by MNNG treatment. Other data indicating the mutagenic potential of O^6MeG was provided by Guttenplan (1990) who showed that when *Salmonella* are treated with MNU, there is a correlation between the level of O^6MeG and the frequency of mutations observed. He further showed that there was no such correlation for the other adducts detected, indicating that O^6MeG is the principal mutagenic lesion generated by MNNG treatment.

Richardson et al. (1987) sequenced MNU-induced mutations in the xanthine guanine phosphoribosyltransferase gene of *E. coli* and found all the mutations were GC to AT transitions. The same results were reported by Sikpi et al. (1990) when a MNU treated shuttle vector replicated in human lymphoblastoid host cells. Most of the mutations obtained were GC to AT. In addition, Zhang and Jenssen (1991) analyzed MNU-induced *HPRT* mutations in Chinese hamster ovary (CHO) cells and found the majority of mutations were GC to AT and occurred in the nontranscribed strand suggesting there is a strand preference for either alkylation damage or repair. However, since the cells they used show only marginal repair (Jenssen 1982) their results may reflect only the poor repair capacity of the cells. The results from Lukash et al. (1991) also showed the majority of premutagenic lesions resulting from treatment of human cells with a

methylating agent were found in the nontranscribed strand. They further showed that the strand distribution of mutants is not affected by inactivation of repair indicating the differences seen were likely to be a reflection of a strand preference by the alkylating agent.

C. Repair of O^6MeG

1. Bacterial

When *E. coli* are exposed to low levels of MNNG for a short period of time (< 30 minutes), they show induction of mutation. However, if the treatment time extends beyond 30 minutes, the induction of mutations stops (Samson and Cairns, 1977). Furthermore, if the MNNG is removed and reapplied a short time later, the mutations return, indicating a reversible and reproducible effect. This adaptive response was not seen in bacteria that lacked the *ada* gene (Jeggo 1979; Sedgwick 1982) indicating *ada* is involved in this response. Jeggo (1979) further showed that the adaptive response is less pronounced when bacteria are treated with an ethylating agent, indicating the response is specific for a methylating agent.

Lawley and Orr (1970) were the first to report that bacteria could repair O^6MeG, but the underlying mechanism of repair was unknown. It was expected to involve enzymatic removal of the adduct based on *in vitro* studies (Lawley and Warren, 1976; Kirtikar and Goldthwait, 1974) that indicated methylated bases are excised from the DNA. However no labeled O^6MeG could be detected in the *in vitro* reactions. Olsson and Lindahl (1980) revealed a crucial aspect in O^6MeG repair when they demonstrated the labeled methyl group is bound to a cysteine

residue of a protein *in vitro*. Lindahl et al. (1982) showed this transfer involved one molecule of protein accepting a single methyl group. A study by Demple et al. (1982) indicated the protein is not regenerated after the transfer was accomplished. They also showed that the protein is specific for O^6MeG adducts, since it had no affect on N-alkylated purines. In addition, their report showed this repair protein exhibits greater activity on double-stranded DNA than single-stranded DNA. The authors proposed this as an explanation for the increased number of mutations seen at replication forks.

Further studies revealed that bacteria contain two repair proteins capable of removing the methyl group from O^6MeG, these proteins are termed alkylguanine-DNA alkyltransferases (AGT) (for a review see Pegg, 1990). The first to be characterized was the Ada protein (Nakabeppu et al., 1985). However, Rebeck et al. (1988) showed that bacteria that lacked *ada* still exhibit repair of methylation damage, indicating the presence of additional alkyltransferase proteins. Furthermore, they showed that these bacteria repair both O^6MeG and O^4MeT. Potter et al. (1987) were the first to clone and characterize this second alkyltransferase, the Ogt protein. They found it to have a fair degree of homology to the Ada protein.

The adaptive response described above is due to activation of the Ada protein that increases several hundred fold in activity in response to DNA damage. The Ogt protein does not appear to be induced (Pegg 1990). The two alkyltransferases exhibit distinct activities towards alkyl adducts of differing size. The Ada protein shows a higher affinity for methyl adducts with a marked

decrease in reactivity towards larger adducts (Pegg 1990). In contrast, the Ogt protein is capable of removing larger adducts in addition to methyl adducts (Pegg 1990). In addition, the study by Sassanfar et al. (1991) found that the Ada protein shows highest affinity for O^6MeG, while the Ogt protein is more active towards O^4MeT.

2. Mammalian

The gene for rat AGT was cloned and characterized as a 209 amino acid, 23 Kd protein by two independent groups (Potter et al., 1991; Rahden-Staron and Laval, 1991). Montesano et al. (1980, 1983) showed that rats chronically exposed to the methylating agent, DMN, show an enhanced repair capacity that is specific for O^6MeG within 10 minutes of treatment. This implies an adaptive response similar to that seen for the bacterial Ada protein (see above). However, the level of induction was much less than that seen in bacteria, only 10 fold at most (Pegg 1990). The level of AGT protein varies from tissue to tissue with liver showing the highest levels. Furthermore, it varies between the different cell types within a tissue (Pegg et al., 1985; Belinsky et al., 1988).

Tano et al. (1990) cloned the coding sequence for the human AGT and showed it to be a 22 kDa protein that has no nucleotide similarity to bacterial alkyltransferases. However, analysis revealed a 61 amino acid sequence of human AGT show a high degree of homology to the acceptor region of bacterial AGTs (Potter et al., 1987). Furthermore, the human protein exhibits over 60% homology to the rat AGT (Potter et al., 1991; Rahden-Staron and Laval, 1991). Yarosh et al. (1985) showed that human cells of a particular tissue contain more

AGT than rodent cells of the same type. Although various studies have investigated whether human AGT could be induced in a manner similar to the induction seen in bacterial and rodent AGT, the results obtained were controversial. Some laboratories were able to induce AGT while others could not find such induction (Yarosh, 1985; Laval, 1990; Pegg, 1990; Fritz et al., 1991).

The major difference that has been found between the bacterial and mammalian AGT proteins is their ability to repair O^4MeT. As noted above, the bacterial AGT proteins are capable of removing the methyl group from O^4MeT, but attempts to duplicate this using rodent or human AGT have not been successful (Dolan et al., 1984; Brent et al., 1988). These early works may have been hindered by problems associated with synthesizing labeled O^4MeT. However Dolan et al. (1988) using an oligonucleotide that contained O^4MeT as the only methylated base found that neither the human nor rat AGT repaired the lesion, although the bacterial AGTs were able to repair it. In contrast to these results, Sassanfar et al. (1991) used a similar substrate and showed that human AGT was inactivated by the O^4MeT adduct, implying that it recognized the lesion and repaired it. Unfortunately, these results required concentrations of O^4MeT too high to be of physiological relevance.

3. Mechanism of repair

It is widely accepted that the mechanism of mammalian AGT repair is identical to bacteria. The report by Demple (1990), indicated that bacterial AGT initiates repair with an interaction between a basic residue in the acceptor site and the cysteine acceptor residue, generating a thiolate ion which then removes

the alkyl group from O^6MeG. Scicchitano et al. (1986) found that the base opposite the lesion does not affect repair of O^6MeG by either bacterial or mammalian AGT. Morgan et al. (1993) showed that the carboxy terminus of AGT, which is highly conserved among mammals, plays a role in the rate of repair at reduced temperatures and also in substrate specificity. Similar to the bacterial AGTs, mammalian AGT is capable of removing adducts as large as a propyl group, while larger adducts are repaired by other mechanisms. However, O^6-benzylguanine (O^6BzG) as a free base is an excellent substrate for AGT (see below).

4. Defects in repair

Human cells that lack AGT have been denoted as Mex- or Mer- (Day et al., 1980). This designation reflects the lack of ability of a cell line to support the growth of adenoviruses treated with a methylating agent since they are unable to repair methylated DNA. Cell lines that have the ability to repair the damaged virus, and therefore allow the virus to replicate are termed Mex+. Most cell strains with this designation are either tumorigenic or transformed (Day et al., 1980), suggesting there may be a growth advantage accompanying the loss of AGT. Analysis of Mex- cells revealed they do not contain AGT protein (Ostrowski et al., 1991; Pegg et al., 1991) or express a detectable level of AGT message (Fornace et al., 1990; Pieper et al., 1990). However, Southern analysis revealed no gross changes in the genomic DNA of the AGT gene in these cells (Pieper et al., 1990; Tano et al., 1990) indicating the Mex- phenotype is not due to loss of DNA. The work by Pieper et al. (1991) indicated that hypermethylation

of the promoter region of the AGT gene may play a role in the phenotype of these cells.

5. Inhibition of repair

As noted above, upon removal of the methyl group from O^6MeG the AGT protein is permanently inactivated. Therefore it should be possible to saturate the repair mechanism for O^6MeG and render a cell unable to repair further damage. This would allow a direct correlation between the cytotoxic and mutagenic effects of O^6MeG and the presence of such adducts in the cells DNA.

Dolan et al. (1985) showed that addition of the free base O^6MeG or O^6-butylguanine to culture medium decreases the AGT activity of HeLa cells. They also showed that O^6MeG is a more potent inhibitor than O^6-butylguanine. Building on these results, Domoradzki et al. (1985) found that O^6MeG-induced depletion of AGT activity in human fibroblasts results in an increase in both the cytotoxic and mutagenic effects of MNNG treatment. O^6MeG was capable of lowering the AGT activity level by 70-80%, leaving the cells with marginal repair capacity.

Dolan et al. (1990a) showed that the free base, O^6BzG, was a more potent of an AGT inhibitor than O^6MeG. A dose of O^6BzG 20 times lower than O^6MeG was able to reduce the amount of AGT in a human tumor cell line by more than 90% in only 10 minutes. Hamden et al. (1992) demonstrated O^6BzG to be effective in the inhibition of AGT in human breast epithelial cells. Pegg et al. (1993) showed that inactivation of human AGT by O^6BzG occurs by the formation of AGT bound to the benzyl group at the same cysteine residue involved in

repair. Crone and Pegg (1993) mutated several amino acids in the acceptor region of human AGT and found that a mutation at codon 140, in which proline was replaced by alanine, results in the AGT being refractory to O^6BzG inactivation. Interestingly, the mutation had no effects on the repair ability of the AGT. Goodtzova et al. (1997) showed that both human AGT and the bacterial Ogt protein react strongly with O^6BzG, both as a free base and in a oligonucleotide. Their results further indicated that the bacterial Ada protein is not inactivated by O^6BzG, and shows only weak affinity for O^6BzG in a DNA template, most likely as the result of steric constraints.

