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MECHANISMS OF HOMOLOGOUS RECOMBINATION INDUCED IN HUMAN FIBROBLASTS BY METHYLATING AGENTS AND INSIGHT INTO GENETIC CONTROL OF CELLULAR SENESCENCE

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By

Hong Zhang

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ABSTRACT

MECHANISMS OF HOMOLOGOUS RECOMBINATION INDUCED IN HUMAN FIBROBLASTS BY METHYLATING AGENTS AND INSIGHT INTO GENETIC CONTROL OF CELLULAR SENESCENCE

By

Hong Zhang

Cancer is a group of diseases that result from a series of changes in genes that control cell growth and behavior. One of the hallmarks of cancer cells is genetic instability, including aberrant homologous recombination. To study the mechanism of methylating agent-induced homologous recombination, I have generated two human fibroblast cell strains which contain duplicated copies of a gene coding for hygromycin resistance, each inactivated by a 10-bp *Hin*d III linker insertion mutation at a unique site. I exposed populations of these strains to O^6-benzylguanine to deplete them of O^6-alkylguanine-DNA-alkyltransferase (AGT), the protein needed for repair of the O^6-methylguanine lesion formed by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). By comparing the frequency of homologous recombination induced by MNNG in cells depleted or not depleted of AGT, I determined that O^6-methylguanine is the lesion principally responsible for MNNG-induced homologous recombination. By repeated exposure of one of the two parental cell strains to the cytotoxicity effect of MNNG and selection for resistant cells, I identified three mismatch repair deficient cell strains. I determined that the induction of homologous recombination by MNNG in these mismatch repair-deficient cells is greatly decreased compared to that in the

repair-proficient parental cells. The lack of MNNG-induced homologous recombination in these mismatch repair deficient cell strains is not the result of general defect in the recombination process. They exhibit normal spontaneous or (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[α]pyrene (BPDE)-induced recombination frequencies. These data support the hypothesis that futile mismatch repair of mismatches containing induced O^6-methylguanine results in persistent discontinuities in DNA, which then initiate recombination.

Another hallmark of cancer cells is immortalization. Most cancer cells escape senescence and can divide indefinitely. Cellular senescence is considered an important tumor suppression mechanism. The molecular mechanism underlying cellular senescence is not well studied. In the course of these studies, I serendipitously generated two cell strains whose senescence/immortal phenotypes are controlled by the tetracycline-regulatory expression system randomly inserted into chromosomes. I hypothesize that a gene controlling senescence is activated by the active tetracycline-regulatory enhancer-promoter inserted adjacent to it, and leads to cellular senescence. I have begun to characterize those genes.

DEDICATION

To my parents, Sulan Wu and Yunchang Zhang,

For the love and the values that you teach me!

To my wife, Jie Song.

For the past, present and future!

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1-NOP: 1-nitrosopyrene

4-NQO: 4-nitroquinoline

ACF: aberrant crypt foci

AGT: O^6-alkylguanine DNA alkyltransferase

AP-1: activator protein 1

APC: adenomatous polyposis coli

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

Cdk: cyclin-dependent kinase

DMN: dimethylnitrosamine

EGF: epidermal growth factor

FAP: familial adenomatous polyposis

FISH: fluorescence *in situ* hybridization

GTBP: G:T binding protein

HNPCC: hereditary nonpolyposis colorectal cancer

HPRT: hypoxanthine-guanine phosphoribosyltransferase

HPV: human papilloma virus

Htk: the herpes simplex virus I thymidine kinase gene

Hyg: the hygromycin phosphotransferase gene

IGF-BP3: insulin-like growth factor-binding protein 3

JPS: juvenile polyposis syndrome

MNNG: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

MNU: *N*-methyl-*N*-nitrosourea

N-AcO-AAF: *N*-acetoxy-2-acetylaminofluorene

NF1: neurofibromatosis type 1

O^6-BzG: O^6-benzylguanine

O^6-MeG: O^6-methylguanine

PCNA: proliferating cell nuclear antigen

SA-β-gal: senescence-associated β-galactosidase

UC: ulcerative colitis

UV: ultraviolet

VHL: von Hippel-Lindau

XP: xeroderma pigmentosum

INTRODUCTION

Cancer is a group of diseases that result from a series of changes in genes that control cell growth and death (Peto, 1977; Bishop, 1991; Hunter, 1997). Carcinogenesis is a multistep process in which, by a series of steps, a normal cell acquires inherited changes in a number of critical genes, e.g., mutations that activate protooncogenes and mutations that inactivate tumor suppressor genes. These genetic changes promote clonal selection of cells with increasingly aggressive growth behavior (Bishop, 1991, Hunter, 1997). The multistep nature of carcinogenesis is well supported by studies of human tumors (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996) as well as studies of neoplastic transformation in culture (McCormick and Maher, 1988; 1994; 1996).

One of the hallmarks of cancer cells is genetic instability. Cancer cells often have increased frequencies of mutation, chromosome abnormality, recombination, loss of heterozygosity, microsatellite instability, etc. (Sandberg, 1984; Solomon et al., 1991; Rabbits, 1994; Sengstag, 1994). Recombination between homologous DNA sequence can result in many genetic changes, including translocation, amplification, deletion, loss of heterozygosity and microsatellite instability, which play a pivotal role in carcinogenesis (Sengstag, 1994). Many carcinogens can induce homologous recombination in human cells (Bhattacharyya et al., 1990; Tsujimura et al., 1990; Benjamin and Little, 1992; Zhang et al., 1996). It is clear that in addition to their mutagenic ability, many carcinogens play an important role in the development of tumors through their

recombinogenic ability. Aberrant homologous recombination contributes significantly to genetic instability found in cancer cells. It has been shown that inappropriate homologous recombination can result in the activation of oncogenes and the inactivation of tumor suppressor genes (Sengstag, 1994). Thus homologous recombination plays an instrumental role in certain neoplasia. The mechanisms of carcinogen-induced homologous recombination are not well understood. DNA strand breaks are considered to be the cause of Ionizing radiation induced homologous recombination (Resnick, 1976). DNA damage caused by bulky chemical carcinogens, ultraviolet radiation, or cross-linking agents, which interferes with DNA replication and causes a delay in replication fork progression, has been shown to induce homologous recombination. It has been suggested that the discontinuities by delayed fork progression are what initiate this recombination (Bhattacharyya et al., 1990; Tsujimura et al., 1990). However, the mechanisms of homologous recombination by alkylating agents, which do not greatly interfere with replication, are unknown.

Another hallmark of cancer cells is immortalization. Normal primary cells have a limited life span. They only can proliferate for a limited number of times in culture before they enter a stage called cellular or replicative senescence (Hayflick and Moorhead, 1961; Hayflick, 1965). Senescent cells do not divide, and are arrested in the G1 phase of the cell cycle (Sherwood et al., 1988). In contrast, most cancer cells have escaped from senescence (Leibovitz, 1986), suggesting that immortalization is a common and early event during carcinogenesis. Cellular senescence is considered to represent a tumor

suppression mechanism (Sager, 1991; Smith and Pereira-Smith, 1996). Cellular senescence is genetically programmed, acting as a dominant trait (Vojta and Barrett, 1995). Cell-fusion studies indicate that there are four complementation groups, suggesting that multiple genes or pathways are involved in cellular senescence (Pereira-Smith and Smith, 1988), and chromosome transfer studies indicate that potential senescence genes are located on chromosomes 1, 2, 3, 4, 6, 7, 11, 18, and X (Vojta and Barrett, 1995; Smith and Pereira-Smith, 1996). However, none of these genes has been cloned.

The objective of my studies was to investigate the mechanisms of alkylating agent-induced homologous recombination in human cells in order to shed light on the relationship between recombination induced by these agents and neoplastic transformation. During the course of my recombination studies, I serendipitously made a very interesting observation, which led me to initiate a study of the genetic control of cellular senescence in human fibroblasts.

In Chapter 1, I review the literature that supports the multistep nature of carcinogenesis and the general mechanisms of homologous recombination, and its relationship with carcinogenesis. Alkylating agents and mismatch repair, as well as the phenomena of cellular senescence, its role in carcinogenesis and the genetic programs that control cellular senescence are also discussed in Chapter 1. Chapter 2 is a manuscript that was published in 1996 in **Carcinogenesis**. It describes the research I carried out on *N*-methy-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-induced homologous recombination. I generated and characterized two MSU-1.2-derived cell strains that carry two mutant genes coding for hygromycin

resistance as the recombination substrate, and showed that O^6-methylguanine, a lesion caused by MNNG, is the principal lesion that is responsible for MNNG-induced homologous recombination. The second and third authors contributed the data in Figure 3 and 4, respectively. Chapter 3 is prepared in the form of a manuscript that will be submitted for publication. In this chapter, I examine the role of mismatch repair in MNNG-induced homologous recombination using three mismatch repair deficient human cell strains that I generated for this study. I show that MNNG-induced homologous recombination is significantly reduced in these three mismatch repair deficient cell strains, whereas spontaneous or (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[α]pyrene (BPDE)–induced homologous recombination is similar to that of the mismatch repair proficient parental cells, suggesting that functional mismatch repair is required for MNNG-induced homologous recombination. The third and fourth authors contributed the data in Figure 3. Chapter 4 is prepared in the form of a manuscript that will be submitted for publication. By serendipity, I generated two human fibroblast cell strains whose senescence/immortal phenotypes are controlled by a tetracycline regulatory expression system stably inserted into a chromosome. In one cell strain, I have determined the location of the inserted tetracycline regulatory enhancer-promoter to be on chromosome 1 in the region 1p31.3-33. I predict that a gene controlling onset of senescence is located in that region. Using the chromosomal walking technique, I determined that genomic DNA sequences flanking the inserted tetracycline regulatory enhancer-promoter in two cell strains differ from each other and share no significant homology with

any genomic sequences in the databases. In the Appendix, I discuss the future plans to clone the potential senescence gene and characterize its function in cellular senescence.

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CHAPTER 1

LITERATURE REVIEW

I. Carcinogenesis is a multistep process

It is generally recognized that cancer is a group of diseases that results from a series of changes in genes that control cell growth and behavior. These genetic changes transform a healthy cell into a cancer cell. Carcinogenesis is a multistep process in which, by a series of steps, a normal cell acquires inherited changes in a number of critical genes, e.g., mutations that activate protooncogenes and/or mutations that inactivate tumor suppressor genes. Identification of these critical genes involved in neoplastic transformation enhances our understanding of the nature of the multistep process of carcinogenesis.

A. Cancer is a group of genetic diseases

1. Mutation hypothesis of carcinogenesis

Accumulation of evidence supports that cancer arises from genetic changes in the DNA that promote clonal selection of cells with increasingly aggressive behavior. It was recognized two centuries ago that chemicals can induce cancer in humans (Miller, 1978). Many chemical carcinogens or their activated metabolites can react with DNA and cause mutations (Maher et al., 1968; Miller and Miller, 1981). The most plausible hypothesis of carcinogenesis is that cancer is caused by mutations in critical genes. Consistent with this mutation theory of

carcinogenesis, carcinogens are often mutagens (Lawley, 1989). Ames and his colleagues tested the mutagenicity of many carcinogens using the Ames test (Ames et al., 1973; Maron and Ames, 1983). According to their studies, about 90% of all carcinogens tested are also mutagens. Moreover, few non-carcinogenic agents show significant mutagenicity using this test system (McCann et al., 1975). There is some quantitative correlation between the mutagenicity and carcinogenicity of specific chemical agents. For example, alkylating agents form O^6-alkylguanine adducts in the DNA. Their carcinogenic potential is directly related to their mutagenicity (Frei et al., 1978; Swenson et al., 1986).

The observation of chromosomal abnormalities in cancer cells further strengthens the mutation hypothesis of carcinogenesis. The Philadelphia chromosome is the first chromosomal abnormality that was found to be definitely associated with human cancer (Nowell, 1965). The Philadelphia chromosome results from a reciprocal chromosomal translocation between long arms of chromosomes 9 and 22 [t(9;22) (q34;q11)] (Rowley, 1973), and it is found in the leukemia cells of more than 90% of patients with chronic myelocytic leukemia. To date, more than 100 commonly occurring translocations have been observed in leukemias, lymphomas and solid tumors (Solomon et al., 1991; Trent and Meltzer, 1993; Rabbits, 1994). In addition to translocation, other chromosomal abnormalities found in human cancer cells include inversion, insertion, deletion, amplification and aneuploidy (Sandberg, 1984). These chromosomal alterations

most often result in the activation of protooncogenes or the inactivation of tumor suppressor genes.

2. Familial cancer syndromes

The vast majority of mutations in cancer are somatic and found only in an individual's cancer cells. Inherited cancers represent a small fraction, about 1% of all human cancers (Knudson, 1977). The individuals with hereditary cancer syndromes carry a particular germline mutation in every cell of their body. About 50 forms of hereditary cancer syndromes have been reported (Li, 1988; Birch, 1994; Fearon, 1997). Genes, for which certain mutant alleles have been demonstrated to cause highly penetrant cancer syndromes, have been identified. Members from the Li-Fraumeni syndrome families develop cancer at abnormally early ages, primarily sarcomas and breast cancers. Brain tumors and leukemias have also been found in these families (Garber et al., 1991). It is now recognized that the *p53* gene is responsible for the Li-Fraumeni syndrome (Srivastava et al., 1990). The gene responsible for the familial retinoblastoma syndrome is the *Rb* gene (Friend et al., 1986; Lee et al., 1987). Patients from familial retinoblastoma families develop primarily retinoblastomas, and to a lesser extent osteosarcomas. The observation of an interstitial deletion of chromosome band 5q21 in familial adenomatous polyposis families led to the discovery of the *APC* gene which is responsible for the development of colorectal cancer in these families (Groden et al., 1991; Nishisho et al., 1991). Patients with familial adenomatous polyposis typically develop hundreds to thousands of colorectal

tumors during their second and third decades of life (Kinzler and Vogelstein, 1996). Collectively, studies of these autosomal, dominantly inherited cancer syndromes suggest that a number of inherited mutations in critical genes contribute to the causation of these cancer (Fearon, 1997).

Further evidence for a causal relationship between mutations and human cancer comes from the studies of certain recessive hereditary cancer syndromes, such as ataxia telangiectasia, Bloom's syndrome, Fanconi's anemia, hereditary nonpolyposis colorectal cancer (HNPCC) and xeroderma pigmentosum (XP). Each of these disorders is characterized by the inability to repair specific kinds of physical or chemical damage in DNA. The incidence of cancer in patients from these syndromes is greatly increased (Setlow, 1978; Lindahl, 1994).

XP patients are characterized by extreme sensitivity of the skin to sunlight and a strong predisposition to sunlight-induced melanocarcinomas and basal cell and squamous cell carcinomas of the skin, in addition to other nonneoplastic cutaneous and ocular abnormalities (Cleaver, 1990). Somatic cell genetics has identified seven complementation groups (XP-A to XP-G) defective in nucleotide excision repair and one group that complement all the others (XP variant) (Cleaver, 1990). The predisposition of XP patients, except for XP variant, to skin cancer is a consequence of defective DNA repair. Cell lines from XP patients are defective in repair of pyrimidine dimers caused by the ultraviolet light from the sun as well as other DNA lesions (Cleaver, 1994). Therefore they are more prone to acquire mutations than normal cells. The association of nucleotide excision

repair deficiency with skin cancer in XP provides a good example of DNA damage and defective repair leading to carcinogenesis.

HNPCC (Lynch syndromes I and II) provides another good example. Lynch I families are susceptible to early onset of colorectal cancer, while Lynch II kindreds are also at risk for extracolonic epithelial tumors of the endometrium, ovary, stomach, small intestine, kidney and ureter (Lynch et al., 1996). The majority of tumors occurring in HNPCC patients contain frequent mutations in the simple repeat sequences $(A)_n$, $(GGC)_n$, or $(CA)_n$. Mutations in these microsatellite repeat sequences are typically tumor-specific, suggesting that they are somatic. Their incidence is dramatic: tumor cells with this characteristic contains thousands of microsatellite mutations (Aaltonen et al., 1993). It was recognized that the microsatellite instability observed in tumors was similar to that observed in bacteria harboring mutations in mismatch repair genes such as *mutS* and *mutL* (Strand et al., 1993). The hypothesis that HNPCC is caused by hereditary mutations of human homologs of *mutS* or *mutL* stimulated the search for such human homologs. Now the majority of HNPCC cases can be attributed to a defect in any one of the multiple loci responsible for DNA mismatch repair. These include the *hMSH2* (Fishel et al., 1993; Leach et al., 1993), *hMLH1* (Bronner et al., 1994; Papadopoulos et al., 1994), *hPMS1* and *hPMS2* genes (Nicolaides et al., 1994). It has been proposed that the initial event in the development of tumors in HNPCC patients is the functional loss of mismatch repair activity (Kinzler and Vogelstein, 1996), resulting in rapid accumulation of mutations in critical genes necessary for neoplastic transformation. For instance, mutations in

the type II TGFβ receptor gene contribute to the development of colorectal cancer. In tumors from HNPCC patients with microsatellite instability, these mutations almost always include frameshift mutations within a microsatellite sequence embedded in the TGFβ receptor II gene (Markowitz et al., 1995).

B. Evidence supporting that carcinogenesis is a multistep process

Cancer is a disease predominantly found in the elderly, with the risk of acquiring the disease increasing with age. Early epidemiological studies have suggested that four to six genetic changes are necessary for tumor formation in adult human (Armitage and Doll, 1954; Peto, 1977; Dix, 1989). Renan (1993) recently addressed the question of the number of mutational changes required for 28 different human malignancies, by plotting the log of the age-specific mortality rate against the log of the age in years of the person affected by cancer. He determined the best-fit linear regression coefficient for each of the 28 tumor types. He concluded that seven to eight genetic changes are required for the formation of tumors in bladder, kidney, lip, pancreas, skin and stomach, and for cancer with very later onset, such as prostate cancer, 12 genetic changes are necessary. These studies provide some evidence for the multistep nature of carcinogenesis.

1. Colorectal tumorigenesis

The multistep nature of carcinogenesis is best illustrated by colorectal tumorigenesis. Colorectal tumors provide an excellent model for studies of the

genetic alterations involved in neoplasia. Most, if not all malignant colorectal tumors (carcinomas) arise from preexisting benign tumors (adenomas) (Sugarbaker et al., 1985). Tumors of various stages of development can be obtained for study. Furthermore, colorectal cancer exists in both hereditary and sporadic forms, allowing the study of both inherited and somatic genetic alterations. Vogelstein and his colleagues studied the hereditary colon cancer syndromes (familial adenoma polyposis and hereditary nonpolyposis colorectal cancer) as well as sporadic tumors, and they have identified the critical genetic changes during each stage of tumor development (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996).

The defect in the *APC* (for adenomatous polyposis coli) gene is responsible for the development of colorectal cancer in familial adenomatous polyposis (FAP) families (Groden et al., 1991; Nishisho et al., 1991). One allele of the *APC* gene is mutated in the germline of the FAP patients (Mandl et al., 1994), and truncation mutations of the *APC* gene have also been identified in about 60% of sporadic colorectal cancers or adenomas (Powell et al., 1992; Ichii et al., 1993). The rate-limiting step in colorectal neoplasia initiation is mutations in the *APC* gene (Levy et al., 1994), which acts as the gatekeeper for colon neoplasia. The alteration of *APC* gene is responsible for the hyperproliferative epithelium present in these patients, and patients develop numerous dysplastic aberrant crypt foci (ACF). Some of the dysplastic ACF progress to early adenomas as they acquire additional mutations. Hypomethylation is present in very small adenomas in

patients with and without polyposis, and this alteration may lead to aneuploidy, resulting in early adenomas.