Dolan et al. (1990b) showed O^6BzG is capable of inactivating AGT in various tissues of hamsters and mice, indicating that O^6BzG could be used *in vivo*. A study by Mitchell et el. (1992) showed that when mice containing human tumor xenografts were treated with the chemotherapeutic drug 1,3-bis(2-chloroethyl)-1-nitrosourea and O^6BzG the tumors showed an increased latency. O'Toole et al. (1993) treated rats with a combination of O^6BzG and a methylating agent and found elevated levels of both O^6MeG and O^4MeT in the liver, indicating that O^6BzG can elevate O^6MeG levels *in vivo*. To investigate the in vivo metabolism of O^6BzG, Dolan et al. (1994) fed rats labeled O^6BzG, and found that there was a higher level of label in the liver than other tissues. In more recent studies utilizing cultured human fibroblast cells, Zhang et al. (1996) showed that depletion of AGT activity by O^6BzG results in increased incidence of intrachromosomal recombination, most probably the result of lack of repair O^6MeG accumulation.

In addition, Boley et al. (1999) showed that inactivation of AGT in human fibroblasts by O^6BzG significantly enhances the transforming effects of MNU.

VI. Recombination

Genetic recombination can be grouped into four main categories (Low and Porter 1978). Homologous recombination occurs between DNA sequences that show little or no heterogeneity between them. Recombination occurring between two specific sites in DNA that does not involve loss of DNA sequence between the two components is termed site-specific recombination. Transpositional recombination describes the mechanism by which transposons are nonspecifically integrated into the DNA. Irregular recombination is defined by recombination between sequences that show little homology. For my review, I will detail the components and mechanisms for homologous recombination although many of the factors involved in homologous recombination are seen in other forms of recombination.

The most common initiating factor in recombination is a break in the DNA. Double strand breaks, which are a common result of ionizing radiation, have been shown to induce recombination (Thaler and Stahl, 1988). To survive the effects of double strand breaks, yeast cells use a recombinational repair pathway to correct the damage and maintain genomic stability. Resnick et al. (1989) showed that introduction of a nonhomologous yeast chromosome into a different strain of yeast results in a four to five-fold increase in ionizing radiation-induced aneuploidy over that seen in yeast containing homologous chromosomes. This

indicates that homologous recombination is involved in the retention of ploidy following ionizing radiation-induced dsDNA breaks. Another initiating event in recombination is ssDNA breaks, such as those that occur randomly in DNA, and these ssDNA breaks are considered to be the primary causal event in spontaneous recombination (Roeder and Stewart, 1988). Other sources of single strand breaks are the repair intermediates resulting from NER and MMR acting on DNA damage (see above).

A. Components

Most of what is known about recombination in human cells has come from studying lower organisms. Since my thesis involves human cells I will discuss what is known about components involved in eukaryotic recombination. I will first discuss the primary components involved in yeast recombination and then discuss the human homologs that have been isolated to date.

1. Yeast

a. *RAD50*

Alteration of this gene was shown to result in yeast that exhibit a increased sensitivity to ionizing radiation (Game and Mortimer, 1974) presumably due to the inability to repair double-strand breaks. In addition, RAD50 is essential for meiotic recombination and loss of *RAD50* results in an increased spontaneous mitotic recombination frequency, suggesting opposing roles for RAD50 in meiotic and mitotic recombination (Malone and Esposito, 1981). In a related study, Malone et al. (1990) showed that although RAD50 is required for double-strand break repair, RAD50 is not required for spontaneous mitotic

recombination, indicating double-strand breaks are not the initiating factor in spontaneous mitotic recombination. Alani et al. (1989) characterized the RAD50 protein as a 153kDa protein that contains a purine nucleotide-binding domain indicating a possible ATPase function for this protein. Purified RAD50 is able to bind double-stranded DNA in a nonspecific ATP-dependent manner, supporting a possible ATPase function (Raymond and Kleckner 1993). In an intriguing study, Kironmai and Muniyappa (1997) showed mutated RAD50 causes yeast to grow slowly and further analysis revealed these mutants had shortened chromosomes.

b. *RAD51*

Mutation of *RAD51* results in yeast that display a lowered frequency of spontaneous and ionizing radiation-induced mitotic recombination (Morrison and Hastings, 1979). In addition, Contopoulou et al. (1987) showed yeast containing mutant *RAD51* are defective in double-strand break repair. Expression of *RAD51* is induced by ionizing radiation (Aboussekhra et al., 1992), supporting a role for RAD51 in the repair of dsDNA breaks. The RAD51 protein has structural similarities to the regions of the bacterial RecA protein (Shinohara et al., 1992) that are involved in oligomerization and recombination (Ogawa et al., 1992) and RAD51 has DNA binding properties similar to those of RecA (Shinohara et al., 1992). Studying recombination *in vitro*, the study by Sung (1994) indicated RAD51 is able to pair homologous DNA and catalyze strand exchange. In a related study, Petukhova et al. (1998) found that the interaction of RAD51 with RAD54 enhances this pairing. In addition, Clever et al. (1997) showed that

RAD51 interacts with RAD54 *in vitro* and that this interaction is involved in the repair of the dsDNA breaks.

c. *RAD52*

Resnick and Martin (1976) showed yeast defective for *RAD52* exhibit increased sensitivity to ionizing radiation as the result of an inability to repair dsDNA breaks. Yeast containing mutant *RAD52* possess reduced mitotic (Jackson and Fink 1981) and meiotic (Malone and Esposito 1980) recombination rates, suggesting RAD52 functions as a general recombination factor. Milne and Weaver (1993) used a two-hybrid system to show RAD52 interacts with RAD51. Sugiyama et al. (1998) showed that RAD52 facilitates the annealing of ssDNA bound by RPA and the report by New et al. (1998) indicated the interaction of RAD52 with RAD51 stimulates the strand exchange function of RAD51. In addition, RAD52 complexes with RPA, RAD55 and RAD57 at meiotic recombination loci in yeast, establishing these proteins as being involved in recombination *in vivo* (Gasior et al., 1998).

d. *RAD54*

Cole et al. (1987) showed expression of *RAD54*, like *RAD51* (see above), is induced by DNA damage, implying a role for RAD54 in dsDNA break repair. Further support of a role for RAD54 in double-strand break repair came from the study by Game (1993) who found that *RAD54* mutants are defective in the repair of double-strand breaks resulting from ionizing radiation. RAD54 interacts with RAD51 suggesting a role for RAD54 in recombination, and overexpression of *RAD54* partially reverts the effects of a *RAD51* deletion (Clever et al., 1997).

e. *RAD55* and *RAD57*

RAD55 was characterized by Lovett (1994) and shows similarities to both RAD51 and RecA. The study by Hays et al. (1995) demonstrated RAD55 and RAD57 complex with RAD51 and RAD52 and they further showed that overexpression of *RAD51* or *RAD52* reverts the X-ray sensitivity of *RAD55* and *RAD57* mutants suggesting the proteins are functionally interchangeable. In a recent study, Sung (1997) showed RAD55 and RAD57 form a stable heterodimer and that this heterodimer is able to stimulate the strand exchange property of RAD51, indicating a role for RAD55 and RAD57 in recombination.

2. Human

As described above, the components of yeast recombination play a role in the repair of ionizing radiation-induced double-strand breaks. Therefore, the efforts to identify mammalian homologs centered on genes involved in double-strand break repair. In mammalian cells, repair of double-strand breaks occurs more rapidly by end joining and slowly by recombination. Weibezahn et al. (1985) showed that CHO cells sensitive to ionizing radiation are made resistant through the ability to rejoin the DNA ends. Study of ionizing sensitive rodent cells revealed nine complementation groups indicating nine genes involved in repair of double-strand breaks (Collins 1993). Using these studies, human cross-complementing genes have been found and named XRCC (x-ray cross complementing) genes. Although genes for all nine groups have been isolated, I will limit my discussion to those genes that have a possible role in recombination.

a. *XRCC1*

Using cell fusion experiments Thompson et al. (1985) were able to reduce the level of sister chromatid exchange (SCE) in a CHO cell line that has a high level of spontaneous SCE (Thompson et al., 1982). That study lead to the isolation of the *XRCC1* gene and characterization revealed XRCC1 plays a role in the control of SCEs and also is involved in restoring dsDNA break repair (Thompson et al., 1990). XRCC1 copurifies with DNA ligase III (Caldecott et al., 1994) and this interaction is required for activation of DNA ligase III (Caldecott et al., 1995) since CHO cells lacking *XRCC1* show a reduced level of DNA ligase III activity. In addition, the interaction of XRCC1 with DNA ligase III plays a role in base excision repair (Capelli et al., 1997).

b. *XRCC2*

Thacker et al. (1995) showed the *XRCC2* gene is involved in the reversion of the ionizing radiation sensitivity of group 2 hamster cells. The cDNA for *XRCC2* exhibits homology to the yeast *RAD51* gene which is involved in yeast recombination (see above) (Tambini et al., 1997). Further support for *XRCC2* playing a role in DNA repair came from Cartwright et al. (1998) who transfected CHO cells with *XRCC2* and found the transfectants did not show the genomic instability and radiation sensitivity that were characteristic of the parental line.

c. *XRCC3*

Tebbs et al. (1995) showed *XRCC3* partially restores the ionizing radiation resistance to hamster cells belonging to complementation group 3. The study by Liu et al. (1998) demonstrated XRCC3 interacts with the human RAD51 protein

in vitro, implying a role for XRCC3 in recombination. Further support of these results came from the study by Bishop et al. (1998) who transfected group 3 cell with *ERCC3* and found ERCC3 and Rad51 form complexes *in vivo* in response to DNA damage.

d. *XRCC5*

Jeggo and Smith-Ravin (1989) showed that reversion of the ionizing radiation sensitivity of CHO cells from complementation group 5 correlates with an increased rate of recombination as well as double-strand break repair. Getts and Stamato (1994) showed the ability to bind dsDNA ends correlates with the ability of mammalian cells to repair ds breaks. Using antibodies, they were further able to show the protein responsible for binding the dsDNA ends, and restoring ds break repair was related to the human Ku antigen. Rathnell and Chu (1994) found this DNA end-binding factor is lacking in complementation group 5, and revertants from this group are proficient at DNA end-binding and V(D)J recombination. The identification of the *XRCC5* gene came from the work by Smider et al. (1994) who showed transfection of group 5 cell with Ku80 cDNA restores the ability of the cells to repair dsDNA breaks and recombination. Taccioli et al. (1994) compared the Ku80 protein with the XRCC5 protein and found them to be identical.

e. *XRCC4*, *XRCC6*, and *XRCC7*

Li et al. (1995) showed *XRCC4* restores the ability of complementation group 4 cells to perform V(D)J recombination and repair double strand breaks. XRCC4 interacts with DNA ligase IV (Grawunder et al., 1997) and is proposed to

be involved in the completion of V(D)J recombination. In a recent study, Leber et al. (1998) demonstrated XRCC4 is a target for DNA-dependent protein kinase (DNA-PK), which is also involved in dsDNA break repair. They further showed that XRCC4 facilitates the binding of DNA-PK to DNA.

Gu et al. (1997) engineered murine cells that lacked the Ku70 subunit of DNA-PK and found the cells are sensitive to ionizing radiation and also deficient in V(D)J recombination. Their results supported the Ku70 as a new complementing gene, *XRCC6*. Blunt et al. (1995) showed mouse and hamster cells defective in V(D)J recombination lack the catalytic subunit of DNA-PK. They further showed that by introducing the XRCC7 gene (the catalytic subunit) the recombination ability is restored. Therefore three XRCC genes (*XRCC5*, *XRCC6* and *XRCC7*) make up the intact DNA-PK complex, and deficiencies in any one of these genes has been shown to affect recombination, illustrating the importance of these genes in recombination.

f. Homologs to the RAD genes

The human homolog of *RAD51* exhibits a high degree of homology to the yeast protein (Shinohara et al., 1993). Vispe and Defais (1997) showed human RAD51 facilitates strand transfer *in vitro*, but at a lower efficiency than the bacterial RecA protein. These results indicate that the human protein requires cofactors to efficiently complete strand transfer. Overexpression of the homologous hamster *RAD51* gene in CHO cells increases the level of homologous recombination (Vispe et al., 1998), suggesting a role for human RAD51 in recombination. Overexpression of human *RAD52* results in enhanced

homologous recombination in monkey cells (Park 1995) further supporting a role for human RAD52 in recombination. In a subsequent study, Park et al. (1996) showed RAD52 forms complexes with RPA both *in vitro* and *in vivo*, and both proteins are necessary for recombination in monkey cells. Human RAD52 exhibits DNA binding properties similar to the RecA protein and human RAD52 is also able to promote strand exchange and branch migration *in vitro* (Reddy et al., 1997). Yamaguchi-Iwai et al. (1998) demonstrated that chicken cells lacking *RAD52* exhibit decreased homologous recombination. The initial characterization of the human homolog to *RAD54* by Rasio et al. (1997) found the protein contained sequences that indicate RAD54 is a helicase. A subsequent study by Swagemakers et al. (1998) showed RAD54 contains a DNA dependent ATP-ase domain and proposed this may function in the branch migration of recombination (see below).

B. Mechanism

It is widely accepted that the basic mechanism of recombination has been conserved from bacteria to man based on the high degree of homology of the components involved. I will therefore present a review of the general mechanism of bacterial recombination since many of the processes involved have been determined. This same basic mechanism should apply to higher organisms through the action of homologous proteins.

1. Initiation and presynapsis formation

The initiation of recombination in bacteria requires ssDNA. In an *in vitro* system this is easy to produce but *in vivo* the most likely source of ssDNA is

nicked DNA that, through the dynamic nature of DNA, is able to form transient regions of ssDNA. In bacteria, the initial phase of recombination involves the ssDNA being coated with the RecA protein (for a review on RecA, see Cox and Lehman 1987). For yeast and humans the homologous protein is RAD51 and possibly XRCC2. *In vitro*, ssDNA is often comprised of secondary structure that inhibits RecA binding. Muniyappa et al. (1984) showed that *E. coli* ssDNA binding protein removes this secondary structure and facilitates RecA binding to ssDNA. The RecA protein then polymerizes on the ssDNA to form a nucleoprotein filament. Tsang et al. (1985) found that RecA bound to ssDNA forms aggregates with dsDNA *in vitro*, and aggregates do not form if the components are added separately. These results indicate RecA contains two binding sites for DNA. Once the filament forms, it makes contact with duplex DNA in search of regions of homology (Shibata et al., 1979) if there is no homology, the filament dissociates and binds again in a different region. Once a region of homology is found the filament invades the duplex (Shaner and Radding, 1987).

As stated, the filament requires homology between the ssDNA coated with RecA and the duplex to initiate recombination. The minimum size requirement for homology in bacteria is estimated at between 20 to 100 base pairs (Watt et al., 1985). Longer regions of homology result in an increased rate of recombination. In addition, Watt et al. (1985) demonstrated that the presence of mismatches within these homologous regions, while not prohibiting

recombination, have significant effects on the rate of recombination. Once the filament finds a region of homology, the presynaptic phase is over.

2. Strand exchange

The exchange of DNA between the RecA coated ssDNA and the duplex begins by RecA unwinding the duplex in the region of homology in an ATP-dependent manner (Wu et al., 1983). The RAD54 protein in humans is the most likely candidate to fulfill this function by virtue of its potential helicase activities (Rasio et al., 1997). Khan and Radding (1984) showed strand exchange is a slow process, averaging only a few base pairs per second and Radding (1982) found the presence of ssDNA binding proteins increases this rate by approximately 3-fold. The strand exchange extends past regions of heterologous DNA such as DNA mismatches and DNA damage such as thymine dimers, although at a reduced rate (Livneh and Lehman, 1982). Strand transfer results in a three stranded structure referred to as a D loop. Shibata et al. (1979) demonstrated RecA is capable of forming the D loop structure *in vitro*. The conversion of a D loop structure to the primary recombination intermediate, termed a Holliday junction, is not entirely clear. It is likely that the RecBCD enzyme, which has a ssDNA exonuclease activity (Wright et al., 1971), cleaves this structure leading to the formation of the Holliday junction. To facilitate formation of the Holliday junction it is possible that a RecA-type protein may direct the invasion of the displaced strand in a reciprocal fashion.

3. Branch migration

Branch migration, which is the movement of the junction along the DNA, begins once the Holliday junction is formed. Cox and Lehman (1981) showed that branch migration requires the continued presence of RecA, most likely due to its ability to unwind DNA. The proteins responsible for branch migration in *E. coli* are the RuvA and RuvB proteins. Parsons et al. (1995) showed these proteins promote branch migration even in the presence of heterologous DNA. The reaction requires the presence of *E. coli* ss DNA binding protein. In the study by Mitchell and West (1996) efficient branch migration requires the continued presence of RuvA.

4. Resolution

West et al. (1983) showed the presence of RecA is not required for resolution of a Holliday junction. The RuvC protein, which is activated by the RuvA and RuvB proteins (Zerbib et al., 1998), accomplishes the resolution of the Holliday junction. Ishioka et al. (1998) showed that E. coli containing mutated *Ruv* genes are incapable of segregating DNA following UV irradiation. Further analysis revealed that the bacterial DNA exhibited numerous unresolved recombination intermediates.

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CHAPTER II

Malignant Transformation of Human Fibroblast Cell Strain MSU-1.1 by Methylnitrosourea: Evidence of Homologous Recombination to Eliminate *p53*[1]

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[3] The abbreviations used are:

AGT, O^6-alkylguanine-DNA alkyltransferase

AI, anchorage independence

BPDE, (±) - 7β, 8α - dihydroxy - 9α, 10α - epoxy – 7, 8, 9, 10 – tetrahydrobenzo-[*a*] pyrene

DMSO, dimethyl sulfoxide

EtBr, ethidium bromide

FCS, fetal calf serum

HPRT, hypoxanthine phosphoribosyltransferase

LOH, loss of heterozygosity

MAMA, mismatch amplification mutation assay

MNU, N-methylnitrosourea

MNNG, N-methyl-N'-nitro-N-nitrosoguanidine

MEM, minimal essential media

O^6BzG, O^6-benzylguanine

O^6MeG, O^6-methylguanine

PBS, phosphate buffered saline

PCR, polymerase chain reaction

RFLP, restriction fragment length polymorphism

RT, reverse transcription

SCS, supplemented calf serum

UV, ultraviolet light

ABSTRACT

To determine whether the methylating agent methylnitrosourea (MNU) can induce transformation of human cells and whether the O^6-methylguanine adduct (O^6MeG) is causally involved, two populations of the infinite life span, karyotypically stable, human fibroblast cell strain MSU-1.1 differing only in their level of O^6-alkylguanine-DNA alkyltransferase were exposed to MNU and assayed for focus formation. There was a dose-dependent increase in foci in both groups, but in the cells that lacked the ability to repair O^6MeG, the frequency of foci was significantly higher, indicating a causal role for O^6MeG. A transgenic yeast assay used to determine the transactivating ability of the p53 gene in 40 completely independent focus-derived cell strains indicated that 15 (37.5%) lacked functional *p53*, and one cell strain was heterozygous for *p53*. The tumorigenic potential of 35 strains was determined by subcutaneous injection into athymic mice: None of the 19 strains with wild type p53 formed tumors, whereas the heterozygous strain and 10 of the 15 strains (67%) with mutant p53 formed malignant tumors. The data indicate that loss of p53 transactivating ability is not sufficient for the malignant transformation of MSU-1.1 cells, but significantly increases the chance of a cell strain becoming tumorigenic. Sequencing the coding region of the p53 gene in several focus-derived cell strains revealed that each contained the identical mutation, an A-to-G transition that changed codon 215. This same mutation was found in the mutant allele of the focus-derived cell strain that was heterozygous for p53. These data strongly suggest that the MSU-1.1 population used for this study contained a pre-existing

subset of cells heterozygous for the p53 mutation at codon 215. Our data indicate that the mere presence of this mutant allele does not result in focus formation. However, if the wild type *p53* allele is eliminated or replaced by the codon 215 mutant allele spontaneously or as a result of exposure to MNU, this can render the cells able to form foci. A series of focus-derived cell strains lacking wild type p53 activity, 14 from MNU-treated populations, and 1 from an untreated population, was assayed for evidence of mitotic homologous recombination. Analysis of DNA for loss of two restriction fragment length polymorphisms and six microsatellite markers on chromosome 17 revealed seven different patterns of loss of heterozygosity among the MNU-induced p53 mutant cell strains. Cell strains derived from foci induced by ionizing radiation exhibited only two of these patterns. These data suggest that the specific patterns were induced by carcinogen treatment, rather than developing spontaneously.

INTRODUCTION

It is generally accepted that transformation of normal cells into tumorigenic cells involves mutation of critical genes involved in the control of cellular proliferation, and that mutations occur as the result of replication of a DNA template that has been damaged by either endogenous or exogenous factors. An important area in cancer research is determination of the genetic change(s) associated with this transformation of normal cells into tumorigenic cells. As a model system for such studies, McCormick and colleagues have developed and characterized the infinite life span, karyotypically stable, nontumorigenic, human fibroblast cell strain designated MSU-1.1 (1). MSU-1.1 cells can be transformed into tumorigenic cells by transfection of H-*ras* or N-*ras* oncogene expressed at a high level (2,3) or by a single exposure to a carcinogen, benzo(a)pyrene diol epoxide (4) or ionizing radiation (5,6). Focus formation was used as the assay for detecting cells with a transformed phenotype. Approximately one third of the cell strains clonally derived from prominent foci form malignant tumors in athymic mice after a relatively short latency period, and these malignant strains have recently been shown to have lost wild type p53 transactivating ability.