K-Ras, as an oncogene, requires only one genetic event for its activation. A mutation in the *K-Ras* gene appears to occur in one cell of a preexisting small adenoma, and through clonal expansion produces a larger and more dysplastic tumor (Vogelstein et al., 1988). Mutations in *K-Ras* are considered to play an important role in the development of intermediate adenomas from early adenomas. The progression from intermediate adenomas to later adenomas is accompanied by the allelic loss of chromosome 18q21, which is lost in more than 70% of carcinomas and in almost 50% of later adenomas (Vogelstein et al., 1988). It has been proposed that this chromosomal region may contain several different tumor suppressor genes involved in colorectal neoplasia, with *DCC*, *DPC4* and *JV18-1* as the candidates (Fearon et al., 1990; Kinzler and Vogelstein, 1996).

The loss of a large portion of chromosome 17p, through chromosome loss or mitotic recombination, has been seen in more than 75% of colorectal carcinomas (Vogelstein et al., 1988), but such loss is relatively infrequent in adenomas of any stages. This suggests that the loss of the chromosome 17p gene is responsible for the progress from adenomas to carcinomas. The common region of loss on chromosome 17p in colorectal tumors has been identified and contains the *p53* gene (Baker et al., 1989). The loss of the wild type *p53* gene is often associated with the progression from adenoma to carcinoma. Additional alterations, such as losses of chromosomes 1q, 4p, 6p, 6q, 8p9q and 22q, have been observed in 25-

50% of the cases studied. These additional changes may be responsible for the ability of carcinomas to metastasize and cause death (Fearon and Vogelstein, 1990).

Colorectal cancer arises from a series of mutational events in critical oncogenes and tumor suppressor genes. Mutations in at least four to five genes are required for the formation of a malignant tumor. This can be translated into at least seven genetic events since both alleles of the tumor suppressor genes must be inactivated (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996). It was initially thought that the total accumulation of these genetic changes, rather than their order with respect to one another, is most important (Fearon and Vogelstein, 1990). Now it is recognized that it is not simply the accumulation of mutations, but rather it is also their order that determines the propensity for neoplasia. Changes in only a subset of the genes that can affect cell growth can actually initiate the neoplastic process (Kinzler and Vogelstein, 1996). In the case of colorectal tumorigenesis, the *APC* gene is the gatekeeper. Tumors from patients with HNPCC go through a similar (Huang et al., 1996), but not identical series of mutations (Markowitz et al., 1995). Mismatch repair deficiency in HNPCC speeds up this mutation and subsequent clonal expansion process.

2. Transformation studies in culture: MSU-1 lineage

Not all human malignancies can be studied in such detail as colorectal cancer since many common occurring tumors either lack the defined stages of tumor development, or tumors of different stages are not available easily for study. To

overcome this limitation, McCormick and Maher and their colleagues (McCormick and Maher, 1988; 1994; 1996) have taken an alternative approach to study the number and kind of genetic changes required for the malignant transformation of human cells. They began with normal human fibroblasts in culture and by a stepwise process succeeded in converting them to cancer cells (see below). The advantage of their approach is that one can recapitulate the *in vivo* neoplastic transformation process in culture, and at many stages, the relevant genetic change is known. Their studies suggest that six or more genetic changes are required, which is in keeping with the colorectal tumorigenesis (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996).

Normal human primary cells can only proliferate for a limited number of times in culture before they enter senescence (Hayflick and Moorhead, 1961). For human fibroblasts to be fully transformed into malignancy, several oncogenes need to be transfected into the cells in a step-wise fashion. McCormick and Maher soon recognized that it would be necessary to use infinite life span cells in their transformation studies. They successfully generated the infinite life span MSU-1 lineage following transfection of a *v-Myc* oncogene into a diploid human fibroblast cell, LG1 (Morgan et al., 1991). The clonally-derived *v-Myc*-expressing LG1 population underwent senescence, and as the result of some spontaneous, as yet unidentified genetic changes, an infinite life span, diploid cell strain arose, designated as MSU-1.0. Most likely the *v-Myc* expression contributed to the immortalization process since no immortal cells arises from populations without expression of the *v-Myc*. The results of cell fusion between LG1 and MSU-1.0 or

its derivative cell strain, MSU-1.1, indicate that the spontaneous, as yet unidentified genetic changes in the immortalization of MSU-1.0 probably involve the loss of a tumor suppressor gene which is critical for immortalization. Therefore, at least two genetic changes, if not three were involved in the immortalization step of MSU-1.0.

From the diploid MSU-1.0 cells, a spontaneous variant strain with a growth advantage arose. This cell strain is designated MSU-1.1. MSU-1.1 cells are not tumorigenic but have an alteration in growth control. This cell strain has a stable karyotype with 45 chromosomes including two unique marker chromosomes (Morgan et al., 1991). This suggests that at least two additional genetic changes were involved in the progression of MSU-1.1 from MSU-1.0. MSU-1.1 cells can be transformed into full malignancy by overexpression of an *H-Ras* (Hurlin et al, 1989) or an *N-Ras* oncogene (Wilson, 1990). Expression of the same *Ras* oncogenes at the level found for the endogenous *H-Ras* or *N-Ras* does not cause malignant transformation of MSU-1.1 cells. Lin et al. (1995) showed that transfection of a *v-Fes* oncogene into MSU-1.1 cells expressing *H-Ras* at the normal level was able to cause these cells become malignant, indicating that MSU-1.1 cells require at least two genetic changes to be malignant. These studies indicate that from the normal fibroblasts, LG1, to the fully malignant cells, at least six genetic changes are required, and similar to tumor development *in vivo*, neoplastic transformation of human cells in culture is indeed a multistep process.

In summary, carcinogenesis is a multistep process in which, by a series of steps, normal cells acquire inherited changes in a number of critical genes, e.g., mutations that activate protooncogenes and/or mutations that inactivate tumor suppressor genes. Such mutations frequently give cells a proliferative advantage, which results in the clonal expansion of mutant cells. Clonal expansion increases the chance that a second genetic change could occur in one of the progeny cells. This process is repeated until a cell has acquired all the necessary genetic changes. Clonal expansion of this cell gives rise to tumor.

C. Cancer genes

As discussed above, genes critical for cancer development are generally classified into two categories: oncogenes and tumor suppressor genes. Oncogenes act dominantly in neoplastic transformation, and they only require mutations in one of the two alleles to be activated. The wild type forms of oncogenes are referred as protooncogenes. The products of protooncogenes are involved in the regulation of normal cellular growth and differentiation. Tumor suppressor genes are recessive. To be functionally inactivated, both alleles of a tumor suppressor gene need to be altered, except for certain mutant alleles that act in a dominant negative fashion.

1. Oncogenes

To date, more than 100 oncogenes have been identified. With the increasing diverse functions uncovered for oncogenes, the complexity of cellular growth

control is beginning to be understood. New principles of cell regulation and regulatory networks have begun to be delineated. The deciphering of growth control signaling pathways has furthered our understanding of the mechanisms underlying neoplastic transformation. The positioning of multiple oncogenes on the same pathway has underscored the importance of the signaling pathways in transformation.

1.1. Oncogenes in plasma membrane-to-nucleus signaling pathways

Several signaling pathways are involved in transducing growth control signals from plasma membrane to nucleus. One of the most important pathways is the mitogen-activated protein (MAP) kinase module, which consists of three protein kinases in series, a MAPK, a MAPKK and a MAPKKK (Brunet and Pouyssegur, 1997). The MAPK module has been adapted to transmit responses from many different types of surface receptors, including receptor protein tyrosine kinases, G protein-coupled receptors, and cytokine receptors (Hunter, 1997). Activated MAPKs are translocated into the nucleus, and constitutive activation of MAP kinase-mediated signaling pathways elicits neoplastic transformation. For example, the ERK MAPK pathway, which can be activated by oncoproteins Ras, Raf or Tpl-2, has been shown to be involved in transformation (Cowley et al., 1994; Mansour et al., 1994; Patriotis et al., 1994).

Another important membrane-to-nucleus signaling pathway is the PI3 kinase pathway. Many types of extracellular signals, especially those involving activation of receptor protein tyrosine kinase (Hawkins et al., 1997; Hunter, 1997), stimulate

PI3 kinase activity. PI3 kinase generates 3' phosphoinositides, PI3,4,5,P_3, PI3,4P_2 and PI3P. These molecules act as second messengers, although their effector proteins have not been completely identified.

1.2. *Ras* family

Mutations in the Ras family of oncogenes, including *H-Ras*, *K-Ras*, *N-Ras*, *R-Ras* and *TC21*, are the most frequent in human cancers (Bos, 1989). They represent a class of oncogenes: membrane associated G-proteins. These G-proteins normally serve as signal transducers for multiple growth factor receptor tyrosine kinases (Egan and Weinberg, 1993; McCormick, 1993; Marshall, 1995). When a ligand such as platelet-derived growth factor (PDGF) binds to its receptor on the membrane, the receptor is autophosphorylated and becomes active. The activated receptor binds to adapter proteins Shc and Grb2, which subsequently recruit Sos (for son of sevenless) to the plasma membrane where Ras is bound. Sos is a guanine nucleotide exchange factor. Its interaction with Ras leads to the release of GDP and binding of GTP to Ras (Feig, 1993). The GTP bound form of Ras is active. The oncogenic forms of *Ras* genes have mutations in codon 12, 13, 59, or 61, which reduce their intrinsic GTPase activity and their ability to interact with GAPs, and in that way keep the mutant Ras in the GTP-bound, active form (Tong et al., 1989; Krengel et al., 1990). A number of effectors for Ras have been identified, which bind preferentially to Ras in the GTP-bound state. These include Raf, PI3 kinase, PKC, MAPK, Mek (also known as MAPKK). All of these have been implicated in tumorigenesis (Hunter, 1997).

1.3. Protein tyrosine kinases

The role of mutant activated receptor protein tyrosine kinases (PTKs) in tumorigenesis is well established (Hunter, 1987). Growth factor stimuli are transmitted into cells through specific transmembrane receptors which modify key regulatory proteins in the cytoplasm. The activation of receptor PTKs is ligand-mediated dimerization. Increasing evidence indicates that oncogenic activation of receptor PTKs occurs through mutations that lead to constitutive dimerization and activation of the cytoplasmic catalytic domain. The constitutive activation of receptor PTKs results in unregulated cell growth (Schlessinger and Ullrich, 1992; Hunter, 1997). The best studied PTKs include *EGF* receptor family: *EGFR*, *HER2*, *erb-3* and *erb-4* (Schlessinger and Ullrich, 1992; Plowman et al., 1993) and *Src* gene family (Bolden et al., 1992; Brown and Cooper, 1996).

1.4. Transcription factors

Modulation of gene expression is frequently an important ramification of intracellular signaling and plays a critical role in the control of cell proliferation and differentiation. A number of nuclear transcription factors and a variety of transcriptional regulators have been implicated as oncogenes (Lewin, 1991; Forrest and Curran, 1992). One example is the transcription activator protein 1 (AP-1). AP-1 serves as the nuclear target of many oncogenic signal transduction pathways (Ransone and Verma, 1990; Angel and Karin, 1991). This multigene family includes *Fos* genes (*c-Fos*, *Fos B*, *Fra-1* and *Fra-2*) and *Jun* genes (*c-Jun*,

Jun B and *Jun D*). AP-1 complex binds to specific DNA sequences and regulates the transcription of genes containing the *cis* element in their promoters. Altered activity of AP-1 complex affects mitogenic control and promotes neoplastic transformation (Vogt, 1994).

1.5. Oncogenes in cell cycle control

Direct connections between aberration of the cell cycle and cancer have been evident for some time (Sherr, 1996). Progression through the cell cycle is strictly controlled by a family of cyclin proteins, cyclin-dependent kinases (Cdks), and cdk inhibitors (Hartwell and Kastan, 1994; Hunter and Pines, 1994). In general, activation of Cdks requires binding of cyclins, and can be constrained by at least two families of Cdk inhibitors (Sherr and Roberts, 1995). Unique combinations of cyclins and Cdks assemble during each phase of the cell cycle, and their association allows the subsequent activation of the complex and drives cell proliferation forward by phosphorylating specific substrates. For instance, the D type cyclins (D1, D2, and D3) and Cyclin E are the primary G1 phase cyclins in mammalian cells (Sherr, 1993). Cyclin D is associated with Cdk4 and Cdk6 during G1 phase. The retinoblastoma protein, Rb and related family members (p107, p130) are critical targets for cyclin D-Cdk4 and cyclin D-Cdk6 complexes. Rb protein is phosphorylated by these complexes during mid-G1 phase, and its phosphorylation enables the release of a family of heterodimeric transcription factors, collectively termed the E2Fs, which can activate the transcription of genes required for S phase entry and progression (Weinberg, 1995). These

genes include most notably dihydrofolate reductase, thymidine kinase, *Cdc2* and *c-Myc* (Nevins, 1992; Lathangue, 1994). Phosphorylation of Rb is initially triggered by the cyclin D-dependent Cdks (Sherr, 1994), and then accelerated by the cyclin E-Cdk2 complex (Weinberg, 1995).

1.6. Oncogenes in apoptosis

An emerging scheme in carcinogenesis is the acquired ability of tumor cells to avoid undergoing apoptosis in response to DNA damage or other conditions that would induce a normal cell to commit apoptosis. Bcl2 family proteins are key regulators of apoptosis. These proteins have been categorized as either pro-apoptotic (Bax or Bad) or anti-apoptotic (Bcl2 or Bcl-X_L). The balance of pro- versus anti-apoptotic Bcl2 family members dictates the cellular response (Reed, 1995). Two of the genes transactivated by p53, *Bax* and *IGF-BP3* (insulin-like growth factor-binding protein 3), can also influence the decision to undergo apoptosis. Bax binds to Bcl2 and antagonizes its ability to block apoptosis (Miyashita and Reed, 1995). IGF-BP3 blocks the IGF mitotic signaling pathway by binding to IGF and preventing its interaction with its receptor (Buckbinder et al., 1995). The blocking of IGF activity is considered to enhance apoptosis.

2. Tumor suppressor genes

To date, about a dozen tumor suppressor genes have been identified. Some of these genes have been extensively studied. I will discuss the *Rb*, *p53* and *Cdk*

inhibitor genes as examples, and then introduce the concept of gatekeepers, caretakers and landscapers.

2.1. The *Rb* and *Cdk* inhibitor genes

Progression through the cell cycle is strictly controlled by a family of cyclin proteins, cyclin-dependent kinases (Cdks), and Cdk inhibitors (Hartwell and Kastan, 1994; Hunter and Pines, 1994). As discussed above, unique combinations of cyclins and Cdks assemble during each phase of the cell cycle, and their association allows the subsequent activation of the complex and drives cell proliferation forward by phosphorylating specific substrates. The retinoblastoma protein, Rb, and related family members (p107, p130) are critical targets for cyclin D-Cdk4 and cyclin D-Cdk6 complexes. As mentioned earlier, Rb protein is phosphorylated by these complexes during mid-G1 phase, and its phosphorylation enables the release of a family of heterodimeric transcription factors, collectively termed the E2Fs, which can activate the transcription of genes required for S phase entry and progression (Weinberg, 1995). Phosphorylation of Rb is initially triggered by the cyclin D-dependent Cdks (Sherr, 1994), and then accelerated by the cyclin E-Cdk2 complex (Weinberg, 1995).

In general, activation of Cdks requires binding of cyclins, and can be constrained by at least two families of Cdk inhibitors (Sherr, 1994; 1996; Sherr and Roberts, 1995). INK4 proteins, including p15, p16, p18, and p19, inhibit the kinase activities of Cdk4 and Cdk6, in that way negatively regulate cell cycle

progression through G1 phase (Kamb et al., 1994; Nobori et al., 1994). Another family of Cdk inhibitors, which inhibit cyclin D-, E-, and A-dependent kinases, includes at least three proteins: p21, p27 and p57 (Harper et al., 1993; Xiong et al., 1993; Polyak et al., 1994). p21 is also capable of binding to proliferating cell nuclear antigen (PCNA), and can thereby affect DNA replication (Waga et al., 1994).

2.2. The *p53* gene

As discussed above, apoptosis plays an important role in carcinogenesis. One important apoptosis regulator is p53 protein. More than 50% of human tumors contain mutations in the *p53* gene (Hollstein et al., 1994). p53 has been shown to play an important role in triggering apoptosis in response to some signals (Ko and Prives, 1996; Levine, 1997). p53 is activated by different types of DNA damage, and this activation results in a rapid increase of the protein level in cells and activation of p53 as a transcription factor (Cox and Lane, 1995; Ko and Prives, 1996; Levine, 1997). In some cell types, or under some circumstances, p53-mediated apoptosis requires its transactivation activity (Haupt et al., 1995). Two of the genes transactivated by p53 could influence the decision to apoptosis: *Bax* and *IGF-BP3*. Another possible downstream event of p53 activation is to direct signals through unidentified mediators to initiate apoptosis independent of its transactivation activity.

In addition to its role in apoptosis, p53 is also involved in DNA repair and cell cycle control. p53 transactivates the transcription of *GADD45* gene in response

to DNA damage (Kastan et al., 1992). The GADD45 protein interacts with the replication and repair factor PCNA and inhibits the entry of cells into S phase (Smith et al., 1994). p53 protein also interacts directly with proteins that are involved in DNA replication and repair, such as RP-A (Dutta et al., 1993), TFIIH components (XPD, XPB and p62) and CSB (Wang et al., 1995). *p21* is the most well studied p53 responsive gene (El-Deiry et al., 1993; Xiong et al., 1993). In response to DNA damage, the activated p53 protein transactivates the transcription of *p21*, whose gene product in turn inhibits cyclin D-, E- and A-dependent Cdks (Harper et al., 1993; Xiong et al., 1993). p53-dependent G1 arrest is mediated, at least in part, through p53's induction of p21 (El-Deiry et al., 1993).

2.3. Gatekeepers, caretakers and landscapers

As discussed above, in general, oncogenes have relatively well defined functions, working as either growth factors, protein tyrosine kinases (both receptors and non-receptors), Thr/Ser kinases, transcriptional regulators, cyclins, Cdks, or involved in regulation of apoptosis. However, tumor suppressor genes are not so well studied. Some of the tumor suppressor genes control cell proliferation directly, such as *p53*, *Rb*, *APC* (adenomatous polyposis coli), *VHL* (von Hippel-Lindau), *NF1* (Neurofibromatosis type 1) and *p16*. It has become clear that mutations in genes that maintain the integrity of the genome may be the more frequent causes of inherited predisposition to cancer. These include genes responsible for xeroderma pigmentosum (*XPA-XPG*), whose gene

products are involved in nucleotide excision repair (Cleaver, 1994), genes responsible for hereditary nonpolyposis colorectal cancer (HNPCC) (*hMSH2*, *hMLH1*, *hPMS1*, and *hPMS2*), whose gene products are involved in mismatch repair (Kolodner, 1996), *BRCA1* (Scully et al., 1996; Ludwig et al., 1997), *BRCA2* (Milner et al., 1997; Sharan et al., 1997), *ATM* (ataxia telangiectasia) (Rotman and Shiloh, 1997) and *BLM* (Bloom's syndrome) (Ellis et al., 1995). Another class of indirectly acting tumor suppressor genes is suggested by recent studies of juvenile polyposis syndromes (JPS). The genes responsible for JPS are identified as *PTEN* and *SMAD4* (Lynch et al., 1997; Howe et al., 1998; Olschwang et al., 1998). The increased cancer susceptibility of JPS seems to be the product of an abnormal stroma environment, which affects the development of adjacent epithelial cells. Kinzler and Vogelstein (1997; 1998) recently proposed to classify tumor suppressor genes or tumor susceptibility genes into three categories: gatekeepers, caretakers and landscapers. The meaning of these terms is discussed below.