The product of the *p53* gene, a potent transcription factor, has been shown to be inactivated in over 50% of all human tumors studied (7,8). p53-responsive genes play critical roles in cell cycle control, DNA repair, and apoptosis (for reviews see 9,10). In addition, inactivation of *p53* has been shown to result in the progression of fibroblasts in culture to a transformed phenotype (11,12).

Alkylating agents covalently attach alkyl groups to cellular macromolecules. Reaction of a methylating agent with DNA results in the formation of up to 13 different adducts, with methylation of the O^6 position of guanine (O^6MeG) being the most potentially mutagenic lesion, inducing primarily G:C to A:T transitions (13-16). Repair of O^6MeG in human cells is accomplished primarily through the action of AGT, which transfers the methyl group from O^6MeG in DNA to an interior cysteine residue of AGT (17-19). This results in the inactivation and subsequent degradation of the AGT protein. O^6BzG is a strong electrophile that can serve as a substrate analog for AGT (20), resulting in the transfer of the benzyl group from O^6BzG to the same cysteine of AGT that would be involved in removal of a methyl group from DNA. Therefore, cells in culture can be depleted of AGT by addition of low levels of O^6BzG to the medium. This ability to manipulate the cells' capacity to repair O^6MeG allows one to determine the specific cellular effects of O^6MeG.

The present study was designed to answer four issues: whether a simple methylating agent, viz., MNU, can transform MSU-1.1 cells to focus formation; whether O^6MeG plays a causal role in this transformation; whether MNU treatment can result in the malignant transformation of MSU-1.1 cells; and what role mutations in the *p53* gene play in focus formation and/or transformation. For this purpose, two populations of MSU-1.1 cells were prepared, with one group being depleted of AGT activity by O^6BzG and the other not depleted. Both sets were exposed to MNU and assayed for focus formation. The transactivating

ability of the *p53* gene of the focus-derived strains was determined (21,22), and representative strains were assayed for their tumorigenic potential.

The results showed that a single exposure to MNU induces a dose dependent increase in focus formation by MSU-1.1 cells. Pretreatment with O^6BzG very significantly increased the frequency of focus formation per dose MNU, indicating that O^6MeG is the lesion principally responsible for this transformation. A substantial fraction of the focus-derived cell strains assayed in athymic mice formed tumors (11/35). Of these, ten lacked wild type p53; the eleventh was heterozygous for p53. Interestingly the tumors produced by the heterozygous strain were found to lack wild type p53. DNA sequencing analysis of amplified *p53* DNA and PCR amplification using mismatched primers strongly suggested that the MSU-1.1 population used for these studies contained a low frequency of cells, i.e., a pre-existing subpopulation heterozygous for a specific p53 mutation. Analysis using informative RFLP and microsatellite markers on the p arm of chromosome 17 strongly support the hypothesis that treatment with MNU causes the heterozygous cells to undergo homologous mitotic recombination resulting in loss of the wild type allele, which greatly facilitates malignant transformation of MSU-1.1 cells.

MATERIALS AND METHODS

Cell Culture. Unless otherwise noted, the MSU-1.1 cells and all focus-derived cell strains were cultured in Eagles' MEM (Life Technologies, Gaithersburg, MD) (pH 7.2), supplemented with 0.2 mM L-aspartic acid, 0.2 mM L-serine, 1.0 mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100 μg/ml), hydrocortisone (1 μg/ml), and 10% SCS (HyClone, Logan, UT) (complete medium). Cells were grown in a 5% CO_2 humidified incubator and subcultured before they reached confluence.

Carcinogen Treatment. Cells to be treated were plated in complete medium at 10^4 cells/cm^2. After ~16 h, the medium was removed, the cells were rinsed twice with PBS and covered with Eagles' MEM lacking serum and buffered with 20 mM HEPES (pH 7.25) (treatment medium). MNU (Sigma Chemical, St. Louis, MO) was dissolved in anhydrous DMSO immediately prior to use. The appropriate amount of the MNU-DMSO solution was added to each dish to give the designated concentrations. The total concentration of DMSO in the medium was less than 0.5%. Control populations were treated the same way, but received DMSO only. The cells were incubated for 30 min at 37° C in a humidified incubator with 5% CO_2, after which the treatment medium was removed, the cells were rinsed twice with PBS, and complete medium was added.

Elimination of AGT Activity using O^6BzG. O^6-BzG (a generous gift from Dr. A. E. Pegg) was dissolved in DMSO at a concentration of 25 mM and stored at -20° C under nitrogen gas. For the population of cells to be depleted of

AGT by O^6BzG the compound was added at a final concentration of 25 μM 2 h prior to treatment with MNU. A set of control cells also received O^6BzG. O^6BzG (25 μM) was also present in the treatment medium, and at the end of the MNU treatment, these cells were refed with complete medium containing O^6BzG. After 48 h, the medium for these cells and also for the other population was changed to complete medium lacking O^6BzG.

Cytotoxicity Assay. Immediately following treatment, cells were rinsed with PBS, dislodged with trypsin, suspended in complete medium, with or without O^6BzG, and plated at densities designed to yield ~40 clones per 100-mm diameter dish. A minimum of three dishes were used per group. All cells were re-fed with complete medium at 48 h post-treatment and after one week. After two weeks, the cells were stained with crystal violet. The clones were counted, and the surviving fraction was calculated using the cloning efficiency of the treated cells and expressed as a fraction of the cloning efficiency of untreated cells.

Focus Assay. The focus assay was used as a means of screening for transformed cells was performed essentially as described (6). Briefly, cells to be assayed for the ability to form foci were kept in exponential growth following MNU treatment, subculturing as needed. After 8 days, the cells were dislodged with trypsin, pooled, and suspended in complete medium supplemented with 20 mM HEPES (pH 7.5) and with the serum level reduced to 0.5% SCS. For each dose, 10^6 cells were assayed at 5 x 10^4 cells per 100-mm diameter dish. Cells were refed weekly with this medium. After 4-6 weeks, the dishes were scanned for

foci, i.e., densely piled, clonal proliferations or colonies of cells that exhibited an altered morphology on a confluent monolayer, visible when the dishes were illuminated from beneath with a focused beam of light. Representative foci were isolated using trypsin and subcloned twice to eliminate background non-focus forming cells. The remaining cells in the dishes used to assay for focus formation were fixed with methanol and stained with methylene blue, and the foci were counted to determine the frequency.

***p53* Transactivational Activity Assay.** To determine the transactivating ability of the p53 protein expressed in the parental MSU-1.1 cell strain and in focus-derived cell strains, a transgenic yeast assay, described by Scharer and Iggo (21) was performed essentially as described (22) with the noted exceptions. Briefly, total RNA was isolated from cells using the RNAzol B method (TEL-TEST, Friendswood, TX) according to the manufacturers' instructions. Reverse transcription of mRNA was performed using 2.5 μg total RNA denatured at 65°C for 15 min in a 20 μl volume containing: 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 500 nM each dNTP, 400 ng oligo$(dT)_{12-18}$, and 40 U MuLV reverse transcriptase (Life Technologies, Gaithersburg, MD) and incubated at 37° for 90 min. The *p53*-specific primers used for PCR were described previously (21). The RT reaction was diluted 1:10 in water and 5 μl was used as template in a 50 μl reaction containing: 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 20 mM Tris-HCL (pH 8.2), 2 mM $MgCl_2$, 0.1% Triton X-100, 10 μg/ml BSA, 400 nM each dNTP, 400 nM each primer, and 2.5 U *Pfu* polymerase (Stratagene, LaJolla, CA). The PCR reaction was carried out in a HyBaid

thermocycler (Woodbridge, NJ) for 40 cycles (94°C for 30s, 55° for 30s, 72° for 90s), and the amplified *p53* coding sequence was purified using a silica-gel column (QIAGEN, Valencia, CA). A gapped plasmid carrying a selectable marker and the purified PCR product (p53 cDNA) were electroporated into yeast using the Cell-Porator system (Life Technologies, Gaithersburg, MD) set at 400V, 10μF and low resistance, and yeast that incorporated a p53 cDNA into the gapped plasmid were selected. The yeast also contain a resident plasmid harboring a p53-responsive reporter gene. Yeast cells that incorporated the p53 DNA were selected for expression of this reporter gene, which requires wild type p53 for transcriptional activation.

Nucleotide Sequencing of the *p53* Gene. The sequence of the *p53* RT-PCR product was determined by automated dye terminator sequencing (Applied Biosystems Division, Perkin Elmer, Foster City, CA). Primers specifically designed to bind to *p53* cDNA (Table 1) were used for sequencing.

Mismatch Amplification Mutation Assay (MAMA). To determine whether various populations of MSU-1.1 cells in this study contained a subpopulation of cells altered at codon 215 in the p53 gene, we utilized the MAMA protocol (23). Briefly, this protocol utilizes a primer designed to amplify a specific sequence, which in our case was a template containing the codon 215 mutation. The primers used were as follows: primer 1 (control) CCT GGG CAT CCT TGA GTT; primer 2 (control) TCA CAG CAC ATG ACG; primer 3 (mismatch) CAG AAA CAC TTT TCG ACA GG. The mismatch primer was designed so that the two terminal bases at the 3' end would not bind, and

Table 1. Sequencing primers for p53 RT-PCR product

Primer Sequence	Binding Site[a]
GCT GTC CCC GGA CG	267-280
GCA GCT ACG GTT TCC G	449-464
TCA CAG CAC ATG ACG	631-645
CTG ACT GTA CCA CCA T	815-832
CCG GCG CAC AGA GG	978-991
TGA ATG AGG CCT TGG	1166-1180
AGC TTC ATC TGG ACC TGG G	324-306
ATG CAA GAA GCC CAG	480-465
CTC ATG GTG GGG GC	675-662
GCC GCC CAT GCA GGA A	870-855
TCC CCT TTC TTG CGG	1016-1002
CCT GGG CAT CCT TGA GTT	1338-1315

[a] The top six primers are forward primers and the rest are reverse primers. The sequences represent primer binding locations on the p53 cDNA where 1 represents the beginning of exon 1.

therefore could not amplify the wild type p53 sequence. However, the terminal base of the mismatch primer was able to bind to the mutant p53 sequence and therefore amplification was possible. PCR was carried out using 100 ng of purified p53 cDNA (see above) in a 50 μl reaction containing: 50 mM Tris-Cl (pH 9.0); 20 mM $(NH_4)_2SO_4$; 1.5 mM $MgCl_2$; 400 nM each dNTP; 80 nM each primer; and 2.5 U *Tfl* polymerase (Epicentre Technologies, Madison, WI) using a PCR profile of 94^0 C for 3 min followed by 25 cycles of (94° C for 30 s, 60° C for 30 s, 72 $^\circ$ C for 60 s). Each template was used in two reactions, one containing the control primers, and another containing the mismatch primer in place of control primer 1. Results were obtained by running 5 μl of PCR product on a 1% agarose gel containing 0.5 μg/μl EtBr and visualized with UV light.

Restriction Enzyme Analysis. Analysis of *p53* RT-PCR product involved digestion of 1 μg of purified *p53* coding sequence in a 20μl reaction volume using *Nla*III restriction enzyme (New England Biolabs, Beverly, MA) following the manufacturers' recommended procedure. The entire reaction was resolved on a 3% agarose gel containing 0.5 μg/ml ethidium bromide, and the resultant DNA bands were visualized using UV light.

Southern Blotting Analysis. DNA was isolated using the Puregene kit (Gentra Systems, Research Triangle Park, NC). DNA (15μg) was digested with the designated restriction enzyme (Boehringer Mannheim, Indianapolis, IN) (5U/μg DNA) following manufacturers' instructions. The reactions were carried out at 37°C for 16 h and the products were purified by phenol: chloroform extraction. Purified digest (10 μg) was resolved on a 1% agarose gel by

electrophoresis at 35 volts (constant) for 16-18 h. DNA was transferred to a Zeta Probe membrane (BioRad, Hercules, CA), crosslinked using the UV Stratalinker 2400 (Stratagene, LaJolla, CA), and analyzed. For RFLP analysis, probe pUC10-41 (American Type Culture Collection, Rockville MD), that is homologous to D17S71, and pYNZ22.1 (ATCC, Rockville MD), that is homologous to D17S5 were used. To determine the number of *p53* alleles, *p53* DNA (RT-PCR product) was used as a probe for *p53*, and an intron 1 DNA sequence of the HPRT gene (24) was used as a probe for the *HPRT* gene, which served as a loading control. The random primer labeling of probes and hybridization conditions used were as described (25). Blots were exposed to PhosphoImaging cassettes, and the data were analyzed using Image Quant 3.1 software (Molecular Dynamics, Sunnyvale, CA).

Microsatellite Analysis. The sequences for the primer pairs (Whitehead Institute database) used to amplify the region surrounding the microsatellite repeats are given in Table 2. PCR was performed using 250 ng genomic DNA in a 25 μl mixture containing: 50 mM Tris-Cl (pH 9.0); 20 mM $(NH_4)_2SO_4$; 1.5 mM $MgCl_2$; 400 nM each dNTP; 150 ng each primer; and 2.5 U *Tfl* polymerase (Epicentre Technologies, Madison, WI). Amplification was performed using a HyBaid thermocycler (Woodbridge, NJ) and a PCR profile of 94° C for 3 min followed by 35 cycles of 94° C for 10 s, X° for 10 s and 72° C for 15 s. The value "X" is specific for the individual primers sets and is given in Table 2. The PCR products were analyzed by 15% nondenaturing polyacrylamide gel

Table 2. Microsatellite PCR primer information

Locus[a]	T_m	Location (cM)[b]	Annealing Temp	Primer Sequence
D17S1828	54 54	9.8	54	1)TTA AGC CAG TTC GGA TTT G 2)TGC ACT CAC AGA TTT GCC
D17S796	60 58	14.8	60	1)AGT CCG ATA ATG CCA GGA TG 2)CAA TGG AAC CCA ATG TGG TC
D17S960	52 54	16.5	55	1)TGA TGC ATA TAC ATG CTG G 2)TAG CGA CTC TTC TGG CA
D17S952	58 52	18	58	1)ACC TTA CCA TGC ACA CAG TT 2)TCC CCA GGA GAC AGC A
D17S945	54 56	22	56	1)CCT GAA GCC TGA CCC C 2)AAC CAA TCT GGA CTC CCC
D17S947	58 52	32.8	56	1)GAC AAG AAT TTC CCA AGA TAG 2)TGT CCC AGA GTT TCG ATA
D11S1338	62 68	14.9	60	1)TAA TGC TAC TTA TTT GGA GTG TG 2)GAC GGT TTA ACT GTA TAT CTA AGA C
D11S4083	58 60	50.7	56	1)TTT AAC CCA AGG GCA GGA C 2)CAT GTG TAC CCA AGG GCA G
D11S1344	60 60	62.5	56	1)CCC TGA ACT TCT GCA TTC AC 2)GCG CCT GGC TTG TAC ATA TA
D18S68	54 60	94.4	57	1)ATG CTG CTG GTC TGA GG 2)ATG GGA GAC GTA ATA CAC CC

[a] The top six primer sets are for microsatellites located on the p arm of chromosome 17, the next three are for microsatellites located on chromosome 11 and the last is for a microsatellite located on chromosome 18

[b] Distance from the top of the linkage group as determined by Genethon mapping

[c] Sequences, written 5' to 3', were obtained from the Whitehead Institute database

electrophoresis [100 volts (constant) for 16-18h], stained with EtBr, and visualized using UV.

Anchorage Independence. For each cell strain, 50,000 cells were assayed as described (6). Briefly, 5000 cells in 1.5 ml of McM medium containing 0.33% Seaplaque agar and 2% FCS, were plated on top of 5 ml of a solidified base layer made up of McM medium containing 4% SeaPlaque agar (FMC Bioproducts, Rockland, ME) and 2% FCS. The top agar was allowed to solidify overnight, then covered with 3.5 ml of McM medium containing 2% FCS and antibiotics, and buffered with 20mM HEPES. Cells were incubated for 3 weeks at 37° C with 3% CO_2 and 97% humidity, with weekly refeeding. The cells were then fixed with 2.5% glutaraldehyde and stored at 4° C until analyzed.

Tumorigenicity Assay. The tumorigenicity assay was performed as described (6). Tumors were measured weekly using calipers and removed when they reached 1 cm in diameter. A portion of the tumor was returned to culture, while the remainder was fixed with formalin and prepared for histological evaluation. Slides of tumor tissue were examined to determine the histological classification of the tumor

RESULTS

Evidence that O^6MeG Is the Principal Lesion Involved in the Transformation of MSU-1.1 Cells to Focus Formation by MNU. To determine whether MNU could transform MSU-1.1 cells into focus forming cells and whether the O^6MeG lesion played a causal role in this transformation, we prepared two populations of MSU-1.1 cells, with one depleted of AGT activity by being treated with 25μM O^6BzG for 2 h prior to MNU treatment and the other not receiving O^6BzG. These two populations of cells were exposed to various doses of MNU for 30 min in medium containing or not containing O^6BzG, respectively. One set of dishes of treated cells and their untreated control population were used immediately to assay cell survival. After a 7-8 day expression period, during which the cells were maintained in exponential growth, the cells in the other dishes were assayed for the frequency of focus formation. In each instance, the cells that had been depleted of AGT were maintained in medium containing O^6BzG for an additional 48 h. [(Lukash et al., (15) in this laboratory showed that this treatment reduces AGT protein in human fibroblasts to undetectable levels and that AGT remains low for at least 24 additional hours after removal of O^6BzG from the medium).] As shown in Figure 1A, there was a dose-dependent decrease in survival, and this was significantly greater in the cells that had been depleted of AGT activity. Figure 1B shows that MSU-1.1 cells exhibited a corresponding dose-dependent increase in focus induction and that O^6-BzG pretreatment significantly enhanced this induction.

Figure 1. Cytotoxicity and focus formation induced in MSU-1.1 cells as a function of MNU dose. A, cytotoxicity as determined by loss of colony forming ability. The dashed line represents MNU-treated MSU-1.1 cells that received 2 h pretreatment with 25μM O^6BzG, had O^6BzG present during MNU treatment, and then were exposed to O^6BzG for an additional 48h. The solid line represents MSU-1.1 cells that received MNU treatment only. B, frequency of focus formation. The lines are the same as outlined above. The background frequencies of focus formation have been subtracted. These background frequencies per 10^6 cells assayed were: + (8); o (36); ● (9); □ (72); ■ (88); Δ (20); ▲ (15). Only foci taken from populations in which the frequency was 4-fold over background were used to derive cell strains for further study.