2.3.1. Gatekeepers

Gatekeepers are genes that directly regulate the cell growth or apoptosis. Each cell type only has one or a few gatekeepers, and inactivation of a given gatekeeper leads to a very specific tissue distribution of cancer. For example, *APC*, *Rb* and *VHL* and *NF1* genes are the gatekeepers for cells of colon, retina and kidney, and Schwann cells, respectively. Inactivation of these genes is rate limiting for the initiation of neoplasia. People with hereditary mutations in a

gatekeeper gene are at much greater risk (>10^3 fold) of developing tumors than the general population since they only need one additional mutation, whereas the general population needs both copies of the relevant gatekeeper gene to be mutated. In colorectal tumorigenesis, general population has a 5% risk of developing colon cancer, while families of familial adenomatous polyposis (FAP) have a 95% risk (Kinzler and Vogelstein, 1998). This is consistent with Knudson's hypothesis (Knudson, 1971). Challenge remains to identify the gatekeepers for the most common cancers, such as breast and prostate cancers.

2.3.2. Caretakers

In contrast to gatekeepers, inactivation of caretakers does not initiate neoplasia directly. The function of caretaker genes is to maintain the integrity of genome. Inactivation of these genes leads to genetic instability which results in increased mutation rate of all genes, including gatekeeper genes. In members of families with an inherited cancer predisposition syndrome involving caretaker type genes, such as HNPCC and XP, at least three independent mutations are needed to initiate neoplasia: mutation in the normal caretaker allele inherited from the unaffected parent, and mutations in both copies of the relevant gatekeeper genes. Therefore, the risk of cancer in affected families at about 70% is generally only 5-50 fold greater than in the general population (Kinzler and Vogelstein, 1998). However, once such a tumor is initiated by the inactivation of a gatekeeper, it may progress rapidly due to the accelerated mutation rate in other genes which directly control cell growth or apoptosis. Inactivation of

indirectly acting caretakers is not required for neoplasia, and most sporadic tumors will evolve without their inactivation (Kinzler and Vogelstein, 1997).

2.3.3. Landscapers

Studies of JPS and ulcerative colitis (UC) suggest another class of indirectly acting tumor susceptibility genes. Their functions are quite different from those of caretakers. Individuals with JPS or UC have a risk of 10-20% developing colorectal cancer (Kinzler and Vogelstein, 1998). At a young age, the stroma cells in these patients' colon develop into multiple hamartomatous polyps, which are markedly different from adenomatous polyps. Polyps from JPS or UC patients have a low potential to become malignant, and comprise a mixture of mesenchymal and inflammatory elements in which epithelium is entrapped. However, the entrapped epithelial cells have an increased risk becoming malignant. It is possible that the regeneration of epithelial cells to replace the damaged ones increases the probability of acquiring mutations in this abnormal microenvironment. This raises the question whether solid tumors are simply composed of neoplastic epithelial cells, or whether interactions between different cells in that tumor mass can influence the neoplastic transformation process. The increased cancer susceptibility due to inherited mutations in genes responsible for JPS or UC is known to result from the abnormal stromal environment. This altered terrain for epithelial cell growth can be thought as a landscaper defect (Kinzler and Vogelstein, 1998).

II. Mitotic homologous recombination in carcinogenesis

Genetic recombination was recognized in the early 20th century as a key feature in generating species diversity in both prokaryotic and eukaryotic organisms (Low, 1988). In this process, a series of nucleotides are rearranged within a single DNA molecule or between two or more DNA molecules in a new combination. Based on their fundamental differences in the relationships of the series of nucleotides in the DNA undergoing recombination, the recombination process has been divided into three basic types: site-specific recombination, illegitimate recombination, and homologous recombination (Low, 1988).

Numerous organisms have developed specialized mechanisms that direct recombination to occur at specific chromosomal sites that share limited or no sequence homology. The hallmark of site-specific recombination is that all of the nucleotides in the two parental recombining sites are conserved in a simple reciprocal event (Grindley, 1988). These site-specific recombination events include insertion of certain viruses into prokaryotic and eukaryotic chromosomes (Miller, 1988), the movement of transposable elements (Grindley, 1988), and the rearrangement of vertebrate immunoglobulin genes (Perry, 1988). Site-specific recombination is important for the generation of new arrangements of genes in chromosomes and for the proper timing of gene expression during the development of numerous organisms (Grindley, 1988; Miller, 1988).

In illegitimate recombination, DNA recombines without apparent regard to special sites or extensive homology (Allgood and Silhavy, 1988; Roth and Wilson, 1988). Such recombination generates deletions, insertions and other

rearrangements. Although its biological role is unclear, illegitimate recombination may reflect errors of replication or "fail-safe" measures that cells use to rescue otherwise "dead" chromosomes (Allgood and Silhavy, 1988; Roth and Wilson, 1988).

Recombination requiring extensive nucleotide sequence homology in the DNA of the chromosomes is referred to as homologous recombination. In this process, homologous DNA molecules align, and the exchange of genetic information at one or more regions along the DNA molecules produces new combinations of genes or parts of genes. It is important not only in generating species diversity, but also in the repair of damaged DNA molecules, and in eukaryotes, for the faithful segregation of homologous chromosomes during meiosis (Low, 1988).

A. Homologous recombination

1. General mechanisms

Although the existence of homologous recombination has been known for over 60 years, it has only been in the last ten years that its mechanisms have begun to be understood at the molecular level. One of the unifying themes in the study of homologous recombination during the last three decades has been a particular recombination intermediate envisioned by Holliday in 1964 (Holliday, 1964). The Holliday model uses as its centerpiece an intermediate in which the two recombining DNA molecules are covalently held together at a region of homology through a crossover connection (Holliday junction) formed by the

reciprocal exchange of two of the four strands in the participating DNA molecules. The mechanism is shown in Figure 1. When two homologous double helices are aligned, each of the positive strands, or the negative strands, are nicked open in a given region. The resulting free ends leave the complementary strands to which they had been hydrogen bonded and become associated instead with the complementary strands in the homologous double helix (Fig. 1a-f). The result of this reciprocal single-strand exchange is to establish a tentative physical connection between the two DNA molecules which will recombine. This linkage can then be stabilized through a process of DNA repair, which in this case can be as simple as the formation of two phosphodiester bonds by DNA ligase (Fig. 1e). The structure shown in Figure 1e is the Holliday recombination intermediate, with the point of crossover being known as a Holliday junction. In this structure, steric hindrance has been shown to be minimal, and virtually all of the bases can be paired (Sigal and Alberts, 1972). The Holliday junction can be moved along the DNA to generate symmetric heteroduplex DNA, either by spontaneous or enzyme-catalyzed branch migration (Meselson, 1972; Tsaneva et al., 1992). The heteroduplex structure may undergo a reversible isomerization which can make the two pairs of homologous strands at the site of exchange both susceptible to an endonucleolytic attack (Fig. 1g-j). The production of mature recombinant DNA molecules requires resolution of the Holliday junctions. In *E.coli*, such a process is carried out by the RuvC protein via a dual incision mechanism (Connolly et al., 1991; Bennett et al., 1993). The nicks introduced into the homologous strands of similar polarity can then be sealed by DNA ligase.

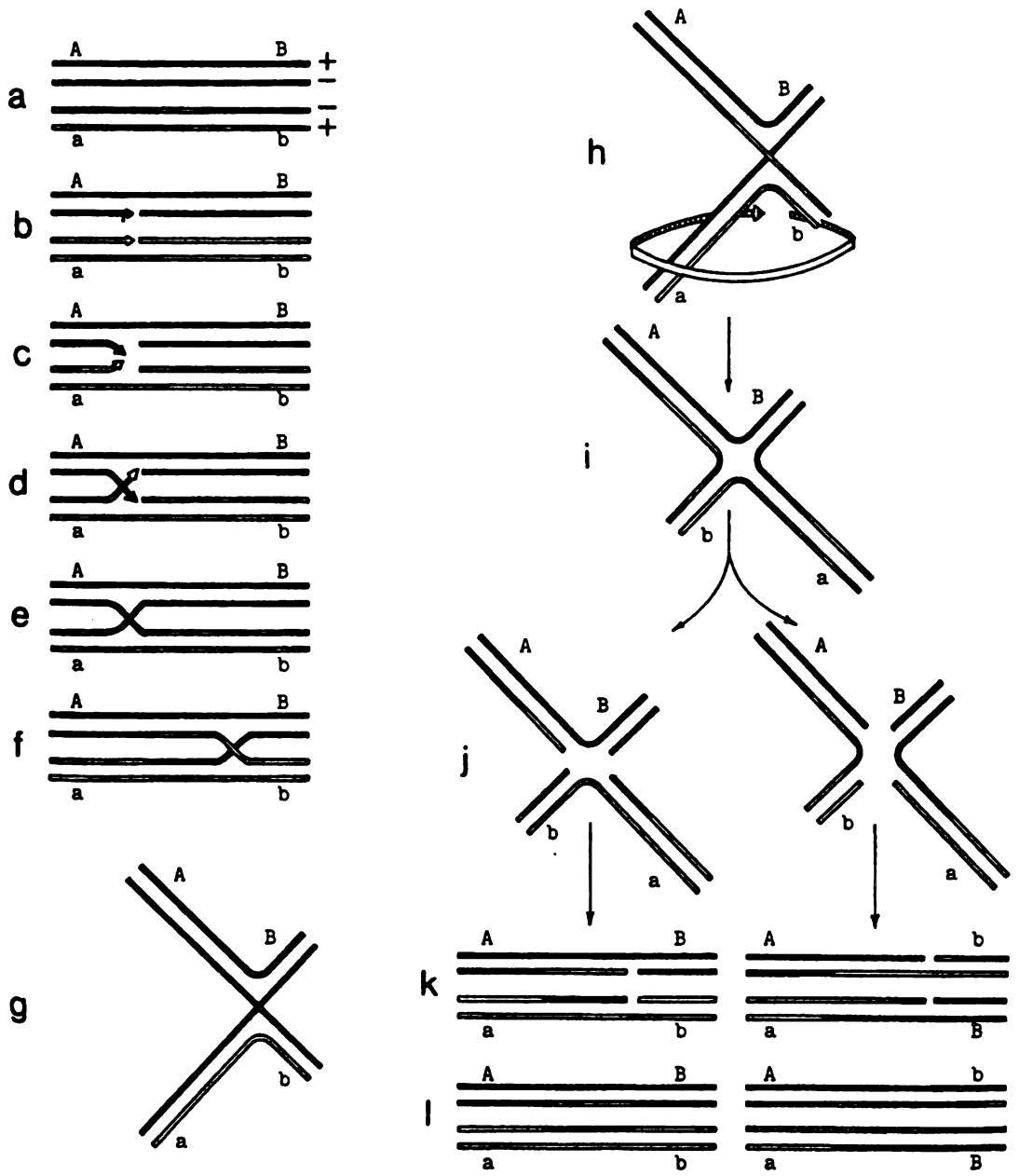

Figure 1. Holliday model of homologous recombination.

The resolution of a Holliday junction can result in either crossing-over or gene conversion (Fig. 1k, i). Crossing-over arises from the reciprocal exchange between two DNA molecules, while gene conversion is the result of the repair of DNA mismatches in a region of symmetric heteroduplex DNA.

After the proposal of the Holliday model, the results of several experiments demonstrated that not all heteroduplex DNA was symmetric (Stadler and Towe, 1971). In 1975, Meselson and Radding proposed a two-step initiation mechanism for the formation of the Holliday structure (Meselson and Radding, 1975). In their model, as shown in Figure 2, recombination is initiated by a single-strand nick on one of the two interacting DNA molecules. The 3' end of the nicked strand acts as a primer for DNA synthesis, which displaces the strand ahead of it (Fig. 2). The displaced single strand invades the other duplex at a homologous site, displacing a "D loop" and forming a small region of asymmetric heteroduplex DNA (Fig. 2b, c). The single-stranded D loop is degraded and the invading strand is ligated in place (Fig. 2d). The limited region of asymmetric heteroduplex is expanded by DNA synthesis of the donor duplex, and by exonucleolytic degradation on the recipient duplex. The Holliday structure is formed by bringing the 5' and 3' single-stranded ends into opposition so that they can be ligated. The resulting Holliday junction can move along the duplex by the process of branch migration, generating symmetric heteroduplex DNA. Resolution of the Holliday junction can yield either the crossing-over or the gene conversion configuration, as shown in Figure 1j-l.

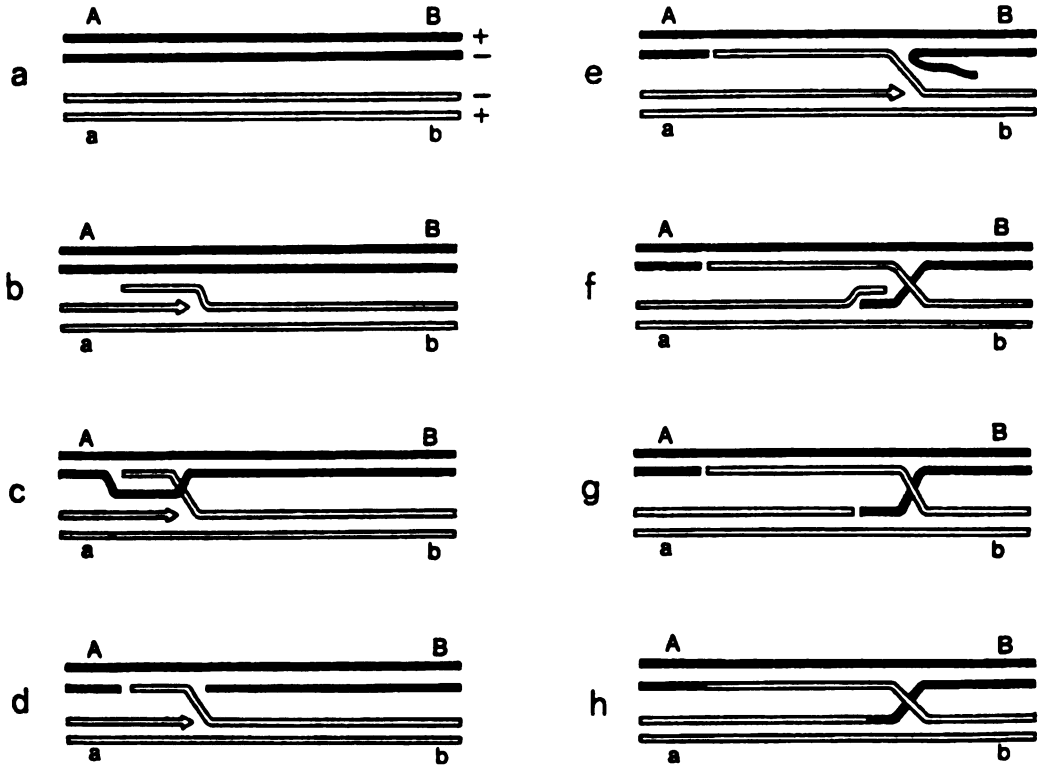

Figure2. Meselson and Radding model of homologous recombination.

Until the 1980s, the single-strand break initiation mechanism was the common feature of various recombination models proposed. Based on their work, Szostak and his colleagues proposed a new initiation mechanism in 1983 (Szostak et al., 1983). Their model differs from the Holliday model and the Meselson-Radding model in that a double-strand break in one of the two participating DNA molecules initiates recombination. The double-strand break serves to generate two free ends which will play a direct role in forming Holliday junctions, as shown in Figure 3. The free ends become partially digested by exonucleases to yield single-stranded tails. In a manner reminiscent of the previously discussed models, the exposed single-stranded tails can now serve to invade an intact recipient double helix at a region of homology. The invading single strand DNA is immediately extended by DNA polymerase to create a growing D loop (Fig. 3d). The displaced single strand in the D loop eventually makes contact with the other free end formed during the original double-strand break, as shown in Figure 3e, f. The invasion and extension of the two free ends provide a basis not only for establishing a firm connection between the two participating DNA molecules and converting the initial D loop structure into completely duplex DNA, but also for the "repair" of the break in the donor DNA molecule. Another major difference between single-strand break model and double-strand break model is that there are two standard Holliday junctions formed in double-strand break model (Fig. 3f). The resolution of the two Holliday junctions can result in crossing-over and gene conversion.

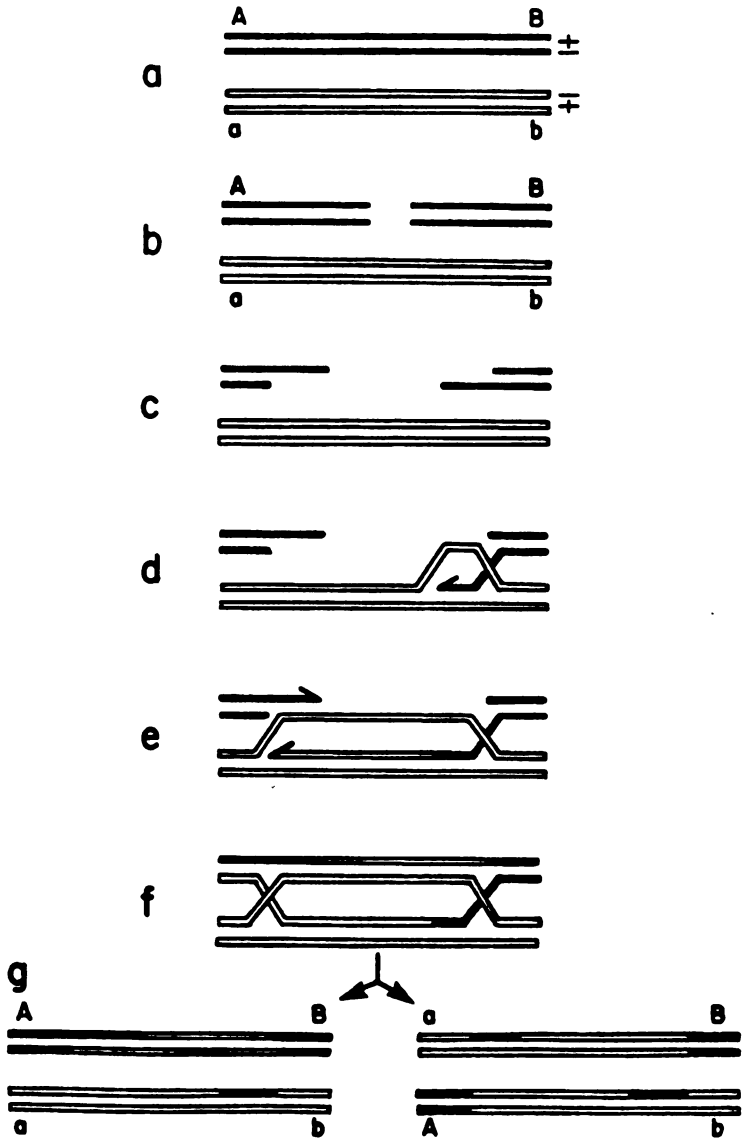

Figure 3. Double-strand DNA break model of homologous recombination.