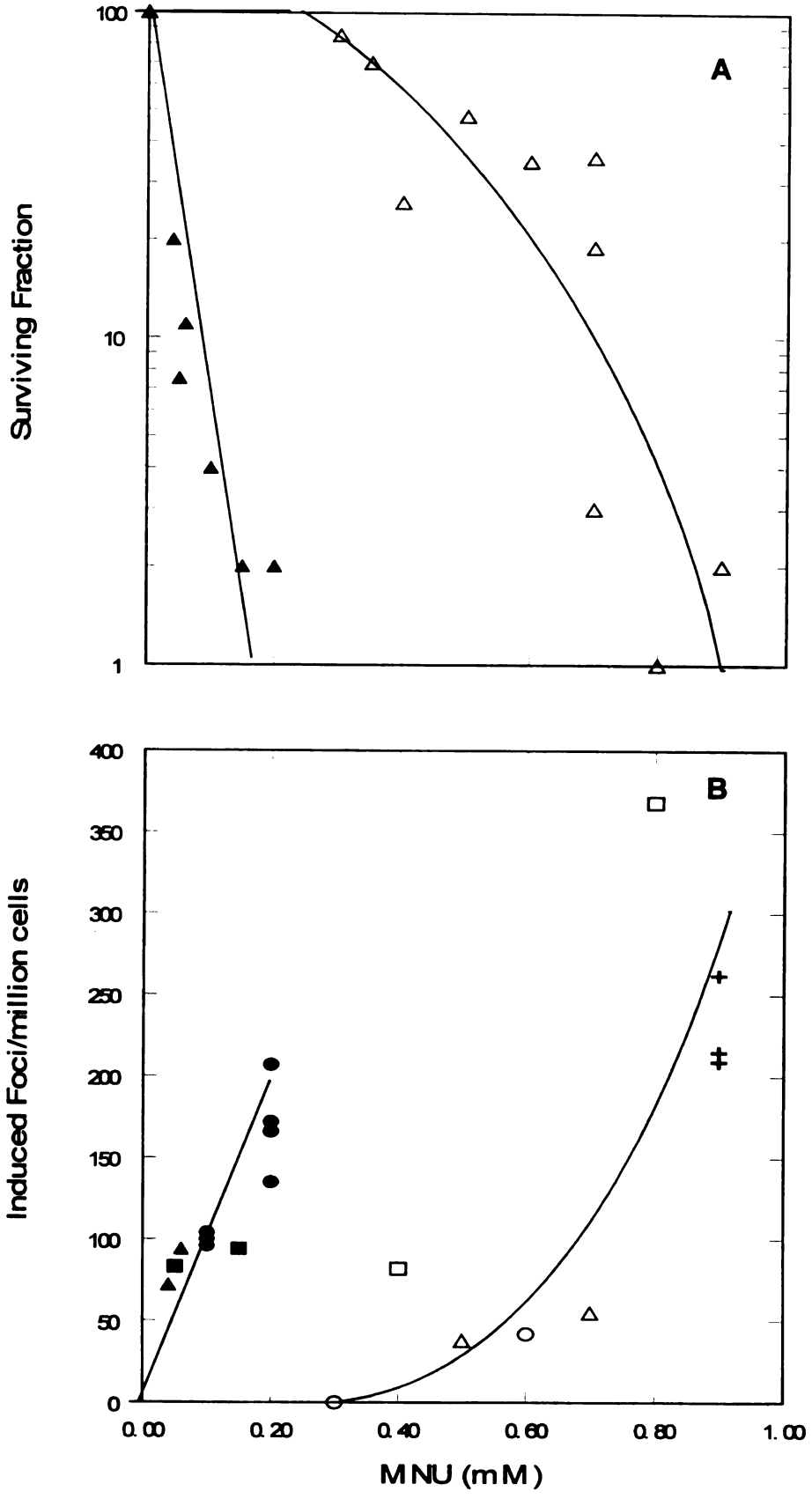
A
100
10
1
Surviving Fraction
B
400
350
300
250
200
150
100
50
0
0.00
0.20
0.40
0.60
0.80
1.00
Induced Foci/million cells
MNU (mM)

Figure 1

Tumorigenic Potential of Focus-Derived Cell Strains. Because earlier studies from this laboratory show that a significant proportion of unequivocally independent foci induced by treating of MSU-1.1 cells with benzo(a)pyrene diol epoxide (4) or ionizing radiation (6) form malignant tumors in athymic mice, representative MNU-induced foci were isolated, sub-cloned twice, and after expansion, the focus-derived cell strains were assayed for the ability to form tumors. These foci were taken from experiments in which the frequency was at least four times higher than background, the majority was from experiments giving frequencies 6- to 30-fold above the background. A total of 35 independent focus-derived cell strains were assayed for the ability to form tumors in athymic mice, 28 from MNU-treated populations and seven from untreated control populations. Ten of the strains formed malignant tumors that reached a diameter of 1 cm in a relatively short period (6-16 weeks). One of these ten was from a focus taken from a untreated population. An additional cell strain, derived from a focus from an MNU-treated group, gave rise to tumors after a somewhat longer period (11-31 weeks) (Table 3, columns 6 and 7).

p53 Status of Focus-Derived Cell Strains. O'Reilly et al. (6) recently reported that 78.5% of the cell strains derived from foci induced by a single exposure of MSU-1.1 cells to cobalt 60 radiation showed loss of *p53* transactivating function. To see if loss of wild type p53 was also found in cell strains from MNU-induced foci, 39 cell strains derived from unequivocally independent foci were assayed for p53 transactivating ability. Seven were derived from foci in the control populations, and 32 were from MNU-treated

Table 3. Characterization of MNU-induced focus-derived cell strains

Cell strain	MNU (mM)	O^6BzG[a]	% AI[b]	Functional status of p53 gene[c]	Tumors per injection site	Tumor latency (weeks)[d]
MSU-1.1	-	-	0	WT	0/50	-
MA0-1	-	-	37	-/-	6/8	6-10
MB5-1	0.7	+	17	-/-	6/6	6-10
MA4-1	0.9	-	51	-/-	3/4	7-9
MA3-3	0.8	-	47	-/-	3/4	8-12
MB3-1	0.3	+	30	-/-	4/4	8
MB4-2	0.5	+	41	-/-	1/8	9
MB4-3	0.5	+	51	-/-	4/4	11
MA3-2	0.8	-	0	-/-	2/4	13
MB2-2	0.2	+	27	-/-	2/4	14
MA5-1	1.3	-	ND	-/-	2/8	16
MA2-1	0.7	-	4	+/-	4/8	11-31
MA2-1T[e]	0.7	-	25	-/-	-	-
MA3-1	0.8	-	19	-/-	0/8	-
MA5-2	1.3	-	6	-/-	0/4	-
MB2-3	0.2	+	2	-/-	0/8	-
MB4-5	0.5	+	13	-/-	0/4	-
MB5-2	0.7	+	7	-/-	0/8	-
MA0-2	-	-	ND	+/+	0/4	-
MA0-3	-	-	ND	+/+	0/4	-
MB0-1	-	+	ND	+/+	0/4	-
MB0-2	-	+	ND	+/+	0/4	-
MBO-3	-	+	ND	+/+	0/4	-
MB0-4	-	+	ND	+/+	0/4	-
MB1-1	0.1	+	ND	+/+	0/4	-
MB1-2	0.1	+	ND	+/+	0/4	-
MB2-1	0.2	+	14	+/+	0/8	-

Table 3 (cont'd)

Cell strain	MNU (mM)	O^6BzG[a]	% AI[b]	Functional status of p53 gene[c]	Tumors per injection site	Tumor latency (weeks)[d]
MB2-4	0.2	+	ND	+/+	0/4	-
MB3-2	0.3	+	ND	+/+	0/4	-
MB4-1	0.5	+	8	+/+	0/4	-
MB4-4	0.5	+	ND	+/+	0/4	-
MA3-4	0.8	-	72	+/+	0/4	-
MA3-5	0.8	-	36	+/+	0/4	-
MA3-6	0.8	-	ND	+/+	0/4	-
MA4-2	0.9	-	ND	+/+	0/4	-
MA4-3	0.9	-	ND	+/+	0/4	-
MA4-4	0.9	-	ND	+/+	0/4	-

[a] Treatment protocol from which the focus -derived cell strain was isolated. +, cells received 2 h pretreatment and 72 h post-treatment of 25 μM O^6BzG in addition to MNU, -, cells received MNU treatment only.

[b] Ability to form colonies in soft agar as measured against positive and negative controls with the frequency of colonies that were larger than 80 μm.

[c] Transactivational ability of p53 as determined by a transgenic yeast assay or *Nla*III digestion, as described in Materials and Methods. -/- denotes cell strains that express only mutant p53 and contain two alleles as determined by Southern blot analysis, +/+ denotes cell strains that express only wild type p53 but were not verified by Southern blot, +/- denotes cell strains that are heterozygous for *p53*. There are an additional five independent cell strains that expressed only wild type p53 that are not listed.

[d] Time required for tumors to reach 1 cm in diameter (500 mm^3).

[e] MA2-1T represents the tumor-derived cell strain of MA2-1.

populations. These 32 included the 28 that were assayed for tumorigenicity. Initially, to determine if the p53 alleles in these strains were functional, we employed the transgenic yeast assay developed by Scharer and Iggo (21). This was also used to confirm that the parental MSU-1.1 cell strain has wild type p53 function. Because of the design of the assay, it can only detect inactivating mutations that are located within exons 4 through 10. It offers the advantage of assaying each allele separately and, therefore, can determine that a cell is heterozygous for p53. However, the assay cannot reveal that a cell is hemizygous for p53.

Nucleotide sequencing of the coding region of *p53* from four cell strains that expressed only mutant p53 showed that each contained an AT-to-GC transition at the first position of codon 215, which changes the amino acid from serine to glycine. Finding a common mutation was highly unexpected. This is because each strain had been derived from a focus that developed in an independent population of MNU-treated MSU-1.1 cells in which the frequency of foci was significantly higher than background, and it has been shown that the coding region of p53 has hundreds of sites that, when mutated, eliminate its transactivating function (7). Moreover, an AT-to-GC transition is not commonly induced by methylating agents (15). Therefore, we hypothesized that the four strains we had analyzed for p53 mutations by DNA sequencing were not independent, but instead reflected the presence of a pre-existing subpopulation in the target MSU-1.1 population.

The AT-to-GC base change at codon 215 creates a recognition site for the restriction enzyme *Nla*III. This allowed us to make use of restriction enzyme digestion and gel electrophoresis to screen rapidly for the presence of this specific base substitution in additional focus-derived cell strains that the yeast assay indicated had lost p53 transactivating ability. As shown in Figure 2, wild-type *p53* gives the following restriction pattern: 477bp, 414bp, 173bp and 5 fragments less than 30bp. The AT to GC transition at codon 215 results in the 173bp fragment being further digested to yield fragments of 107bp and 66bp (Fig. 2). The results of the *Nla*III digestion confirmed that all the focus-derived cell strains that exhibited loss of wild type p53, as judged by the yeast assay, contained the same codon 215 mutation. In total, 39 focus-derived cell strains were analyzed for the transactivating ability of the product of the p53 cDNA using a variety of methods. Of these, 23 expressed only wild type p53, 15 expressed only mutant p53, and one strain, MA2-1, the one that produced tumors only after a longer latency than the rest of the cell strains, proved to be heterozygous for p53. Analysis of the cells derived from a tumor formed by the cell strain MA2-1 for their p53 status using the yeast assay revealed that they no longer contained a wild type p53 allele. The result of these studies of the status of the p53 alleles is included in Table 3 as column 5.

Evidence of Homologous Recombination in Focus-Derived Cell Strains That Have Lost p53 Transactivating Function. The presence of a focus-derived cell strain that was heterozygous for *p53*, with one wild type allele and one containing the codon 215 mutation, the common mutation found in all

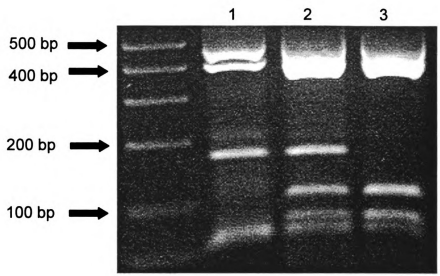

Figure 2. Gel electrophoresis of *Nla*III restriction digests of *p53* RT-PCR product. *Lane 1*, wild type p53 product, *lane 2*, p53 RT-PCR product heterozygous at codon 215, *lane 3*, p53 RT-PCR product fully mutant at codon 215.

the focus-derived cell strains that did not have wild type p53, strongly supported our hypothesis that a subpopulation of cells, altered at codon 215, were present in the population of parental MSU-1.1 cell strain used for this study. Whether these cells are heterozygous, homozygous, or hemizygous for the codon 215 mutation is not easily determined. None of the three assays employed, i.e., the yeast assay, DNA sequencing, and *Nla*III digestion, can distinguish between the latter two possibilities. To test the hypothesis that the subpopulation is heterozygous for the *p53* codon 215 mutation and that MNU treatment converts it to the homozygous mutant state by inducing homologous recombination or gene conversion; or to the hemizygous state by inducing loss of part of the chromosome containing the wild type *p53* allele, we used RFLP analysis for LOH of informative markers, combined with Southern blotting to determine the number of p53 alleles in a cell strain.

Preliminary investigation in MSU-1.1 cells revealed two informative RFLP markers on the p arm of chromosome 17. One locus, located between *p53* and the telomere, is detected by the pYNZ22.1 probe, produces multiple bands in the range of 0.5 kb to 1.3 kb in *Msp*I-digested DNA. The other locus, located between the centromere and *p53* and detected by the pUC10-41 probe, yields bands of 2.4 and 1.9kb in *Msp*I-digested DNA. These two markers were used to assay each of the focus-derived cell strains that had been derived from unequivocally independent sets of MNU-treated target cells and that had lost p53 transactivating function. Three patterns of LOH were found: loss of the telomeric marker, loss of both markers, and no

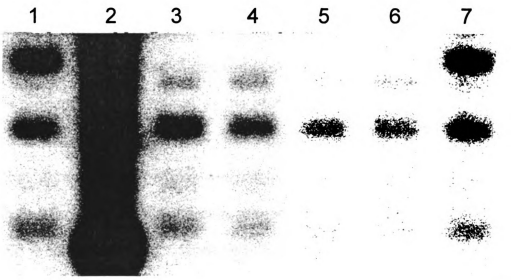

Figure 3. Representative RFLP analysis of an informative marker on 17p of MSU-1.1 cells using the pYNZ22.1 probe. *Lane 1*, MSU-1.1 cells, *lane 2*, molecular weight standards *lanes 3-7*, focus-derived cell strains that express mutant p53. The pattern shown for MSU-1.1 (lane 1) indicates retention of heterozygosity; alterations from this pattern denote LOH.

LOH (data not shown). A representative RFLP Southern blot is shown in Figure 3.

The status of the p53 gene in the heterozygous focus-derived cell strain MA2-1 confirmed that the parental MSU-1.1 cells contain two *p53* alleles. To determine the copy number of *p53* in the other focus-derived cell strains, Southern blotting analysis of *Eco*RI-digested DNA was carried out, using an RT-PCR product from p53 mRNA as the probe for the *p53* gene. The *HPRT* gene, used as a loading control for the DNA blots was probed with an intron 1 segment of that gene (24). The blot was initially probed for *p53*, then stripped and reprobed for *HPRT*. MSU-1.1 cells are derived from a male donor and contain a single X chromosome (1). Since *HPRT* is located on the X chromosome, *HPRT* represents a single copy gene for these male cells. The intensities of the resulting bands from the different probes were normalized using DNA from parental MSU-1.1 cells as the standard. The results from the comparative Southern analysis showed that all 15 focus-derived cell strains that expressed only mutant p53 contained two copies of *p53*. Therefore, the loss of heterozygosity at the *p53* locus was not caused by loss of chromosomal material, and must have resulted from mitotic recombination or gene conversion. These data are included where appropriate in the characterization of the strains in Table 3.

Microsatellite Analysis of p53 Mutant Cell Strains. To determine whether the 15 focus-derived cell strains that had lost *p53* transactivating

function of both p53 alleles differed from one another in their pattern of LOH at the RFLP markers, we mapped the extent of LOH revealed by the RFLP analysis using a set of microsatellite markers. Six informative microsatellite markers on the p arm of chromosome 17 were identified in the parental MSU-1.1 cell strain (Fig 4). Three are located between *p53* and the telomeric RFLP marker; the other three are located between *p53* and the centromeric RFLP marker. A representative microsatellite analysis gel is shown in Figure 5. Analysis of the 15 cell strains using these informative microsatellite markers revealed seven distinct patterns of LOH (Fig 6). To determine whether the LOH seen in these strains for 17p resulted from some kind of generalized genomic instability caused by the loss of *p53* function, we examined the status of informative microsatellite markers on chromosomes 11 and 18. This analysis revealed no LOH of these markers for any cell strain tested (data not shown).

The results from the RFLP analysis, taken together with the comparative Southern blotting data indicating that focus-derived cell strains that contain two copies of mutant p53, support the hypothesis that homologous recombination is involved in the generation of the MNU-induced, focus-derived cell strains that express two mutant p53 alleles. The multiple patterns of LOH indicate that recombination occurred within these focus-derived cell strains, but does not indicate that such recombination could not have arisen spontaneously. To see if such patterns are also found in a fraction of the focus-derived strains, we analyzed ionizing radiation-induced focus-derived cell strains isolated from the

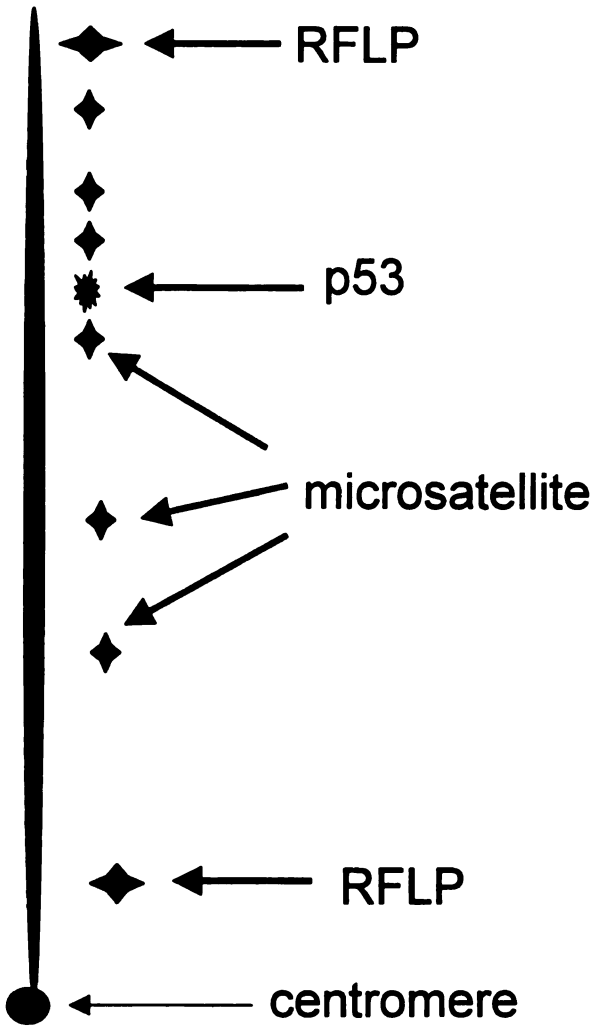

Figure 4. Relative location of informative RFLP and microsatellite markers on the p arm of chromosome 17 in MSU-1.1 cells

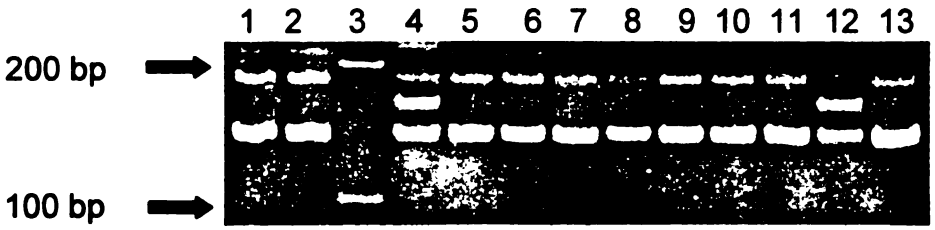

Figure 5. Representative microsatellite analysis of the informative D17S796 marker on the p arm of chromosome 17. PCR was carried out as described in Materials and Methods, with 10 μl of PCR product being loaded per sample. *Lanes 1-10, 13*, focus-derived cell strains that express only mutant p53, *lane 12*, MSU-1.1 cells

Figure 6. Patterns of LOH at informative markers on the p arm of chromosome 17 in MNU-induced focus-derived cell strains that express only mutant p53. The top line represents the p arm of chromosome 17 and the relative distance between each marker and the telomere, expressed in centiMorgan units. ○, retention of heterozygosity at a marker, ●, loss of heterozygosity. The number of independent focus-derived cell strains representing each pattern is as follows: patterns 2, 3 and 4, one strain each, patterns 1 and 6, two strains, pattern 5, three strains, pattern 7, five strains.