Although these models are based on the findings in bacteria, it is generally believed that eukaryotes including human cells also use similar mechanisms. Since the proposal of Holliday model, it has guided the field for the past three decades and has survived every challenge (Stahl, 1994).

Many of the proteins involved in homologous recombination in bacteria have been isolated and extensively studied (Cox and Lehman, 1987; West, 1992; Kowalczykowski and Eggleston, 1994). The best-investigated recombination system is that of *E. coli*, where more than 20 genes cooperate in order to catalyze homologous recombination. These genes code for nucleases (RecB, RecC, RecD, RecE, RecJ, and RuvC) (Taylor, 1988; Lovett and Kolodner, 1989; Takahagi et al., 1991; Connolly et al., 1991), DNA-binding proteins (RecF, RecG and SSB) (Griffin and Kolodner, 1990; Lloyd and Sharples, 1993), DNA helicases (RecB, RecC, RecD, and RecQ) (Umezu et al., 1990), DNA topoisomerases (GyrA, GyrB, and TopA), DNA ligase (Lig) (Clark, 1991), and proteins mediating branch migration (RuvA and RuvB) (Tsaneva et al., 1992). In the center of this system is the RecA protein. The first step in homologous recombination is homologous pairing followed by strand exchange. This step requires that sequence similarity between two DNA molecules is searched, homology is recognized, and individual DNA strands are mutually exchanged. Several proteins have been shown to be able to carry out this step, with RecA the best studied (Kowalczykowski and Eggleston, 1994). RecA protein binds to DNA and polymerizes to form a helical filament of indefinite length on DNA. This nucleoprotein complex interacts with naked duplex DNA, leading to the

establishment of homologous contacts (Radding, 1991). If a free end is present in one of the two interacting DNA molecules, RecA carries out the strand exchange reaction, forming a Holliday junction structure (DasGupta et al., 1980; West et al., 1981). ATP is required for filament assembly and homologous alignment of DNA, but not for homology search and strand exchange (Kowalczykowski and Eggleston, 1994). RecA is a DNA-dependent ATPase (Roberts et al., 1979). Hydrolysis of ATP by RecA is the driving force of filament assembly and homologous alignment of DNA (Roca and Cox, 1990). RecA is also involved in branch migration (Cox and Lehman, 1981) together with RuvA and RuvB (Tsaneva et al., 1992). The resulting Holliday junctions are subject to resolution to yield recombination products. The enzyme that carry out this endonucleolytic cleavage, RuvC, has been well studied (West, 1992).

Considerable progress has been made in understanding the enzymatic mechanisms of homologous recombination in eukaryotes. In *S. cerevisiae*, most of the genes required for homologous recombination fall into the *Rad52* epistasis group, which includes *Rad50* through *Rad57* (Game, 1993). Proteins having sequence and structural homology to RecA have been identified (Kowalczykowski and Eggleston, 1994). In yeast four RecA homologs have been found: Dmc1, Rad51, Rad55 and Rad57 (Aboussekhra et al., 1992; Basile et al., 1992; Bishop et al., 1992; Shinohara et al., 1992; Lovett, 1994). Rad51 shares significant sequence homology to RecA protein (Aboussekhra et al., 1992; Shinohara et al., 1992). Purified Rad51 protein has ATP-dependent double- and single-stranded DNA-binding activities and a single-stranded DNA-dependent

ATPase activity (Shinohara et al., 1992). In the presence of ATP, Rad51 protein forms a helical filament with double-stranded DNA, which is almost identical to that formed with RecA protein (Ogawa et al., 1993). Like RecA, Rad51 catalyzes strand exchange between homologous DNA in an ATP-dependent reaction (Sung, 1994; Sung and Robberson, 1995). Furthermore Rad51 and Dmc1 interact to form multiple nuclear complex prior to chromosome synapsis (Bishop, 1994). Several homologs of yeast Rad51 have been isolated from low and higher eukaryotes (Heyer, 1994), including mouse and human (Shinohara et al, 1993). Human Rad51 homolog has been shown to form helical nucleoprotein filaments on single-stranded and double-stranded DNA (Benson et al., 1994), and promote ATP-dependent homologous pairing and strand transfer reaction *in vitro* (Baumann et al., 1996). Human Rad51 protein forms nuclear foci which are located at selected sites in chromatins in the synaptonemal complexes (Haaf et al., 1995; Plug et al., 1996).

2. Homologous recombination and carcinogenesis

Certain rare genetic recombination events are instrumental in producing human diseases, including certain types of cancer (Adams, 1985; Lee et al., 1987; Dean et al., 1987; Turc-Carel et al., 1987). One of the hallmarks of cancer cells is genetic instability, with high frequencies of mutation, recombination, loss of heterozygosity, chromosomal abnormality, etc. Homologous recombination could result in many genetic changes, including translocation, amplification, deletion, loss of heterozygosity and microsatellite instability (Sengstag, 1994).

Aberrant homologous recombination contributes significantly to genetic instability found in cancer cells. It has been shown that inappropriate homologous recombination could result in the activation of oncogenes and inactivation of tumor suppressor genes (Sengstag, 1994).

Carcinogenesis is a multistep process which involves not only the activation of dominant oncogenes, but also the inactivation of tumor suppressor genes. In the latter case, usually the function of both alleles has to be eliminated. Loss of heterozygosity is a common characteristic of many kinds of tumors (Cavenee et al., 1983; Mulligan et al., 1990; Fearon and Vogelstein, 1990). There is genetic evidence indicating that mitotic recombination between homologous genes is the mechanism responsible for the cells becoming homozygous for a particular recessive allele (James et al., 1989). Cavenee et al. (1983) showed that mitotic homologous recombination is one of the mechanisms that lead to the inactivation of a functional heterozygous *Rb* allele in retinoblastomas. The tumor suppressor gene, *p53*, is often affected by loss of heterozygosity in various cancers (Baker et al., 1989), including colorectal cancer (Baker et al., 1990; Fearon and Vogelstein, 1990).

Homologous recombination could lead to deletion. In this case, recombination occurs within identical or similar sequences on a given chromosome, thereby deleting all genetic information containing between these sequences. This is an important mechanism for gene inactivation. The *Alu* sequence is a 300-bp element belonging to a highly repetitive sequence recurring $3x10^5$ to $5x10^5$ times in human DNA (Schmid and Jelinek, 1982). *Alu* elements have been shown to

mediate deletions in various genes, such as the *LDL* receptor gene (Lehrman et al., 1985), the apolipoprotein B gene (Huang et al., 1989), the C1 inhibitor gene (Ariga et al., 1990), and the *ADA* gene (Berkvens et al., 1990). For its role in cancer development, deletions have been found in the *Rb* gene in retinoblastomas and osteosarcomas (Canning and Dryja, 1989), and in the *NF-1* tumor suppressor gene in neurofibromatosis type 1 (Legius et al., 1993).

One mechanism of protooncogene activation is the amplification of its respective genomic locus. This increases, as a consequence, the copy number of the protooncogene and may result in elevated gene expression. Homologous recombination could lead to duplication of a particular locus (Sengstag, 1994). Amplifications of several protooncogenes have been documented in different types of neoplasia. For instance, *c-Myc* has been shown to be amplified 2-15 fold in human primary breast carcinomas (Escot et al., 1986), and the epidermal growth factor (*EGF*) receptor gene has been recognized to be amplified in primary brain tumors (Libermann et al., 1985). Other protooncogene amplifications found in human tumors include *Neu* in breast cancer (Slamon et al., 1987), *N-Myc* in neuroblastoma (Amler and Schwab, 1989), and *L-Myc* in small-cell lung cancer (Makela et al., 1992). All these amplifications of protooncogenes cause the activation of oncogenes required for neoplasia.

3. Carcinogen-induced homologous recombination

Many carcinogens can induce homologous recombination. It is clear that in addition to their mutagenic ability, many carcinogens play an important role in the

development of tumors through their recombinogenic ability.

Homologous recombination models suggest that strand exchange is preceded by single- or double-strand breaks (Holliday, 1964; Meselson and Radding, 1975; Szostak et al., 1983). X-rays can induce homologous recombination in human cells (Benjamin and Little, 1992; Xia et al., 1994). It is well known that X-rays induce single- and double-strand breaks in DNA (Dikomey and Franzke, 1986), and this provides an explanation for the recombinogenic effect of X-rays (Resnick, 1976).

Ultraviolet (UV) radiation (Wang et al., 1988; Bhattacharyya et al., 1990; Tsujimura et al., 1990; Deng and Nickoloff, 1994), cross-linking agents such as mitomycin C (Wang et al., 1988), and polycyclic aromatic carcinogens, such as (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[α]pyrene (BPDE), 1-nitrosopyrene (1-NOP), *N*-acetoxy-2-acetylaminofluorene (N-AcO-AAF), and 4-nitroquinoline 1-oxide (4-NQO) (Bhattacharyya et al., 1989; 1990) can all induce homologous recombination in mammalian cells. UV radiation generates DNA photoproducts in DNA (Pfeifer, 1997). The DNA damages caused by BPDE, 1-NOP, N-AcO-AAF and 4-NQO are all bulky chemical adducts (Ikenaga et al., 1976; Weinstein et al., 1976; Heflich et al., 1986). This DNA damage as well as that caused by mitomycin C interferes with DNA replication, causing a delay in DNA replication fork progression (Reardon et al., 1990; Basu et al., 1993; Chary and Lloyd, 1995). Maher and colleagues (Bhattacharyya et al., 1990; Tsujimura et al., 1990) found that unrepaired DNA damage, rather than excision repair, is responsible for the induction of homologous recombination by these carcinogens.

They further proposed that unrepaired DNA lesions block DNA replication, leading to the generation of discontinuities, and the single-stranded DNA regions initiate homologous recombination (Bhattacharyya et al., 1990; Tsujimura et al., 1990). Evidence supporting this hypothesis comes from the study of the relationship between transcription and recombination (Deng and Nickoloff, 1994). Preferential repair of UV DNA damage in highly transcribed DNA diminishes UV-induced homologous recombination, further strengthening the hypothesis that the unrepaired DNA damage by blocking DNA replication generates single stranded DNA, which initiates recombination.

Alkylating agents cause small DNA adducts and they can also induce homologous recombination (Wang et al., 1988; Zhang et al., 1996). Alkylating agents do not physically cause DNA strand breaks, and alkylating adducts do not block DNA replication (Reha-Krantz et al., 1996). The mechanism of alkylating agent-induced homologous recombination differs from that of ionizing radiation, UV radiation and bulky chemical carcinogens. It has been found that O^6-methylguanine is the lesion principally responsible for the homologous recombination induced by methylating agents (Zhang et al., 1996). The induction level of homologous recombination is directly linked to the amount of unrepaired O^6-methylaguanine left in DNA (Zhang et al., 1996). These investigators proposed that futile repair of mismatches containing O^6-methylaguanine by mismatch repair generates single-stranded DNA regions which in turn initiate homologous recombination.

B. Alkylating agents

1. Mutagenic mechanisms

Alkylating agents represent one general class of mutagens. Alkylating agents are compounds able to transfer an alkyl group to cellular macromolecules either when placed in aqueous solution, as in the case of nitrosamides such as *N*-methy-*N'*-nitro-*N*-nitrosoguanidine (MNNG), or after cellular metabolic activation, as in the case of nitrosamines such as dimethylnitrosamine (DMN). Despite the differences by which these compounds are activated, both MNNG and DMN share a common reactive species, the methyl carbonium ion. The consequence of their action is the covalent attachment of alkyl groups to RNA, proteins, and most importantly, DNA. The sites of addition vary from compound to compound, with some compounds linking alkyl groups to the bases and/or phosphates of DNA (Singer et al., 1978). The common end result of these actions is the damage to the DNA, which can either be repaired, be bypassed without error, or result in a mutation in the process of DNA replication.

An alkylating agent commonly used for various studies is MNNG. Under physiological conditions, MNNG can methylate DNA at various sites. Methylation takes place on most of the ring nitrogen atoms of the bases: the N7 position of guanine and adenine and the N3 position of thymine, cytosine, guanine and adenine. Sites of methylation on the oxygen atoms are: the O^6 position of guanine, the O^4 position of thymine and the O^2 position of cytosine and thymine (Singer and Kusmierek, 1982). Of the various adducts formed by MNNG, the most abundant lesion is N7-methylguanine (N7-MeG), but it is not mutagenic.

The ratio of O^6-methylguanine (O^6-MeG) to N7-MeG induced by MNNG is approximately 0.1 (Singer and Kusmierek, 1982). O^6-MeG has been shown to miscode very frequently, resulting in G:C to A:T transition mutations (Swann, 1990; Lukash et al., 1991). These mutations arise from O^6-MeG being mistaken for an adenine by the replication complex because of the similar hydrogen bonding angles and lengths between them (Swann, 1990). The replication complex puts a dTMP across from O^6-MeG during replication (Swann, 1990). In the next round of replication, the complex puts a dAMP across from the thymine, completing the G:C to A:T transition.

2. Repair mechanisms

Cells repair O^6-MeG through the action of the O^6-alkylguanine DNA alkyltransferase (AGT) (Pegg and Byers, 1992). The protein directly transfers the methyl group from O^6-MeG to an interior cysteine residue in the active site of AGT. AGT can also transfer larger alkyl groups such as ethyl and propyl, but at a much slower rate. The removal of the methyl group from O^6-MeG results in the restoration of the DNA, and the irreversible inactivation of the AGT protein resulting from the covalent attachment of the methyl group to the cysteine residue.

The compound O^6-benzylguanine (O^6-BzG) is extremely potent in inactivation of AGT, such that the addition of 25 μM of O^6-BzG to the growth media of human cells in culture reduces cellular AGT protein activity to an undetectable level (Lukash et al., 1991). O^6-BzG exerts its effect by acting as a substrate analog for

AGT, with the benzyl group of O^6-BzG being covalently attached to the cysteine residue of an AGT molecule (Dolan et al., 1990). This results in no active AGT protein being available for repair of O^6-MeG in DNA. This inhibitory effect can only be reversed by removing O^6-BzG from the medium and allowing *de novo* synthesis of new AGT molecules.

If O^6-MeG is left unrepaired in DNA by AGT, or the cells lack AGT activity, DNA replication on the methylated template will produce an O^6-MeG:T mismatch. Even in the absence of replication, the O^6-MeG:C base pair constitutes what looks like a mismatch. Both O^6-MeG:T and O^6-MeG:C are recognized as mismatches by the mismatch repair system (Duckett et al., 1996). The mismatch repair system recognizes the mismatches and removes a stretch of the DNA strand that contains the T or C. The resulting gap is filled in by DNA synthesis which has the same probability of inserting a T across from the O^6-MeG again. The repair of mismatches containing O^6-MeG by mismatch repair is thus futile. Such abortive repair events have been hypothesized as being responsible for the decreased cytotoxic effect of methylating agents in cells that lack mismatch repair compared to mismatch repair proficient cells. The cytotoxicity is attributed to the futile turnover of newly synthesized DNA (Karran and Marinus, 1982; Goldmacher et al., 1986).

Support for this hypothesis comes from the observation that inactivation of mismatch repair renders cells tolerant to methylating agents. *In vitro* selection for MNNG or *N*-methyl-*N*-nitrosourea (MNU) resistance has led to identification of several methylation-tolerant mammalian cell lines (Goldmacher et al., 1986;

Branch et al., 1993; Aquilina et al., 1995). All these tolerant cells proved to be defective in mismatch repair (Branch et al., 1993; Kat et al., 1993; Aquilina et al., 1994; Drummond et al., 1995; Papadopoulos et al., 1995). As would be expected of cells that have defective mismatch repair, these cells exhibit high mutation frequencies, either spontaneously or after treatment with methylating agents (Goldmacher et al, 1986; Branch et al., 1993; Kat et al., 1993; Aquilina et al., 1994; Papadopoulos et al., 1995). Furthermore, tumor cells lines from HNPCC patients are defective in mismatch repair, and they also display methylation tolerance (Karran and Bignami, 1994; Branch et al., 1995). Restoration of mismatch repair activity by chromosome transfer renders these cells as sensitive to methylating agents as normal cells and reduces their mutation frequencies to a normal level (Tindall et al., 1998).

C. Mismatch repair

Both prokaryotes and eukaryotes are capable of repairing mismatches in their DNA, and the repair process is well conserved through evolution. Mismatches in DNA can arise by several processes. One of the most important is by replication errors. In this case, the correct base of the mismatches is located in the parental strand, and the incorrect base of the mismatch is in the newly synthesized strand. Proper corrections maintain the fidelity of the genetic information. Another mechanism that produces mismatched base pairs is the formation of heteroduplexes in the Holliday recombination intermediates that are made during homologous recombination. If the two recombining DNA strands differ slightly in

their sequences, mismatches will be formed. The manner in which these mismatches are repaired influences the nature of the recombination products (Detloff et al, 1991). A third and more specialized way that mismatches can arise is by the deamination of 5-methylcytosine. This modified base is present in the DNA of many organisms, including humans. Deamination of 5-methylcytosine converts it to thymine, resulting in a G:T mismatch. Proper correction is important to maintain the fidelity of the genetic information. In addition, mismatches can arise when one of the bases is modified chemically, such as O^6-MeG. As mentioned above, both O^6-MeG:C and O^6-MeG:T have been shown to be recognized as mismatches (Duckett et al., 1996).

1. In prokaryotes

The mismatch repair in bacteria is also celled methyl-directed long-patch system. This pathway is characterized by broad mismatch specificity and is believed to be responsible for correction of replication errors and mismatches in the Holliday recombination intermediates. In *E. coli*, inactivation of mismatch repair results in a strong mutator phenotype (Nevers and Spatz, 1975; Lu et al., 1983; Pukkila et al., 1983). The methyl-directed pathway has a broad mismatch specificity. It has been reported that it can recognize all the base-base mismatches except for C:C mispairs (Modrich, 1991). This system also repairs insertion/deletion mismatches in which one strand contains one, two, three or four extra nucleotides (Dohet et al., 1986; Learn and Crafstrom, 1989; Parker and Marinus, 1992). Insertion/deletion mismatches with more than four extra

nucleotides are not recognized in bacteria by the mismatch repair system (Parker and Marinus, 1992; Carraway and Marinus, 1993). This mismatch specificity is consistent with the nature of mutations that occur in the mismatch repair deficient bacteria, point mutations and short frameshift mutations (Levinson and Gutman, 1987; Schaaper and Dunn, 1987).

The basic repair reaction catalyzed by this pathway is understood in considerable detail. In fact, it has been reconstituted *in vitro* with DNA substrates containing mismatches, the three mismatch repair proteins, i.e., MutS, MutL, and MutH, UvrD helicase, DNA polymerase III holoenzyme, DNA ligase, single-strand DNA-binding protein (SSB), and any one of the three single-stranded DNA exonucleases: Exo I, Exo VII or RecJ protein (Modrich, 1991; Kolodner, 1995; 1996; Modrich and Lahue, 1996). The reaction involves mismatch-dependent nicking at hemimethylated GATC sites of the unmethylated strand and degradation from the nick past the mismatch followed by DNA resynthesis. The roles of many proteins involved have been elucidated. The MutS protein recognizes and binds to mismatches in DNA in the presence of ATP. The mismatch-bound MutS recruits MutL protein, whose exact function is not clear, but which is required for the activation of MutH protein. MutH protein is an endonuclease that cleaves the unmethylated strand at the hemimethylated GATC site when activated by MutS and MutL in the presence of mismatches, with cleavage occurring 5' to the G in the GATC site. The resulting strand break serves as the primary signal that directs correction to the unmethylated strand (Langle-Rouault et al., 1987; Lahue et al., 1989). The repair reaction can utilize

hemimethylated GATC sites located either 5' or 3' to the mismatch, reflecting the bidirectional capacity of the system. The activated MutH endonuclease can make a nick in the GATC site, either 3' or 5' to the mismatch. The polarity of the nick will be such that it provides the shortest distance between the mismatch and the nick (Grilley et al., 1993). Depending on whether the nicked, unmethylated GATC site is 5' or 3' to the mismatch, degradation requires UvrD and one of the exonucleases: Exo I (3' exonuclease), Exo VII (3' and 5' exonuclease) and RecJ (5' exonuclease) (Lahue et al., 1989). DNA polymerase III holoenzyme and DNA ligase then fill in the resulting gap to complete mismatch repair (Lahue et al., 1989).

To preferentially correct the incorrect base in the newly synthesized strand, a mechanism to distinguish the parental and daughter strands is required. In bacteria, the strand specificity is provided by patterns of adenine methylation in GATC sequences (Modrich, 1991). Normally, DNA in *E. coli* is methylated at N^6 position of adenine in GATC sequences by Dam methylase. Since this is a postsynthetic modification, newly synthesized sequences exist in a transiently unmethylated state, and the absence of methylation on newly synthesized DNA targets correction to this strand (Lu et al., 1983; Pukkila et al., 1983). A single GATC sequence is sufficient to direct mismatch repair (Lahue et al., 1987; Bruni et al., 1988; Claverys and Mejean, 1988). The distance between the GATC site and the mismatch can be as long as two kilobases (Lahue et al., 1987; Bruni et al., 1988).

2. In eukaryotes

Evidence is rapidly accumulating that eukaryotes are also capable to carry out mismatch repair and that the overall mechanism of this process has been highly conserved in evolution. It further appears that some of the genes that play important roles in mismatch repair in prokaryotes, particularly *MutS* and *MutL*, have been conserved during evolution. Homologs of *MutS* and *MutL* have been identified in yeast (Kramer et al., 1989; Reenan and Kolodner, 1992a; 1992b; New et al., 1993; Prolla et al., 1994; Ross-Macdonald and Roeder, 1994; Hollingsworth et al., 1995; Marsischky et al., 1996), and mammalian cells (Fujii and Shimada, 1989; Linton et al., 1989; Fishel et al., 1993; Leach et al., 1993; Bronner et al., 1994; Horii et al., 1994; Liu et al., 1994a; Nicolaides et al., 1994; Papadopoulos et al., 1994; Varlet et al., 1994; Drummond et al., 1995; Palombo et al., 1995; Watanabe et al., 1996).

Eukaryotes possess a mismatch repair system similar to the *E. coli* methyl-directed MutSLH pathway with respect to both mismatch specificity and bidirectional capacity (Fang and Modrich, 1993). Eukaryotic mismatch repair system recognizes both base mismatches and insertion/deletion mismatches. In yeast, mismatch repair can recognize insertion/deletion mismatches as long as 12 bases (Bishop et al., 1987). hMSH2 protein binds with highest affinity to insertion/deletion mismatches of 8 to 14 bases long (Fishel et al., 1994). Whether human mismatch repair system can correct insertion/deletion mismatches up to 14 bases long is still not clear (Umar et al., 1994; Modrich and Lahue, 1996).

In yeast, there are six *MutS* homologs (*MSH*). *MSH1* is involved mismatch repair in mitochondria (Reenan and Kolodner, 1992b). *MSH4* and *MSH5* are involved in mismatch repair during meiosis (Ross-Macdonald, 1994; Hollingsworth et al., 1995). MSH2, MSH3 and MSH6 proteins are believed to function in a MutSLH-like mismatch repair pathway (Kolodner, 1996; Modrich and Lahue, 1996; Wiesendanger et al., 1998). Genetic analysis in yeast indicates that mismatch recognition involves three MutS homologs: MSH2, MSH3 and MSH6, which form two different heterodimeric complexes (Marsischky et al., 1996). MSH2 protein forms complexes with both MSH3 and MSH6 protein. The MSH2-MSH6 complex is primarily responsible for the recognition of base mismatches and single-base insertion/deletion mismatches, while the MSH2-MSH6 complex recognizes primarily insertion/deletion mismatches (Kolodner, 1996; Marsischky et al., 1996; Modrich and Lahue, 1996). There is some functional redundancy between these two MSH complexes (Marsischky et al., 1996). This view is consistent with the mutation phenotypes of the mutant yeast strains deficient in a specific MSH protein (Reenan and Kolodner, 1992a; Strand et al., 1993; Marsischky et al., 1996). Two MutL homologs have been suggested to be involved in mismatch repair. MLH1 and PMS1 form a complex to carry out the function that is similar to the function of MutL in bacteria. Mutations in these two genes cause mutator phenotypes in yeast (Kolodner, 1996; Modrich and Lahue, 1996).

Mismatch repair in human cells is very similar to that in yeast. Three MutS homologs, hMSH2, hMSH3 and hMSH6 (also called GTBP, for G:T binding

protein), are involved in recognition of mismatches (Acharya et al., 1996). hMSH2 and hMSH6 form a complex to recognize base mismatches and single base insertion/deletion mismatches (Drummond et al., 1995; Palombo et al., 1995), whereas hMSH2-hMSH3 complex recognizes and binds to insertion/deletion mismatches (Palombo et al., 1996; Risinger et al., 1996). The functional redundancy between these two MSH complexes is also been found in human cells (Genschel et al., 1998; Umar et al., 1998). Three MutL homologs have been identified in human cells. hPMS2 (the closest homolog of yeast PMS1) and hMLH1 form a complex and have been shown to function in mismatch repair in human cells (Li and Modrich, 1995). The third MutL homolog in human cells, hPMS1, is also involved in mismatch repair (Nicolaides et al., 1994). But, little is known about the biochemical role of hPMS1 in mismatch repair.

Other components in mismatch repair pathway are not well studied in either yeast or human cells. No *MutH* homolog has been identified (Modrich, 1991; Kolodner, 1995; Modrich and Lahue, 1996). There is little definite information on the helicases, DNA polymerase or DNA ligase involved in mismatch repair, but the DNA polymerase responsible is sensitive to aphidicolin, suggesting that it is one of these three polymerases: α, δ or ε (Holmes et al., 1990; Thomas et al, 1991; Fang and Modrich, 1993). There has been some recent progress in the identification of an exonuclease that might play a role in eukaryotic mismatch repair. RTH1 (also called RAD27 or YKL510) has been implicated to function as a 5' exonuclease in mismatch repair in yeast (Johnson et al., 1995). Mutations in

RTH1 cause a mutator phenotype (Johnson et al., 1995; Reagan et al., 1995). The exact role of RTH1 in mismatch repair needs more careful studies. Another candidate for an exonuclease involved in mismatch repair is EXO1 in *S. cerevisiae* (Tishkoff et al., 1997). It appears to be a homolog of the *S. pombe* 5' exonuclease EXO1 (Szankasi and Smith, 1995; Tishkoff et al., 1997). Mutations in both *EXO1* genes cause a mutator phenotype (Szankasi and Smith, 1995; Tishkoff et al., 1997). The mechanism of strand specificity in eukaryotic mismatch repair is not clear, but available information on the human pathway indicates some similarities between eukaryotes and prokaryotes. A DNA terminus is sufficient to provide strand specificity (Holmes et al., 1990), but unlike in prokaryotes, methylation-dependent MutH cleavage is not utilized in eukaryotes (Modrich, 1991; Kolodner, 1995; Modrich and Lahue, 1996). The process of generating DNA termini for strand specificity is under investigation.

3. Short-patch mismatch repair

Both prokaryotic and eukaryotic cells also possess short-patch mismatch repair systems (Lieb, 1987; Modrich, 1991) which differ from the mismatch repair systems discussed previously in that they generate short excision repair tracts (typically ten nucleotides or less) rather than the 10^3 bases or more generated in long-patch mismatch repair. Short-patch mismatch repair systems have a relatively restricted specificity with respect to the mismatches that they repair. Some of these systems also appear to operate only within a restricted sequence context (Modrich, 1991).

The best characterized short-patch mismatch repair system is the very short-patch (VSP) mismatch repair in *E. coli* (Modrich, 1991). This system efficiently corrects the T in G:T mismatches that occur in sequences resembling CC(A/T)GG. Dcm methylase methylates the internal C in CC(A/T)GG sequences to generate 5-methylcytosine (Marinus, 1984). When deamination of 5-methylcytosine occurs, the resulting G:T mismatch can be corrected by this VSP mismatch repair. Two of the genes required for methyl-directed mismatch repair, *MutS* and *MutL*, are also required for VSP mismatch repair, but two others (*MutH* and *UvrD*) are not (Jones et al., 1987a; 1987b; Lieb, 1987). In addition, the *Vsr* and *PolA* genes are required for VSP mismatch repair. Vsr protein is a strand-specific mismatch endonuclease, which recognizes G:T mismatches in C<u>T</u>(A/T)GG sequence contexts and makes incisions 5' of the <u>T</u> (Hennecke et al., 1991). DNA polymerase I removes the <u>T</u> with its exonuclease activity and then carries out repair synthesis (Dzidic and Radman, 1989). Since the Vsr endonuclease can act in the absence of MutS and MutL proteins, it seems that the latter two proteins only play a role in stimulating or regulating the activity of Vsr endonuclease (Lieb, 1987).

In addition to being able to repair G:T mismatches by the long-patch system discussed above, mammalian cells can also repair G:T mismatches by a short-patch mismatch repair. This system utilizes a G:T mismatch-specific thymine-DNA glycosylase which removes the T from G:T mismatches (Wiebauer and Jiricny, 1990). This glycosylase has been purified (Neddermann and Jiricny, 1993) and the gene encoding for it has been cloned (Neddermann et al., 1996).

This G:T mismatch-specific thymine-DNA glycosylase can recognize and bind to DNA containing O^6-methylguanine:T, and make an incision at this mismatch with the same efficiency as at G:T mismatch (Sibghat-Ullah and Days, 1992; Sibghat-Ullah et al., 1996). The abasic site generated by glycosylase serves as the substrate for AP endonuclease which generates a single-nucleotide gap (Wiebauer and Jiricny, 1990). The resulting gap is filled in by DNA polymerase β (Wiebauer and Jiricny, 1990). The relationship between short-patch mismatch repair and the mismatch repair proteins hMSH2 or hMSH3 or hMSH6 has not been established. It is predicted that cells with a defect in this short-patch mismatch repair system will have a similar phenotype as cells defective in long-patch mismatch repair, i.e., tolerance to methylating agents and hypermutability. However, this question and the exact role of the short patch repair need further study.

4. Mismatch repair and cancer

Loeb et al. (1974) and Nowell (1976) suggested 20 years ago that genetic destabilization would predispose a normal cell to malignancy. Progressive genetic change during the course of tumor development has been documented (Fearon and Vogelstein, 1990, Bishop, 1991). Tumor-specific genetic instability has been observed in hereditary nonpolyposis colorectal cancer (HNPCC) and sporadic colon cancers. A subset of sporadic colon cancer (Aaltonen et al., 1993; Thibodeau et al., 1993) and the majority of tumors from HNPCC patients (Aaltonen et al., 1993) contain frequent mutations in the simple repeat

sequences $(A)_n$, $(GGC)_n$, or $(CA)_n$. Mutations in these microsatellite repeat sequences are typically tumor-specific, suggesting that they occur somatically. Their incidence is dramatic: tumor cells with this characteristic contains thousands of microsatellite mutations (Aaltonen et al., 1993).

Not long ago, it was recognized that the microsatellite instability observed in tumors was similar to that observed in bacteria harboring mutations in mismatch repair genes such as *mutS* and *mutL* (Strand et al., 1993). The hypothesis that HNPCC is caused by hereditary mutations of human homologs of *mutS* or *mutL* stimulated the search for such human homologs. Now the majority of HNPCC cases can be attributed to a defect in any one of the multiple loci responsible for DNA mismatch repair. These include the *hMSH2* (Fishel et al., 1993; Leach et al., 1993), *hMLH1* (Bronner et al., 1994; Papadopoulos et al., 1994), *hPMS1* and *hPMS2* genes (Nicolaides et al., 1994). The majority of HNPCC kindreds harbor mutations in either *hMSH2* or *hMLH1* (Liu et al., 1994b; Nystrom-Lahti et al., 1996). It has been proposed that the initial event in the development of tumors in HNPCC patients is the functional loss of mismatch repair activity (Kinzler and Vogelstein, 1996), resulting in rapid accumulation of mutations in critical genes necessary for neoplastic transformation.

Since the original description of the effect in HNPCC and sporadic colon cancer, microsatellite instability has been found in a significant fraction of sporadic tumors, including bladder (3-21%), breast (0-20%), cervical (15%), colorectal (12-28%), endometrial (17-23%), esophogeal adenoma (22%), nonsmall cell lung cancer (2-34%), ovarian (16%), pancreatic (67%), prostate

(20-38%), small cell lung cancer (45%), squamous cell skin (50%), stomach (18-39%), and testicular (0-18%) (Egawa et al., 1995; Eshleman and Markowitz, 1995; Uchida et al., 1995). Not all the genetic instability is caused by the loss of mismatch repair, but it contributes a significant fraction. Mutations in mismatch repair genes have been found in 10-15% of sporadic colorectal cancer (Ionov et al., 1993; Thibodeau et al., 1993). Genetic instability caused by loss of mismatch repair and other mechanisms results in rapid accumulation of mutations in critical genes necessary for neoplastic transformation.

III. Cellular senescence

More than thirty years ago, Hayflick and Moorhead observed that normal human embryo fibroblasts can only undergo a limited, fixed number of cell divisions after which they cease proliferation (Hayflick and Moorhead, 1961; Hayflick, 1965). The nondividing stage that cells enter is referred to as cellular senescence or replicative senescence. In addition to the studies on fibroblasts, senescence has been described for a variety of cell types, including glial cells (Blomquist et al., 1980), keratinocytes (Rheinwald and Green, 1975), vascular smooth muscle cells (Bierman, 1978), lens cells (Tassin et al., 1979), lymphocytes (Tice et al., 1979), and endothelial cells (Mueller et al, 1980). Cell cultures do not die after entering senescence but remain viable for years if maintained with weekly changes of culture medium (Matsumura et al., 1979). Senescent cells undergo characteristic morphological changes, becoming flattened, enlarged and granular, In addition, nuclear size and the content of

RNA, protein, glycogen, lipids and lysosomes are all increased (Goldstein, 1990). These cells are arrested in G1 phase of the cell cycle and do not enter S phase in response to physiological mitogens (Sherwood et al., 1988; Cristofalo et al., 1989b). The failure of the cells to grow beyond this limit is an inherent property of the cells, not an artifact of cell culture. Morphologically senescent fibroblasts have also been observed *in vivo* (Bruce, 1991; Dimri et al., 1995).

A. Cellular senescence in cancer and aging

1. Cellular senescence as a tumor suppression mechanism

There is substantial evidence that cellular senescence is a tumor suppression mechanism. In contrast to normal primary cells, cell lines derived from tumors often have escaped from cellular senescence, and they can be serially passaged indefinitely in culture (Bruland et al., 1985; Leibovitz; 1986). The escape from senescence is consequently described as immortalization. A simple explanation is that immortalization is an early and common event in the multistep carcinogenesis. This is further supported by transformation studies in culture. Treatment of normal cells with a variety of carcinogens, including radiation, chemical carcinogens, viruses and oncogene transfection, increases the frequency at which such cells escape senescence, suggesting that this escape is important for cancer induction. In addition, immortal cells are more susceptible than normal (mortal) cells to spontaneous and carcinogen-induced transformation into tumor forming cells (Sager, 1986; 1991). Thus, loss of the

constraint of cellular senescence is an important change that predisposes a cell to further neoplastic transformation.

Normal fibroblasts from adult humans can be grown in culture for approximate 14 to 29 population doublings before senescence (Hayflick, 1976). If all the genetic changes required for tumorigenesis of an adult fibroblast were to accumulate without extension of the life span, it would only grow to form a tumor comprised of between $1.6x10^4$ (14 population doublings) and $5.4x10^8$ cells (29 population doublings). It can be estimated that a tumor formed after 30 population doublings would be approximate 1 cm^3, and it is generally expected that a tumor with a size of less than 1 cm^3 would not cause serious harm. For a tumor cell to give rise to a larger mass, it would be necessary for it to acquire additional proliferative capacity. Paraskeva et al. (1988; 1989) have observed that colon adenomas of < 1 cm^3 in size rarely give rise of immortal cell lines in culture, whereas colon adenomas > 1 cm^3 often give rise of immortal cell lines. This further supports the hypothesis that extension of life span is required for tumor growth beyond a certain size.

Further support for this hypothesis is the fact that 30 population doublings may be not long enough for a cell to acquire the necessary mutations to initiate neoplastic transformation. A single cell needs 20 population doublings to propagate to 10^6 cells. Considering that the spontaneous mutation rate in human cells is estimated to be $1x10^{-6}$ per cell generation, two mutations in two cancer causing genes in a single cell occur at a frequency of $1x10^{-12}$. Thus normal cells can only undergo at most two sequential mutation and clonal expansion

selections before they senesce (McCormick and Maher, 1988). Immortalization, whereby the cell escapes cellular senescence, would allow the cell enough time to accumulate the necessary mutations and subsequent clonal expansions.

2. Cellular senescence represents aging at cellular level

During the past several decades, one major question in cellular senescence research is whether cellular senescence observed in culture represents human aging at the cellular level. Three lines of evidence have established a link between aging *in vivo* and cellular senescence in culture.

The maximum cumulative population doubling that normal fibroblasts can achieve reflects the maximum life span of the species from which they are derived (Stanley et al., 1975; Rohme, 1981). Human cells can grow up to 60 population doublings in culture, whereas cells from rodents (life span of 3-5 years) can only achieve 20-40 population doublings. The proliferation capacity of human fibroblasts has been shown to decrease with increasing donor age (Martin et al., 1970; Hayflick, 1976). Fibroblasts from human embryonic tissue can achieve 50-60 population doublings, whereas normal fibroblasts from adult humans can only be grown in culture for many fewer population doublings before senescence (Hayflick, 1976). This inverse relationship between life span in culture and donor age has also been observed in other cell types, including keratinocytes (Rheinwald and Green, 1975), vascular smooth muscle cells (Bierman, 1978), lens cells (Tassin et al., 1979), lymphocytes (Tice et al., 1979), glial cells (Blomquist et al., 1980), and endothelial cells (Thornton et al, 1983).