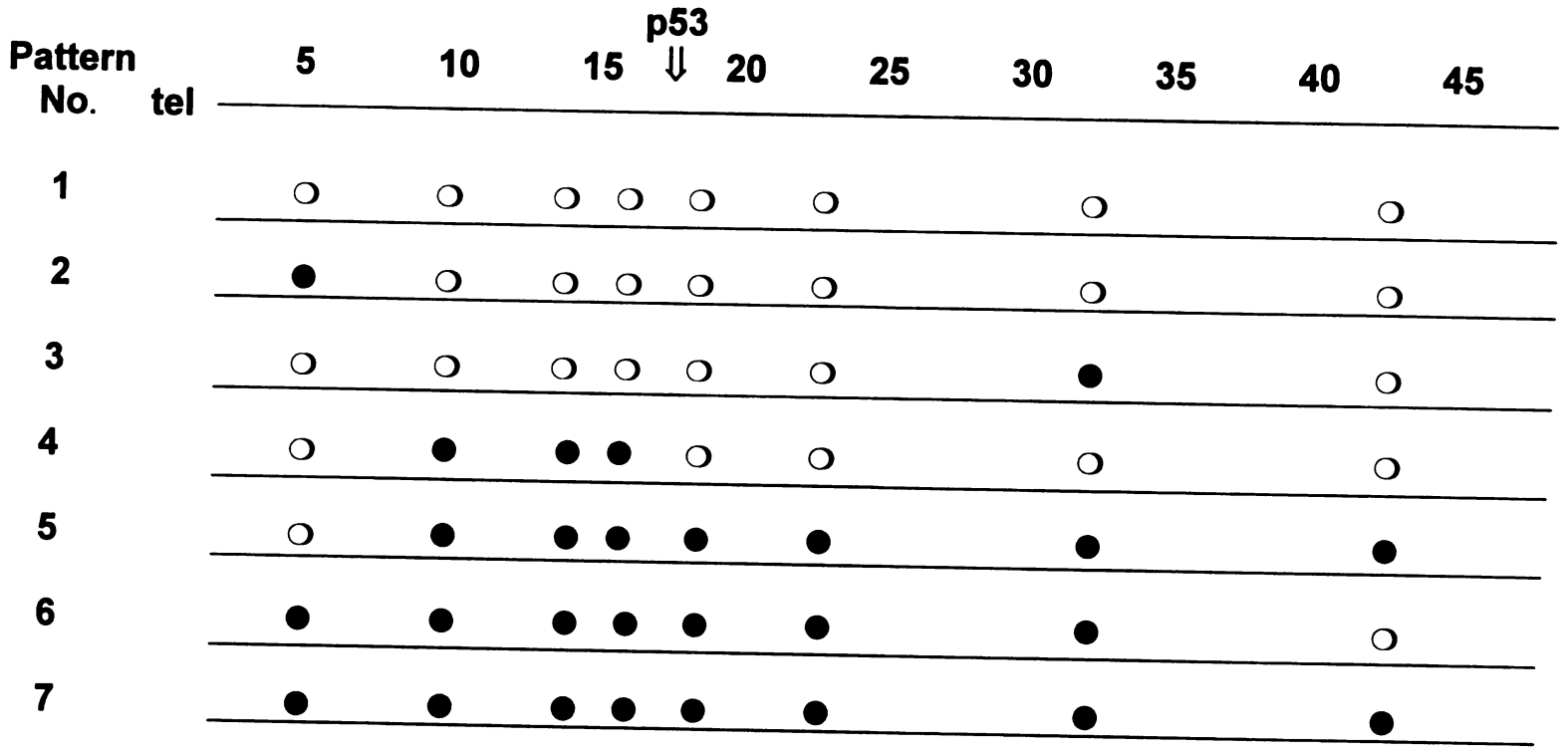

Figure 6.

same population of MSU-1.1 used in our study. The results with ionizing radiation-induced strains showed only two patterns of LOH (data not Shown), viz., pattern 1 (no LOH at any marker) or pattern 7 (LOH at all markers tested) (see Fig.6). These results suggest that the LOH patterns we observed with MNU-treated cell strains are not pre-existing within the subpopulation, but rather, represent MNU-induced LOH.

Attempt to Isolate the Pre-Existing Precursor Containing the Mutation at Codon 215. From the results summarized in Table 3, it is clear that loss of p53 is not sufficient to cause focus-derived cells to be transformed into tumor-forming cells, because only 10 out of 15 strains that lacked functional p53 were malignant. Nevertheless, only those focus-derived cells that had lost all p53 function (or in one case, had lost the function in one p53 allele) formed tumors. These data indicate that although loss of p53 function is not sufficient for carcinogen-induced transformation of MSU-1.1 cells to tumorigenicity, it greatly increases the chance that a strain will be malignant. However, the role that loss of p53 function plays in *focus formation* is less clear. It is evident from Table 3 that such loss is not necessary since 59% (23/39) of the focus-derived cell strains retain wild type p53. Nevertheless, 15 out of 39 have lost p53 function and 1 out of 39 (2.5%) is heterozygous.

Finding that 38.5% of the foci assayed are composed of cells that have lost p53 function, probably as a result of mitotic recombination, raises the question: Does the loss of p53 directly cause focus formation? If it does not, and focus-formation results from an additional, as yet unidentified genetic change

induced by the carcinogen treatment, then cells containing the codon 215 mutations would have to exist in the population at a very high frequency. To examine this question, we plated untreated MSU-1.1 cells at cloning densities and analyzed the p53 status of the cell derived from 80 independent clones, using RT-PCR and *Nla*III digestion. None exhibited an alteration at codon 215. Therefore, the frequency of the subpopulation has to be less than 1.25%. We can estimate that it is not lower than 0.01% because in the course of our studies on the frequency of MNU-induced focus formation we performed a limiting dilution assay to reduce the background frequency of focus formation. For this purpose, the population of MSU-1.1 cells was plated into 10 dishes at 5000 cells per dish and the ten populations were expanded and cryogenically preserved. Progeny cells from five of these limiting dilution sets of cells were assayed for spontaneous focus formation and showed a low background of foci ($< 3 \times 10^{-6}$ cells assayed), and these populations were subsequently used for MNU treatment. Over the next two years or more, focus-derived cell strains devoid of p53 transactivating function as a result of a codon 215 mutation were isolated from each of these five groups of MNU-treated cells.

Assaying Tumorigenic Focus-Derived Cell Strains for Anchorage Independence. Focus-derived MSU-1.1 cell strains transformed to the malignant state by overexpression of a transfected oncogene or by carcinogen treatment form large sized colonies in soft agarose (i.e., exhibit anchorage independence) at a high frequency (4, 6). Therefore, representative MNU-induced, focus-derived, tumorigenic and nontumorigenic cell strains were

assayed for this phenotype. As shown in Table 3, column 4, six of the 11 tumorigenic strains formed large colonies at a high frequency (>30% of colonies measured were larger than 80μm in diameter). The five nontumorigenic cell strains that had lost p53 function did not form colonies at a high frequency. Of the four nontumorigenic strains containing wild type p53 that were assayed, two strains formed colonies at a high frequency, while two did not. These results indicate that cells that can form tumors are likely to be anchorage independent, and this phenotype is not controlled by p53 status.

Discussion

It has been recognized for two decades that methylating agents, including MNU, can induce malignant tumors in animals (27). One of these early studies showed a correlation between the frequency of induction of thymomas in mice by MNU and ethylnitrosourea and the frequency of adducts formed by these carcinogens at the O^6 position of guanine (28). Studies in our laboratory by Domoradzki et al. (29,30) demonstrated that O^6MeG is the principal cytotoxic and mutagenic adduct formed in diploid human fibroblasts by MNNG. This result was confirmed by Lukash et al. (15) who used pretreatment with O^6BzG to deplete populations of such cells of AGT activity and determined the effect on the frequency and spectrum of mutations induced by MNNG in the *HPRT* gene. Zhang et al. (26), using several approaches in a series of human cell strains, including an MSU-1.1 derivative cell strain, MSU-1.2, showed that O^6MeG is the adduct principally responsible for MNNG-induced cytotoxicity and intrachromosomal homologous recombination. The present study shows that O^6MeG is also the MNU adduct principally responsible for transforming MSU-1.1 cells into focus-forming cells (Fig 1B) and converting a substantial number of these into malignant cells (Table 3). Approximately 30% of the representative focus-derived cell strains taken from independent sets of target cells that were assayed for tumorigenicity formed malignant tumors in athymic mice with a short latency. The focus-forming tumorigenic cell strains isolated from populations pretreated with O^6BzG to deplete them of AGT activity were transformed by a significantly lower dose of MNU than was required for those isolated from

populations that received MNU alone. These data strongly suggest that the inactivation of AGT, and subsequent persistence of O^6MeG adducts accounts for the tumorigenic transformation induced in these cells by MNU. These results may have clinical significance since methylating agents are used as chemotherapeutic agents (31,32). Chemotherapy protocols are designed to maximize the cytotoxic effects of specific agents but not at the cost of inducing therapy-related new malignancies. The results of our study indicate that with agents that act through formation of O^6MeG, these two effects can be tightly linked.

Use of the transgenic yeast assay provided a rapid method to determine whether one or the other p53 allele in a specific cell strain coded for p53 protein that lacked transactivating function. With this method we determined that approximately 40% of focus-derived cell strains tested (15/39) had lost p53 transactivating ability (Table 3). Injection of the cells into athymic mice showed that the majority (67%, 10/15) of the focus-derived cell strains that lacked wild type p53 was able to form malignant tumors and the tumors reached a diameter of 1 cm within a relatively short period of time. Taken together, the data in Table 3 indicate that loss of wild type p53 is not required to convert a cell into a focus-forming cell. It is also not sufficient to transform MSU-1.1 cells into tumor-forming cells. However, loss of wild type p53 is causally involved in such transformation because only cell strains that lacked wild type p53 formed tumors.

This conclusion is supported by our finding that focus-derived cell strain MA2-1 which had one wild type and one mutant p53 allele, exhibited a longer

latency period than the strains totally devoid of p53 transactivating ability and that the cells derived from the latter tumors has lost their wild type p53 activity. Unpublished data fom this laboratory shows that the length of the latency period is a function of the number of malignant cells injected into the athymic mice. Therefore, we hypothesize that during the time MA2-1 cells were propagated to obtain sufficient cells for injection, >4 x 10^7 cells, some fraction of the population lost the wild type p53 allele, and it was this subset of cells that eventually gave rise to the tumors observed with this strain.

The frequency of focus-forming cells induced in the MSU-1.1 population by MNU (Fig 1B) is similar to the frequency of 6-thioguanine resistant mutants, i.e., cells with a mutation in the *HPRT* gene, of foreskin-derived diploid human fibroblasts treated with a comparable cytotoxic dose of MNNG (15,29). Although we have not identified the genetic change responsible for allowing the cells to form foci, the frequency is consistent with a single-hit event involving activation of an oncogene or inactivation of a recessive allele that is present as a single copy. The *HPRT* gene on the X-chromosome is an example of the latter case. For the focus-derived cell strains isolated from MNU-treated populations and shown to retain wild type p53 function (~60%), a dominant-acting change such as acquisition of the ability to produce or overexpress a required protein, would be sufficient. But it is difficult to explain why ~40% of focus-derived cell strains have lost all wild type p53 function, unless this loss were contributing strongly to, indeed responsible for, the focus formation.

The identification of a focus-derived strain that had one wild type p53 allele and one allele containing the pre-existing codon 215 mutation, taken together with the data in Figure 5 showing evidence of mitotic recombination or gene conversion between the p arm of chromosome 17 carrying the mutant p53 allele and the p arm carrying the wild type allele, strongly supports the hypothesis that the precursor is heterozygous for the codon 215 mutation, that exposure to carcinogen induces the recombination event, and that loss of p53 function manifests itself in focus formation.

In summary, the molecular analysis of the focus-derived cell strains found to express only mutant p53 strongly suggested that MNU treatment is inducing recombination in a heterozygous precursor cell. This is an illustration of how loss of tumor suppressor gene function occurs *in vivo*; specifically that loss of function is due to a mutation in one allele of a tumor suppressor gene followed by inactivation of the remaining wild type allele through LOH. Therefore, this system mimics *in vivo* conditions in that there are subpopulations of cells within tissues existing in a heterozygous state and these cells may be susceptible to conversion to fully mutant via homologous recombination mechanisms. Furthermore, the data in Fig. 6 indicate that this conversion is greatly facilitated through the action of methylating agents.

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