Werner syndrome is a genetic disease that manifests itself as premature aging. Patients with Werner syndrome display accelerated aging characteristics during the third or fourth decade of life. Fibroblasts derived from Werner patients achieve fewer cell divisions before entering cellular senescence than cells derived from normal individuals of same age (Goldstein et al., 1989). This suggests that premature cellular senescence in culture corresponds to premature aging *in vivo*. Collectively, this evidence supports the hypothesis that cellular senescence represents aging at cellular level. The observation that a fraction of the cells in human skin are able to be stained blue with senescence-associated β-galactosidase activity which is a phenotype characteristic of senescent cells, and that the number of such cells in skin increases strikingly with increasing age of donor (Dimri et al., 1995), further strengthens the relationship between cellular senescence *in vitro* and aging *in vivo*.

B. Cellular senescence is genetically programmed

It is now generally accepted that cellular senescence is genetically programmed (Vojta and Barrett, 1995). A program becomes activated at the end of the proliferative life span of a normal cell, causing the characteristic morphological changes and growth arrest of cellular senescence.

1. Biomarkers of cellular senescence

Accompanying the characteristic morphological changes in senescent cells, there are changes in gene expression in these cells compared to their pre-

senescent counterparts. Many biomarkers have been identified for cellular senescence, including changes in the extracellular environment, growth factors, growth factor receptors, secondary messengers, cell cycle progression, transcription factors, and the fidelity of DNA synthesis (Cristofalo and Pignolo, 1996).

Recent findings suggest that many proteins associated with the extracellular matrix are overexpressed in senescent human fibroblasts. These proteins include alpha 1 procollagen, fibronectin, collagenase, stromelysin and gelatinase-TIMP-2 complex. Also many secretary proteins, such as IGF-1, IGF-BP3, tissue inhibitor of metalloproteinase-1, plaminogen activator inhibitor types 1 and 2, and EPC-1, are differentially expressed, further demonstrating that complex changes occur during senescence. However, changes in extracellular matrix cannot exclusively account for the phenotypic changes in senescent cells (Cristofalo and Pignolo, 1996).

When normal cells approach the end of their life span, they become less and less responsive to serum or growth factors as measured by DNA synthesis and mitosis (Cristofalo et al., 1989a). The basis for this loss of responsiveness can not be attributed to any dramatic reduction in the number of growth factor receptors, nor in the affinities which these receptors bind ligands (Gerhard et al., 1991). However, in some cases, the protein tyrosine kinase activities of these growth factor receptors are decreased in senescent cells (Cristofalo and Pignolo, 1996). Fibroblasts respond to mitogens through the intracellular actions of secondary events including phospholipid turnover, protein kinase C activation

and calcium mobilization. Alterations in these secondary events in senescent cells have been documented. Taken together, senescent cells do not respond to mitogenic stimulations, at least in part due to an unregulated secondary messenger system (Cristofalo and Pignolo, 1996).

Senescent cells have reduced transcription factor-binding activities (Dimri and Campisi, 1994). For example, the activities of the AP-1 (activator protein factor 1) complex, CREBP (cyclic AMP response element-binding protein), E2F-1, ID (inhibitor of DNA binding)-related gene products are severely reduced (Riabowol et al., 1992; Dimri and Campisi, 1994; Dimri et al., 1994; Hara et al., 1994). Of particular interest is the *c-Fos* gene. The expression of *c-Fos* is repressed in late-passage cells, indicating that it is potentially involved in the G1 arrest (Seshadri and Campisi, 1990). The forced expression of an inducible *c-Fos* construct by transfection of senescent cells results in six-fold increase in the number of cells capable of initiating DNA synthesis (Phillips et al., 1992). However, this repression of *c-Fos* is not observed in senescent melanocytes or fibroblasts from Werner syndrome patients, indicating that *c-Fos* is expressed in a cell cycle-dependent manner (Oshima et al., 1995).

One of the hallmarks of senescence in culture is the inability of cells to replicate their DNA. Senescent cells are unable to express PCNA (proliferating cell nuclear antigen), a cofactor of DNA polymerase delta (Chang et al., 1991). In senescent cells, the replication-dependent histones are also repressed and a variant histone polyadenylated RNA is expressed uniquely (Seshadri and Campisi, 1990). Although these findings suggest gross changes in the DNA

synthesis machinery of senescent cells, the observation that SV40 can initiate an additional round of DNA synthesis in senescent cells (Gorman and Cristofalo, 1985), indicates that this machinery is still intact, even though it is nonresponsive.

Because senescent cells are arrested in G1 phase of the cell cycle, it is logical to consider that senescence is the result of impaired cell cycle progression. In fact, two Cdk inhibitors, p16 and p21, are overexpressed in senescent cells, and have been suggested to be involved in cellular senescence (Noda et al., 1994; Alcorta et al., 1996; Hara et al., 1996; Vogt et al., 1998). p16 and p21 act to inhibit G1 cyclin-Cdk complexes, thereby preventing phosphorylation of Rb protein (Sherr, 1996). Rb protein is known to be hypophosphorylated in senescent cells (Stein et al., 1990). Hypophosphorylated Rb in turn sequesters transcription factors E2F, which are required for the transactivation of many genes encoding proteins involved in cell cycle progression, such as cyclin A, CDC2, dihydrofolate reductase, thymidine kinase, DNA polymerase α and ribonucleotide reductase (Weinberg, 1995). p53 protein has also been implicated as being involved in cellular senescence (Kulju and Lehman, 1995; Levine, 1997). However, other evidence has suggested that p21 and p53 are not involved in cellular senescence (Medcalf et al., 1996).

One important question about these biomarkers is which changes in gene expression cause cellular senescence and which merely result from the senescence phenotype. Until this question is answered, the controversies concerning these senescence biomarkers will not rest. Recently, a novel

senescence-associated β-galactosidase activity at pH6.0 has been identified (Dimri et al., 1995). This marker can only be detected in senescent cells, not in presenescent, quiescent or immortal cells. This unique biomarker provides an important tool for the studies of cellular senescence.

2. Senescence genes

Cellular senescence phenotype is dominant. Somatic cell fusions between early and later passage normal fibroblast cells result in hybrid cells of limited proliferative capacity (Muggleton-Harris and Aroian, 1982; Pereira-Smith and Smith, 1982). Somatic cell fusion between normal (mortal) and immortal cells most often leads to mortal hybrid cells, displaying a finite proliferative capacity (Pereira-Smith and Smith, 1981; 1983; Koi and Barrett, 1986; Ryan et al., 1994). These experiments provide evidence that cellular senescence is dominant, and immortalization in culture is a recessive trait.

Somatic cell fusion studies have been used by Pereira-Smith and Smith to assign 40 different immortal human cell lines to four complementation groups (termed complementation groups A, B, C, and D) with no cell line belonging to more than two groups, indicating that at least four genes or gene pathways contribute to cellular senescence (Pereira-Smith and Smith, 1988). Their results also suggest that probably only one senescence gene or pathway has been lost in each immortal cell line. The somatic cell fusion experiments suggest that similar genetic alterations occur in cell lines assigned to the same complementation group, thus fusion between them does not restore their

mortality trait, allowing them to retain their immortal phenotypes and proliferate indefinitely. Immortal cells of different complementation groups have different genetic alterations, and these defects can be complemented when they are fused into one hybrid.

However, the existence of four complementation groups has been challenged by the studies by McCormick and Maher and their colleague (Ryan et al., 1994). When they fused the immortal cell strain MSU-1.1 with representative cell strains from each of the four complementation groups, they found that all of these fusions yield hybrids with infinite life span. One possible explanation is that MSU-1.1 cell belongs to all four complementation groups, and it has lost the functions of all four senescence genes or gene pathways. These results raise the question of the existence of four complementation groups. This challenge is supported by fusion studies of SV40-transformed immortal cells (Duncan et al., 1993). These researchers found that three SV40-transformed immortal cells can be assigned to more than one complementation group. Furthermore, Ryan et al. (1994) found that 38 of 39 hybrids from the fusions between infinite life span cells that have been reported by Pereira-Smith and Smith (1988) to complement each other, failed to exhibit finite life span. After careful examination of the results, they concluded that it is long-term drug selection, rather than complementation, that accounts for the death of the cell hybrids observed by Pereira-Smith and Smith. These results indicate that complementation between different immortal cells is very complicated and careful discrimination among different types of cell death is critically important. A senescence-specific marker able to distinguish senescence

from non-senescence is highly desirable. Senescence-associated β-galactosidase activity (Dimri et al., 1995) provides the unique opportunity to answer this question.

If it is true that different senescence genes or pathways are altered in immortal cells, it is possible to map each senescence gene or pathway to individual chromosomes by microcell-mediated chromosome transfer, in which a single human chromosome is transferred into immortal human cells. These experiments have demonstrated that the senescence pathway(s) in immortal cells can be restored. Senescence activity has been mapped for various immortal cell lines to a number of human chromosomes, including chromosomes 1, 2, 3, 4, 6, 7, 11, 18, and X (Sugawara et al., 1990; Klein et al., 1991; Ning et al., 1991; Koi et al., 1993; Ogata et al., 1993; Hensler et al., 1994; Sandhu et al., 1994; Sasaki et al., 1994; Ohmura et al., 1995; Uejima et al., 1995; Horikawa, et al., 1998; Uejima et al., 1998). More stringent analysis is required to assign the specific chromosome to the corresponding complementation group, that is the chromosome should induce senescence in multiple cell lines that are assigned to the same complementation group, but have no effect on cell lines assigned to other groups. By this analysis, chromosomes 1, 4, and 7 for group C, B and D, respectively, have been shown to carry the putative senescence genes (Ning et al., 1991; Ogata et al., 1993; Hensler et al., 1994).

A lot effort has been devoted to clone these senescence genes; however, little progress has been made in terms of successfully finding these putative senescence genes. There was a meeting report of cloning the senescence gene

on chromosome 4 (Ehrenstein, 1998), but the full paper has not yet been published. Cloning of senescence genes will allow direct investigation of senescence pathways, testing the genetic basis for senescence. Insight into cellular senescence can be predicted to yield important knowledge concerning neoplastic transformation, ultimately leading to new approaches to cancer treatment. Identification of senescence genes and studies of their functions may also help to elucidate the mechanisms of cellular decline of human aging.

3. Immortalization in culture

Normal human cells give rise to spontaneously immortal clones at extremely low frequency (McCormick and Maher, 1988). Human cells are also exceedingly difficult to immortalize by treatment with physical or chemical carcinogens or transfection with oncogenes, although a few successful attempts have been made (Yoakum et al., 1985; Namba et al., 1988; Morgan et al., 1991; Shay et al., 1991b; Kuroki and Huh, 1993). For example, Namba et al. (1988) had to treat human fibroblasts with either gamma-irradiation or 4-nitroquinoline 1-oxide 10 times to immortalize them.

Oncogenes transfection has been shown to be able to achieve immortalization in culture (Faller et al., 1988; Nanus et al., 1989; Morgan et al., 1991). In this laboratory, immortalization of normal human fibroblasts has been successfully achieved by transfection of a *v-Myc* oncogene (Morgan et al., 1991). McCormick and Maher and their colleagues successfully generated the infinite life span MSU-1 lineage following transfection of a *v-Myc* oncogene, derived from

chicken cells, into a diploid human fibroblast cell line, LG1. The *v-Myc* expressing LG1 population underwent senescence, and through some spontaneous, as yet unidentified genetic changes, an infinite life span cell strain arose, designated as MSU-1.0 (Morgan et al., 1991). Most likely the *v-Myc* expression contributed to the immortalization process since no immortal cells arises from populations without expression of the *v-Myc*. Cell fusions between LG1 and MSU-1.0 or its derivative cell strain, MSU-1.1, showed that the hybrid cells were mortal (Ryan et al., 1994). This indicates that the spontaneous, as yet unidentified genetic changes in the immortalization of MSU-1.0 probably involve the loss of a tumor suppressor gene which is critical for immortalization. Therefore, at least two genetic changes were involved in the immortalization of the finite life span cell into MSU-1.0 cells.

Most immortalization studies involve the use of DNA tumor viruses, such as SV40 and human papilloma virus (HPV) (Kuroki and Huh, 1993). SV40 can immortalize human fibroblasts at a frequency of 3×10^{-7} (Shay and Wright, 1989). Following infection of SV40 virus, normal human cells proliferate for approximately 20 to 30 population doublings beyond their normal senescence point. The extended life span population then enters a state of crisis, during which the cell number remains constant or declines as a result of an increase in cell death. From this crisis population arise the rare, immortal clones, presumably as a result of additional genetic changes (Wright and Shay, 1992). This occurs at a frequency of 3×10^{-7} (Shay and Wright, 1989). The large T-antigen protein encoded by SV40 is responsible for immortalization (Radna et al., 1989; Wright

et al., 1989). The large T-antigen of SV40 binds to p53 and Rb proteins and disrupts their functions (Ludlow, 1993). Cells from Li-Fraumeni syndrome patients, who have germline *p53* mutations (Srivastava et al., 1990), are able to escape senescence and become immortal in culture at a relatively high frequency (Bischoff et al., 1990), indicating that loss of p53 function is involved in immortalization. Both p53 and Rb binding functions of SV40 large T-antigen are required for the post-crisis proliferation. Adenovirus E1A and E1B protein or HPV E7 and E6 protein bind to Rb and p53 respectively. The combination of E1A and E1B or E6 and E7 is able to replace SV40 large T-antigen to cause the rare immortalization of human cells, whereas each individual protein by itself fails to do so (Shay et al., 1991a). Furthermore, targeted functional knockout of the *p53* and *Rb* genes with specific antisense oligomers results in an extended life span of normal human fibroblasts (Hara et al., 1991). These experiments establish a causal relationship between immortalization and loss of functions of the *p53* and *RB* genes. The functional loss of the *p53* and *RB* genes is necessary for the extended life span, and additional genetic changes are required for progression beyond crisis, indicating immortalization is a multistep process.

C. Telomere hypothesis

Senescence is determined by the number of times that the cells divide, not by the calendar time (Hayflick, 1976). This suggests that a counting mechanism exists to record cellular divisions, and that proliferation is limited by this "mitotic clock". Telomere length seems to be a good candidate for this parameter.

Telomeres are terminal eukaryotic chromosome sequences consisting of short nucleotide repeats. Human telomeres consist of repeats of the sequence TTAGGG at chromosome ends. Telomeres protect chromosomes, preventing fusion, recombination and degradation (Greider, 1996). Telomeres are synthesized by telomerase, a ribonucleoprotein enzyme composed of an RNA component and the telomerase reverse transcriptase subunit (Blackburn, 1991). The RNA component of telomerase provides the template for telomere synthesis, and the human telomerase RNA component has been cloned (Feng et al., 1995). The telomerase reverse transcriptase recently has been cloned by three independent groups (Kilian et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997). Telomere length can also be maintained by other mechanisms such as recombination between telomeric DNA (Lundblad and Blackburn, 1993; Bryan et al., 1995; McEachern and Blackburn, 1996).

Telomere length has been reported to shorten in normal cells as the proliferative capacity decreases (Harley et al., 1990) and telomere loss is thought to control entry into senescence (Wright and Shay, 1992; Chang and Harley, 1995; Holt et al., 1996). The telomere hypothesis proposes that cells become senescent when progressive telomere shortening during each cell division produces a threshold telomere length (Harley, 1991).

1. Evidence supporting the telomere hypothesis

Telomerase is active in germline cells, and in human, telomeres in these cells are maintained at about 15 kilobase pairs. Telomerase activity is detectable in

80% to 90% of human tumors (Kim et al., 1994; Shay and Bacchetti, 1997). In contrast, telomerase is not expressed in most human somatic cells (Kim et al., 1994; Shay and Bacchetti, 1997), and telomere length is significantly shorter (de Lange et al., 1990). For example, Kim et al. (1994) have examined telomerase activity in a variety of normal (mortal) cell strains and immortal cell lines, in addition to tumor and normal biopsies. In this study, they found that 98 out of 100 immortal cell lines, 90 of 101 malignant tumor biopsies, and all the normal germline tissue biopsies have telomerase activity. In contrast, no telomerase activity was detected in 22 normal (mortal) cell strains and 50 normal or benign somatic tissue biopsies. The correlation between both immortalization and tumor formation with telomerase activity suggest that telomerase activity is required for immortalization, and immortalization is required for tumor growth.

Oshimura and Barrett (1997) transferred a normal chromosome 3 into a renal cell carcinoma cell line RCC23, which has the telomerase activity. They found that senescence is restored in RCC23 cells and is associated with the loss of telomerase activity. Furthermore, the telomere of the introduced chromosome 3 shortens. These investigations suggest that chromosome 3 carries a telomerase repressor and that escape from telomerase suppression is one of the several pathways involved in immortalization (Horikawa et al., 1998).

The most convincing evidence that supports the telomere hypothesis comes from the study by Shay and Wright and their colleagues (Bodnar et al., 1998). They found that expression of the human telomerase catalytic subunit in telomerase-negative normal human epithelial cells and fibroblasts extends the

cells' life span and overcomes cellular senescence. This study establishes a causal relationship between telomere shortening and cellular senescence.

2. Evidence opposing the telomere hypothesis

When two telomerase positive, immortal cells from two different complementation groups are fused together, the hybrid cell senesces (Pereira-Smith and Smith, 1988). Some senescent hybrid cells continue to express telomerase, and their telomerase activity does not correlate with their ability to senesce or proliferate (Bryan et al., 1995). This suggests that the loss of telomerase activity is not required for cellular senescence. Treatment of immortal human B and T cell lines with chemical inhibitors of reverse transcriptase reduces telomerase activity to levels in pre-immortal cells, but has no effect on the immortal phenotypes (Strahl and Blackburn, 1996). Evidence opposing the telomere hypothesis is also provided by chromosome transfer studies. When Oshimura and Barrett (1997) transferred chromosome 7 or 11 into RCC23 cells, these cells did not senesce, and the telomerase activity and telomere length were maintained. One implication is that neither chromosome 7 nor chromosome 11 has a telomerase repressor. However, both chromosomes 7 and 11 have been shown to be able to restore senescence in some other immortal, telomerase positive cells (Koi et al., 1993; Ogata et al., 1993). This suggests that at least chromosomes 7 and 11 restore senescence through pathways other than suppression of telomerase activity. If chromosome 3 carries a telomerase repressor as suggested (Oshimura and Barrett, 1997; Horikawa et al., 1998), and

telomerase activity is sufficient to extend cells' life span (Bodnar et al., 1998), one would expect that chromosome 3 would restore senescence in all the telomerase positive immortal cells. However, this is not the case (Uejima et al., 1995).

One may argue that these senescing cells generated by either cell fusion or chromosome transfer lack telomerase for some unknown reasons or that telomere maintenance mechanisms have been disrupted, and therefore their telomeres keep shortening. In these experiments, the hybrid cells enter senescence after a few population doublings (Sugawara et al., 1990; Uejima et al., 1995). Human telomeres are programmed to undergo gradual shortening by about 100 base pairs per cell division and when several kilobases of the telomeric DNA are lost, cells stop dividing and senesce (Harley et al., 1990; de Lange, 1998). If this is the case, then telomeres are not expected to shorten substantially in a few population doubling time (6-9 population doublings). This suggests that the induced senescence observed in these cells is not due to the critical telomere shortening. The suggestion that telomere length is not so critical in determining cellular senescence is also supported by the study of mouse species, *Mus musculus*. This mouse species has telomeres that are three times longer than those in humans. The rate of telomeric DNA loss in mouse is estimated to be 50-100 base pairs per cell division (Blasco et al., 1997; Zakian, 1997). Therefore, one would expect *Mus musculus* to have a long life span, however, this is not the case (Kipling and Cooke, 1990).

Recently mice lacking telomerase RNA component have been generated (Blasco et al., 1997). Cells from knockout animals do not have detectable

telomerase activity. The knockout mice are viable through at least six generations. Since cells in the mouse germline undergo 62 cell divisions in the male and 25 in the female (Drost and Lee, 1995), each successive knockout mouse generation translates into roughly 40 cell divisions without telomerase. Progressive loss of telomeres occurs in each generation with a shortening rate of 4.8±2.4 kilobases per mouse generation in these knockout mice. The mouse embryonic fibroblasts derived from the second or later generations of knockout mice have substantially shorter telomeres, and some chromosomes in these cells even lack detectable telomeric repeats. However, there is no difference between the first generation of the wild type and the knockout mice primary cultures in the ability to overcome senescence. What is more, these researchers found no difference in the ability of primary cultures of the knockout mice in the first through sixth generation to overcome senescence. These results clearly indicate that telomere length is not a primary determinant of senescence in mouse cells, and telomerase activity is not required in mouse cells to allow escape from senescence. Furthermore, these telomerase-deficient mouse cells can be immortalized in culture, transformed by oncogenes, and generate tumors following transformation, suggesting that the association of telomerase with immortalization may be coincidental rather than of functional significance (Blasco et al., 1997).

The debate over the telomere hypothesis is far from over. It will continue until we identify the genes that are really responsible for onset of senescence and understand their functions. It will be extremely interesting to discover the nature

of the mitotic clock if there is one. To clone senescence genes, it will be very important to understand the criteria for them. The criteria I would use for senescence genes should meet the following standards as modified from proposal by Vojta and Barrett (1995): 1). induction of growth arrest (senescence) following expression in a proliferating cell, with cell viability being preserved; 2). down-regulation, mutation in immortal cells; and 3). induction of immortalization in normal cells by mutation or down-regulation. Cloning of senescence genes will allow direct investigation of senescence pathways and testing the genetic basis for senescence. Insight into cellular senescence will yield important knowledge concerning neoplastic transformation, ultimately leading to new approaches to cancer treatment. Identification of senescence genes and studies of their functions will also help to elucidate the mechanisms of cellular decline of human aging.

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CHAPTER 2

O^6-methylguanine induces intrachromosomal homologous recombination in human cells

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Abstract

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), which alkylates many positions in DNA including the O^6 position of guanine, efficiently induces intrachromosomal homologous recombination in mouse L cells. To investigate the role of O^6-methylguanine in the induction of homologous recombination in human cells, three cell strains containing duplicated copies of the Herpes simplex virus I thymidine kinase (*Htk*) gene and three cell strains containing duplicated copies of the gene coding for hygromycin phosphotransferase (*hyg*) were treated with MNNG. Neither the *Htk* genes nor the *hyg* genes code for a functional enzyme because each contains an insertion mutation at a unique site, *i.e.*, 8-bp *Xho* I linker insertions in the *Htk* genes and 10-bp *Hin*d III linker insertions in the *hyg* genes. These cell strains differ in their level of O^6-alkylguanine-DNA alkyltransferase (AGT), which specifically removes the methyl group from the O^6 position of guanine. Generation of a functional *Htk* or *hyg* gene has been shown to require intrachromosomal homologous recombination between the two mutant *Htk* genes or the two mutant *hyg* genes. In all six cell strains, MNNG induced a dose-dependent increase in the frequency of homologous recombination. In each set, there was an inverse correlation between the frequency of MNNG-induced recombination and the level of AGT activity. To further study the role of O^6-methylguanine in the induction of homologous recombination, we used O^6-benzylguanine to inactivate AGT in two additional human cell strains containing the *hyg* recombination substrate. After depletion of AGT activity by O^6-benzylguanine, both cell strains showed a significantly elevated level of MNNG-induced homologous recombination. These

results indicate that O^6-methylguanine is the principal lesion responsible for the induction of homologous recombination in these human cells by this methylating agent.

Introduction

Homologous recombination has been implicated as one of the mechanisms in the development of certain kinds of cancer (1-4). It represents one method of achieving loss of heterozygosity of critical genes involved in the process of carcinogenesis. For example, homologous recombination in cells from some retinoblastoma patients results in the *Rb* locus on chromosome 13 becoming homozygous (5), and homozygosity of loci on the short arm of chromosome 17 by mitotic homologous recombination has been shown to play a role in the development of astrocytoma (6). Cells from a patient with Bloom's syndrome, which is characterized by an inherited predisposition to various kinds of cancer, have been shown to exhibit an abnormally increased frequency of extrachromosomal recombination between homologous viral genes (7).

Several carcinogens have been shown to induce homologous recombination in mammalian cells (8-11). One of these, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) is particularly efficient in such induction (8). MNNG methylates various sites on DNA, but O^6-methylguanine is responsible for the vast majority of mutations induced in human cells by this agent (12,13). Because of the similar bond angles and lengths between O^6-methylguanine and adenine, the DNA replication complexes misread O^6-methylguanine as adenine and incorporate a thymine

nucleotide across from it, leading to a G:C to A:T transition (14). Human cells use O^6-alkylguanine-DNA alkyltransferase (AGT) to remove the methyl group from the O^6 position of guanine and transfer it to a cysteine residue in the active site of the protein itself. The covalent attachment of the methyl group causes irreversible inactivation of the AGT protein (15).

It has been shown that in cells lacking AGT activity, the frequency of chromosome aberrations induced by methylating agents is abnormally elevated, implicating O^6-methylguanine as an important lesion in induction of such aberrations (16). However, the role of O^6-methylguanine in the induction of homologous recombination is not known. To examine this question, two sets of human cell strains (11, 17) containing duplicated copies of a selectable gene, and each gene containing an insertion mutation at a unique site, were treated with MNNG. The presence of an 8-bp or a 10-bp insertion renders the selectable genes non-functional. Homologous recombination between two defective copies of a selectable gene can generate a functional gene. These cell strains differ in their level of AGT activity. MNNG induced intrachromosomal homologous recombination in a dose-dependent manner in all the cell strains, and in each set there was an inverse correlation between the frequency of MNNG-induced homologous recombination and the level of AGT activity in cells. When the endogenous AGT activity in two additional cell strains was depleted by pretreatment with O^6-benzylguanine, the frequency of MNNG-induced homologous recombination was significantly increased above that seen in the absence of O^6-benzylguanine. These results indicate that O^6-methylguanine is the lesion principally responsible for the induction of homologous

recombination by MNNG in human cells.

Materials and Methods

Plasmids

Plasmid pJS-3 (Figure 1A), containing duplicated copies of the Herpes simplex virus I thymidine kinase (*Htk*) gene and a selectable marker gene coding for aminoglycoside phosphotransferase (*neo*), was kindly provided by Dr. R. Michael Liskay of Oregon Health Sciences University. Details on the construction of the plasmid were reported earlier (18). In brief, the plasmid carries two *Htk* genes, each mutated by an 8-bp *Xho* I linker inserted at different site. Plasmid pTPSN (Figure 1B) contains duplicated copies of the hygromycin phosphotransferase (*hyg*) gene and the selectable marker gene *neo*. Each *hyg* gene is mutated by a 10-bp *Hin*d III linker inserted at different site. Details on the construction of this plasmid were reported earlier (11). We constructed a new plasmid, designated pJH-1 (Figure 1C), in which the *neo* gene from pTPSN was replaced with the *E. coli* xanthine-guanine phosphoribosyl transferase (*gpt*) gene for use in a cell strain containing a *neo* gene (see below). To construct plasmid pJH-1, the fragment from pTPSN containing the duplicated copies of the *hyg* gene was released by digestion with *Xho* I and *Hpa* I and ligated with the fragment from pSV2gpt (19) which contains the *gpt* gene and the gene coding for ampicillin resistance (*amp*). This fragment was linearized by *Bam*H I digestion.

Cell strains and culture conditions

The three tk^- cell strains used in this study, *i.e.*, 143tk^--7, RDtk^--12 and

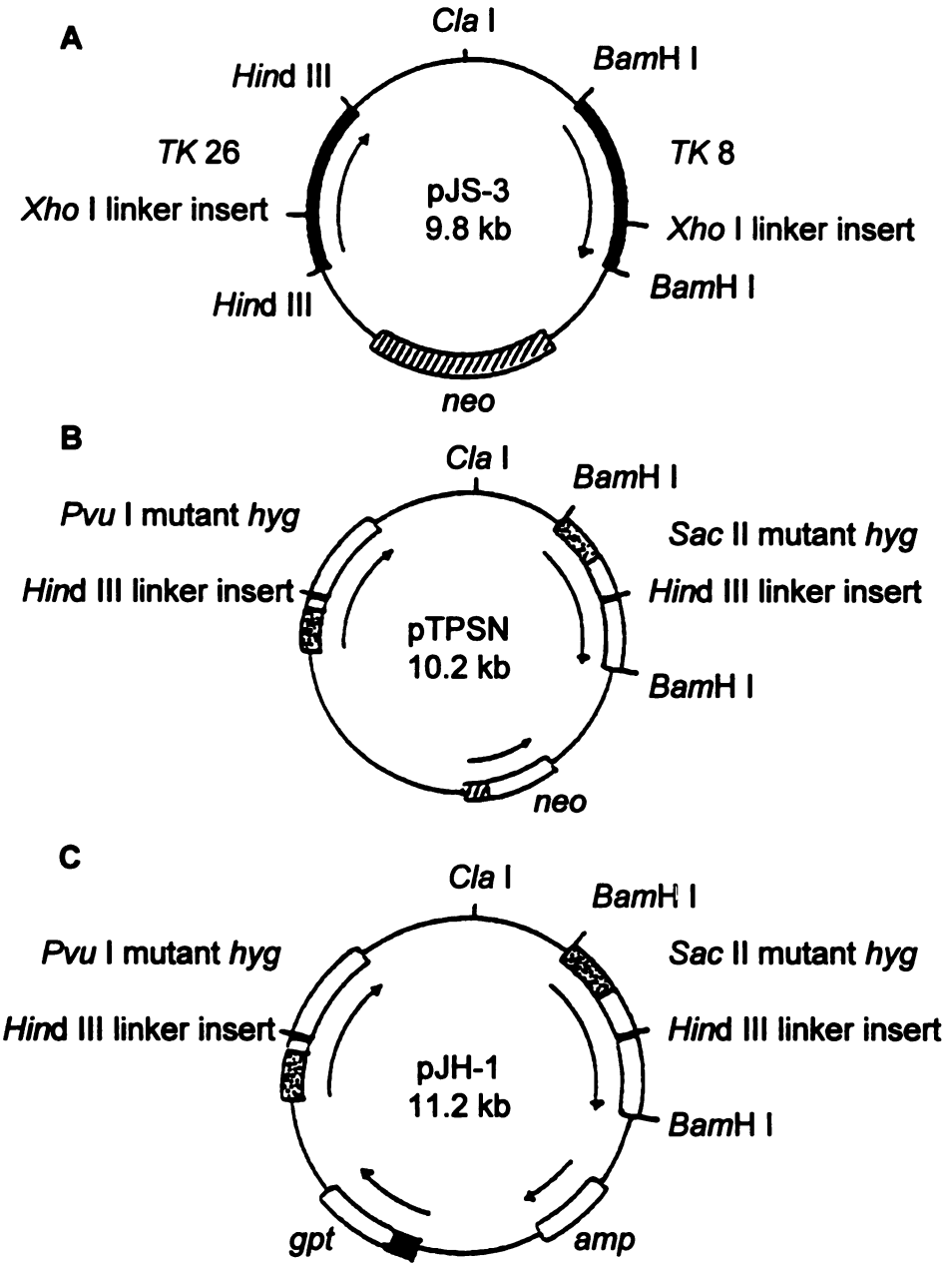


Figure 1. Diagram of the plasmids. (A). pJS-3; (B). pTPSN; (C). pJH-1. The arrows indicate the direction of transcription of the genes.

XP12ROSV40tk$^-$-7, have been fully described previously (17). The strains carry the integrated pJS-3 plasmid containing the duplicated *Htk* genes. Cell strains KMST-6-9, XP2OS(SV)-18 and XP2YO(SV)-65, used in this study, carry the integrated pTPSN plasmid containing the duplicated *hyg* genes. These cell strains, as well as the selection conditions for recombination, have also been fully described previously (11). All six strains were routinely cultured in Eagle's minimal essential medium supplemented with 10% (vol/vol) fetal bovine serum (Hyclone, Logan, UT) and modified as described (8). Cell strain MSU-1.2 is a growth factor independent derivative of the infinite life span, near-diploid, karyotypically stable human fibroblast cell strain MSU-1.1 which was established in this laboratory (20). Cells were routinely cultured in McM medium (21), a modification of MCDB-110 medium (22) containing 10% (vol/vol) supplemented calf serum (Hyclone), 100 U/ml penicillin,100 µg/ml streptomycin and 1 µg/ml hydrocortisone (culture medium).

DNA transfection

MSU-1.2 cells were transfected with plasmid pJH-1 using lipofectAMINE according to manufacturer's (GIBCO) recommendations. Two days later, cells were selected in culture medium containing 25 µg/ml mycophenolic acid, 2 µg/ml aminopterin, 15 µg/ml hypoxanthine and 250 µg/ml xanthine. After two weeks, colonies were isolated and propagated in selective medium.

Polymerase chain reaction (PCR)

Two primers were designed to amplify the *hyg* gene. Primer 1: 5'-CCACTTCGCATATTAAGGTGACGCG-3'. The sequence of primer 1 corresponds to the sequence around the TATA box of the *hyg* gene. Primer 2: 5'-

TCGAAATCAGCTCTTGTTCGGTCGG-3'. The annealing site for primer 2 is located just beyond the stop codon in the *hyg* gene. Either genomic DNA or cell lysate from 2,000-10,000 cells was used as a template for PCR amplification using the conditions described (23).

Southern analysis

Genomic DNA was isolated using a Puregene DNA isolation kit (Gentra System Inc., Research Triangle Park, NC) and digested with restriction enzymes using the supplier's (Boehringer Mannheim) recommended conditions. A 2.2-kb *Bam*H I fragment of pJH-1 containing the *hyg* gene was labeled with [α-^{32}P]-dCTP as a probe using a random-primed labeling kit from Boehringer Mannheim. Southern blotting analysis was carried out as described (17).

Treatment with MNNG

Exponentially growing cells were plated at 5.5×10^5 cells per 100-mm diameter dish. After 14-16 hours, culture medium was removed from the dishes, and the cells were rinsed twice with phosphate-buffered saline (PBS) to ensure that no serum remained to interfere with MNNG. Eagle's minimal essential medium, buffered to pH 7.25 using 20 mM HEPES but without sodium bicarbonate (treatment medium), was added to each dish. MNNG (Pfaltz and Bauer, Flushing, NY), dissolved in fresh dimethylsulfoxide (DMSO) immediately before treatment, was added to the dishes to yield the desired concentrations. Appropriate amounts of DMSO were added to each dish to ensure that all cells, including the control cells, were exposed to the same concentration of DMSO (final concentration less than 0.5%). After the dishes were incubated at 37°C, 5% CO_2 for one hour, the medium was removed and the

cells were refed with the appropriate culture medium.

Depletion of AGT activity

To deplete cells of AGT activity, O^6-benzylguanine (a generous gift from Dr A. E. Pegg, Pennsylvania State University), freshly dissolved in DMSO, was added to culture medium to achieve the concentration of 25 μM. After 2 hours, the medium was exchanged for treatment medium supplemented with O^6-benzylguanine (25 μM), and the cells were treated with MNNG as above. At the end of 1 hour, the treatment medium was exchanged for culture medium containing 25 μM O^6-benzylguanine. Cells remained in this medium for 48 additional hours. Previous studies in this laboratory show that AGT activity remains undetectable for at least 48 hours after removal of O^6-benzylguanine from culture medium (13), giving a total of ~96 hours of depletion.

Assay for cytotoxicity

The cytotoxic effect of the treatment was determined from the relative colony-forming ability of the MNNG-treated cells compared to the untreated control. After MNNG treatment, the cells in one dish from each concentration were trypsinized and plated at cloning densities (*i.e.*, densities adjusted to give 35 to 60 macroscopic colonies per 100-mm diameter dish, four to six dishes per determination). After 14 days with one refeeding with the appropriate culture medium, the colonies were stained and counted, and the survival was calculated from the relative cloning efficiency (8).

Assay for homologous recombination

Sufficient dishes were used for each dose so as to have approximately $2x10^6$

surviving target cells. After MNNG treatment, culture medium was added. Approximately 48 hours after treatment, cells were selected for either hygromycin resistance (hyg^r) in culture medium containing the designated concentrations of hygromycin-B (Calbiochem) or CHAT resistance using culture medium supplemented with $2x10^{-5}$ M deoxycytidine, $1x10^{-4}$ M hypoxanthine, $4x10^{-7}$ M aminopterin and $3x10^{-5}$ M thymidine (CHAT medium). The cells were refed with the selection medium every 5 days for hygromycin resistance or weekly for CHAT resistance. After three weeks, colonies were counted, and representative recombinants were isolated for characterization. The frequency of recombination was determined from the number of hyg^r or CHAT resistant colonies divided by the number of viable cells as determined from the accompanying cytotoxicity assay. The frequency of induced recombination was determined by subtracting the background frequency observed in the untreated control population from the total frequency.

Results

Construction and characterization of the MSU-1.2 cell strains containing the recombination substrate

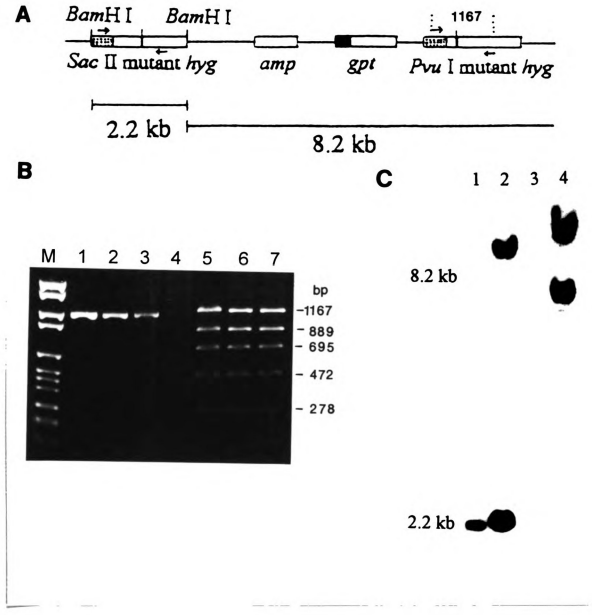
The six human cell strains containing a recombination substrate that were used in previous studies reported from this laboratory (10, 11, 17) are aneuploid and karyotypically unstable. In order to have a near-diploid, karyotypically stable cell strain containing an integrated recombination substrate, we chose MSU-1.2 cells. This clonally-derived infinite life span cell strain was obtained in this laboratory by

selecting MSU-1.1 cells (20) for the ability to form colonies in the absence of exogenous growth factors. The cells are resistant to G418 because their cell of origin was a foreskin-derived neonatal cell line that had been transfected with a plasmid containing a *neo* gene (20).

We transfected MSU-1.2 cells with pJH-1 which had been linearized at its unique *Cla* I site to facilitate the integration of the *hyg* genes in the proper configuration. Representative transfectants were isolated and the nature of the two *hyg* genes was characterized using PCR amplification. The two PCR primers used amplify a 1167-bp fragment of the *hyg* gene (Figure 2A). *Hin*d III digestion of the 1167-bp fragment amplified from the *Sac* II mutant *hyg* gene yields an 889-bp and a 278-bp fragment, whereas a 695-bp and a 472-bp fragment are generated by *Hin*d III digestion of the 1167-bp fragment amplified from the *Pvu* I mutant *hyg* gene. If both copies of the *hyg* gene have been integrated into a chromosome of a transfectant, *Hin*d III digestion of the PCR products generates four fragments, of lengths 278 bp, 472 bp, 695 bp and 889 bp.

Two transfectants, designated MSU-1.2-D10.4 and MSU-1.2-E7.2, were found to contain both copies of the *hyg* gene, as determined by PCR amplification followed by *Hin*d III digestion (Figure 2B). Since extended PCR products from two *hyg* genes can switch strands during the denaturation/renaturation steps, PCR is expected to generate some heteroduplex DNA, *i.e.*, one strand from the *Sac* II mutant *hyg* gene and the other strand from the *Pvu* I mutant *hyg* gene. Such a heteroduplex DNA cannot be digested by *Hin*d III, and therefore, after *Hin*d III digestion, in addition to the four fragments generated by digestion, an uncut 1167-

Figure 2. Analysis of MSU-1.2 transfectants for the integrity of the recombination substrate and the number of integrated copies of the pJH-1 plasmid. (A). Diagram of the sizes of the fragments containing a *hyg* gene after *Bam*H I digestion of *Cla* I linearized pJH-1. Digestion yields a 2.2-kb and a 8.2-kb fragment containing the *Sac* II mutant and the *Pvu* I mutant *hyg* gene respectively. (B). Analysis for integrity of the *hyg* gene using PCR amplification followed by *Hin*d III digestion. Lane M, DNA molecular marker VI from Boehringer Mannheim (pBR328-*Bgl* I DNA + pBR328-*Hinf* I DNA); Lane 1, PCR products amplified from plasmid DNA pJH-1; Lane 2, PCR products amplified from genomic DNA of cell strain MSU-1.2-D10.4; Lane 3, PCR products amplified from genomic DNA of cell strain MSU-1.2-E7.2; Lane 4, PCR amplification using genomic DNA of untransfected MSU-1.2 as an negative control; Lane 5. *Hin*d III digestion of PCR products amplified from plasmid DNA pJH-1; Lane 6, *Hin*d III digestion of PCR products amplified from genomic DNA of MSU-1.2-D10.4; Lane 7, *Hin*d III digestion of PCR products amplified from genomic DNA of MSU-1.2-E7.2. The 1167 bp fragment is the non-digested PCR-dependent heteroduplex described in the Results section. (C). Southern analysis of transfectants from MSU-1.2 cells transfected with pJH-1. DNA was digested with *Bam*H I and probed with the *hyg* gene. Lane 1, plasmid pJH-1 control, showing an 8.2 kb and a 2.2 kb fragment containing DNA that hybridizes to the α-^{32}P labeled *hyg* gene; Lane 2, MSU-1.2-D10.4 cells showing the pattern expected if a single copy of the plasmid has been integrated into the chromosomes of MSU-1.2 cells, *i.e.*, one 2.2-kb and one large junction fragment; Lane 3, non-transfected MSU-1.2 cells (negative control); Lane 4, MSU-1.2-E7.2 cells (for an interpretation, see text).


A
BamH I
BamH I
1167
Sac II mutant hyg
amp
gpt
Pvu I mutant hyg
2.2 kb
8.2 kb
B
M 1 2 3 4 5 6 7
bp
1167
889
695
472
278
C
1 2 3 4
8.2 kb
2.2 kb

bp fragment should also be present. This is the result we obtained with both cell strains (Figure 2B). That such heteroduplex formation is an artifact of PCR was confirmed by using only one or the other copy of the *hyg* genes as the template for PCR. In the latter case, PCR products amplified from either copy of the *hyg* genes were completely digested by *Hin*d III as expected (data not shown), since no heteroduplex can be generated during PCR.

These two MSU-1.2 transfectant cell strains were then analyzed by Southern blotting to determine the number of integrated copies of the plasmid pJH-1 and to confirm the integrity of the *hyg* genes. As shown in Figure 2A, if only a single intact integrated copy of the recombinant substrate is present, digestion with *Bam*H I should produce one 2.2-kb fragment and one large junction fragment containing the other *hyg* gene. If two intact copies of the substrate have integrated into the genome at separate sites, one 2.2-kb fragment and two large junction fragments will be seen. The Southern blotting data (Figure 2C, Lane 2) showed that cell strain MSU-1.2-D10.4 contains only one copy of pJH-1 since one 2.2-kb fragment and one large junction fragment were present. No *hyg* gene was present in the parental MSU-1.2 cells (Figure 2C, Lane 3). As shown in Figure 2C, Lane 4, there are two large fragments in the Southern blot from MSU-1.2-E7.2 cells and no fragment of length 2.2-kb. Because the PCR data (Figure 2B, Lane 7) showed that MSU-1.2-E7.2 cells contain two intact *hyg* genes, the most likely interpretation of Lane 4 is that one *Bam*H I site upstream of the *Sac* II mutant *hyg* gene was destroyed during the process of integration of the plasmid pJH-1 into the chromosome of the recipient cells. Nevertheless, that copy of the *hyg* gene is essentially intact (see below).

The characteristics and in particular the level of AGT activity of these two MSU-1.2-derived cell strains, as well as those of the six human cell strains used in our earlier studies of UV-induced homologous recombination (10, 11, 17), are summarized in Table 1. Productive homologous recombination between either mutant *Htk* genes or mutant *hyg* genes in these cell strains generates a wild type gene, conferring CHAT resistance or hygromycin resistance, respectively. All eight cell strains can undergo spontaneous intrachromosomal homologous recombination (10, 11, 17 and below).

MNNG-induced homologous recombination in the cell strains containing the *Htk* genes as the recombination substrate

Cell strains RDtk$^-$-12, 143tk$^-$-7 and XP12ROSV40tk$^-$-7 were treated with MNNG. The decrease in survival is shown in Figure 3A. The sensitivity of the cell strains to MNNG correlated well with their level of AGT activity in that cells with higher AGT activity were less sensitive to the cytotoxicity effect of MNNG, as expected (12). As shown in Figure 3B, MNNG induced homologous recombination in these three cell strains in a dose-dependent manner. There was an inverse correlation between the frequency of MNNG-induced homologous recombination and the level of AGT activity in the cell strains.

MNNG-induced homologous recombination in the cell strains containing the *hyg* genes as the recombination substrate

The cytotoxicity of MNNG in cell strains XP2YO(SV)-65, KMST-6-9 and XP2OS(SV)-18 is shown in Figure 4A. There was a correlation between the sensitivity to MNNG and the level of AGT activity in cells. After MNNG treatment,

Table 1. Characterization of human fibroblast cell strains.

Cell strain	Recombination substrate	No. of copies of integrated plasmid	Clinical status of donor or tissue	AGT activity [a] (fmoles removed per mg protein)
RDtk$^-$-12	pJS-3 (*Htk*)	1	Rhabdomyosarcoma-derived	221
XP12ROSV40tk$^-$-7	pJS-3 (*Htk*)	2	XP-A, SV40-immortalized	7
143tk$^-$-7	pJS-3 (*Htk*)	1	Osteosarcoma-derived	3
XP2YO(SV)-65	pTPSN (*hyg*)	1	XP-F, SV40-immortalized	166
KMST-6-9	pTPSN (*hyg*)	1	Normal, ^{60}Co-immortalized	55
XP2OS(SV)-18	pTPSN (*hyg*)	2	XP-A, SV40-immortalized	0
MSU-1.2-D10.4	pJH-1 (*hyg*)	1	Normal, *v-myc* transformed	209
MSU-1.2-E7.2	pJH-1 (*hyg*)	1	Normal, *v-myc* transformed	221

a: AGT activity was determined by measuring the decrease of radioactive labeled O^6-methylguanine from DNA using procedures described previously (12). XP-A: xeroderma pigmentosum, complementation group A; XP-F: xeroderma pigmentosum, complementation group F.

Figure 3. Cell killing (A) and induction of homologous recombination (B) as a function of the concentration of MNNG in three cell strains containing the *Htk* gene as the recombination substrate. Squares, RDtk$^-$-12 cells; circles, XP12ROSV40tk$^-$-7 cells; triangles, 143tk$^-$ -7 cells. In the various experiments, the background recombination frequencies per 10^6 cells were 15.5, 22 and 23.5 for 143tk$^-$-7 cells; 4.5 and 14.5 for XP12ROSV40tk$^-$-7 cells; 4.2 and 17.5 for RDtk$^-$-12 cells. These values have been subtracted to give the induced frequencies. The selection conditions used have been described (10, 17).

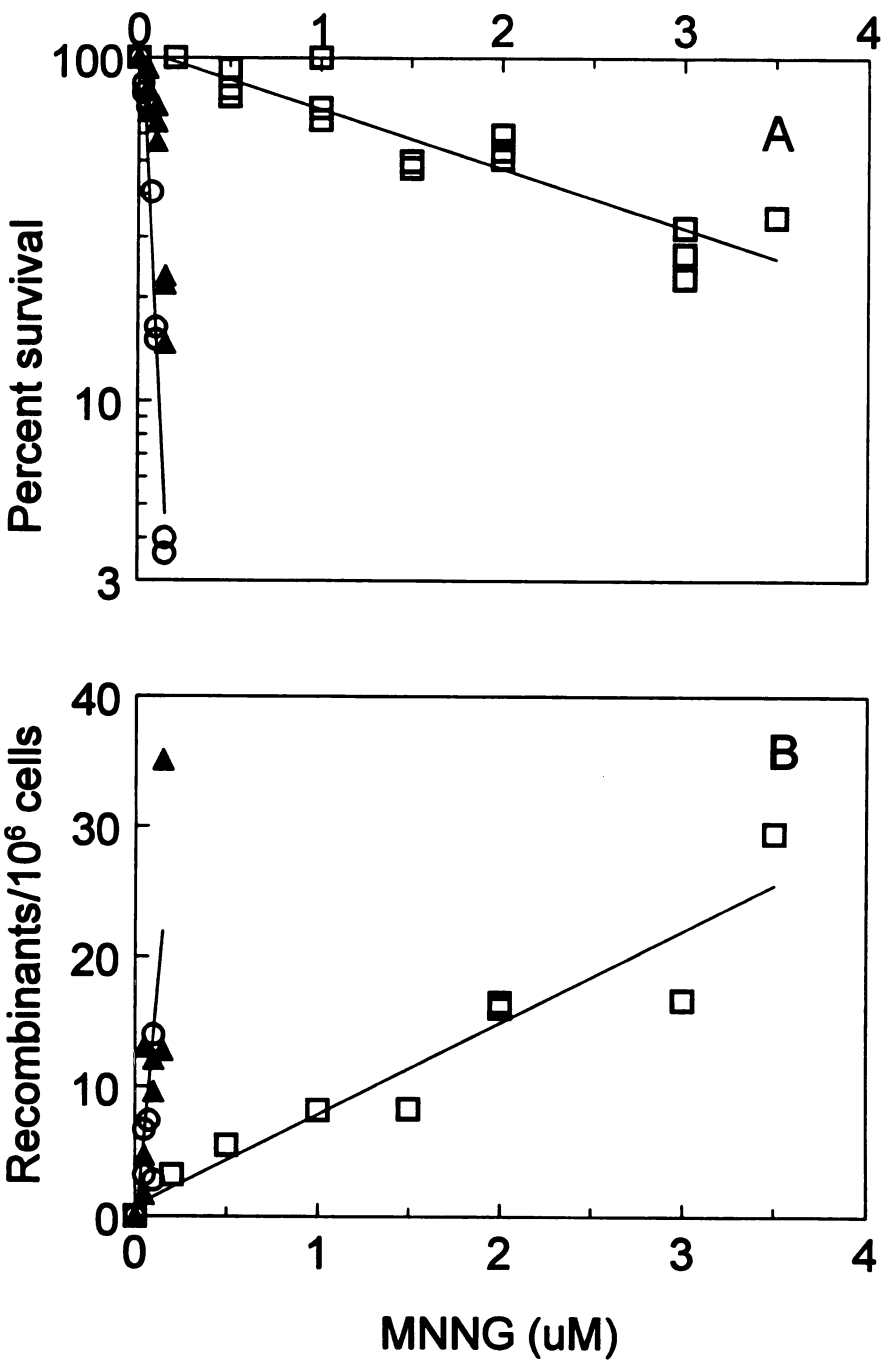

Percent survival
100
10
3
0
1
2
3
4
A
Recombinants/10^6 cells
40
30
20
10
0
B
MNNG (uM)

Figure 4. Cell killing (A) and induction of homologous recombination (B) as a function of the concentration of MNNG in cell strains containing the *hyg* gene as the recombination substrate. Squares, XP2YO(SV)-65 cells; triangles, KMST-6-9 cells; circles, XP2OS(SV)-18 cells. The background recombination frequencies per 10^6 cells in the various experiments were 29.5, 45.5 and 47.1 for XP2OS(SV)-18 cells; 9.3 and 24.5 for KMST-6-9 cells; 3.6, 12, 25 and 36.7 for XP2YO(SV)-65 cells. These values have been subtracted to give the induced frequencies. The selection conditions have been described (11).

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9 cells;

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8 cells;

5 cells.

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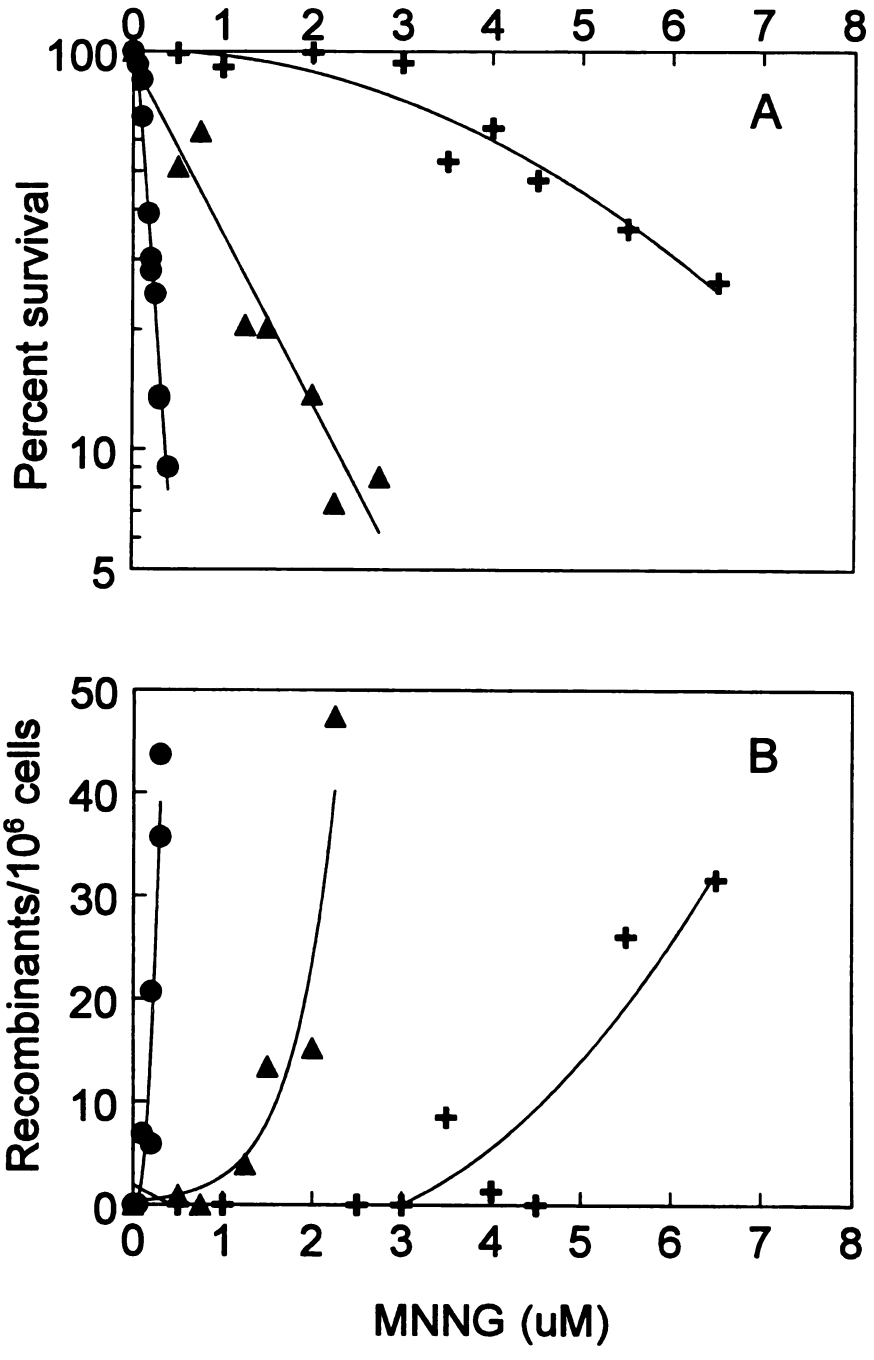


these three cell strains showed a dose-dependent increase in MNNG-induced homologous recombination (Figure 4B). Cells with higher AGT activity were less sensitive to the induction of homologous recombination by MNNG. Since AGT protein removes the methyl group from the O^6 position of guanine and restores the integrity of the DNA, the inverse correlation between the frequency of MNNG-induced homologous recombination and the level of AGT activity strongly suggests that O^6-methylguanine in DNA is the lesion that can induce homologous recombination.

The effect of O^6-benzylguanine on MNNG-induced homologous recombination

To test this hypothesis in isogenic cells, we treated one half of a population of MSU-1.2-E7.2 cells and MSU-1.2-D10.4 cells with O^6-benzylguanine (25 μM) for 2 hours to deplete them of AGT activity. The other half was left untreated. O^6-benzylguanine acts as the substrate analog to irreversibly inactivate the AGT protein (15, 24). As shown in Figures 5 and 6, depletion of AGT activity by O^6-benzylguanine greatly enhanced the cytotoxicity of MNNG as well as the frequency of induction of homologous recombination by MNNG in both MSU-1.2-E7.2 cells and MSU-1.2-D10.4 cells. A similar result was found when MSU-1.2-E7.2 cells were treated with *N*-methyl-*N*-nitrosourea in the presence and absence of O^6-benzylguanine (data not shown). These data indicate that O^6-methylguanine in DNA is the principal lesion responsible for inducing homologous recombination in human fibroblasts.

Characterization of recombination products

Hyg^r recombinants can be generated through three types of homologous recombination events (11). A single reciprocal exchange between two mutant *hyg*

Figure 5. Cell killing (A) and induction of homologous recombination (B) as a function of the concentration of MNNG in cell strain MSU1.2-E7.2 with and without O^6-benzylguanine (25 μM) pretreatment to deplete the AGT activity. Circles, with O^6-benzylguanine; triangles, without O^6-benzylguanine. The background recombination frequencies per 10^6 cells in the various experiments were 2.7, 9.4 and 11.7 for cells without O^6-benzylguanine, and 7.8 for cells with O^6-benzylguanine. These values have been subtracted to give the induced frequencies. The concentration of hygromycin-B used for selection was 100 U/ml, the highest that could be used without a cytotoxic effect on the recombinants.

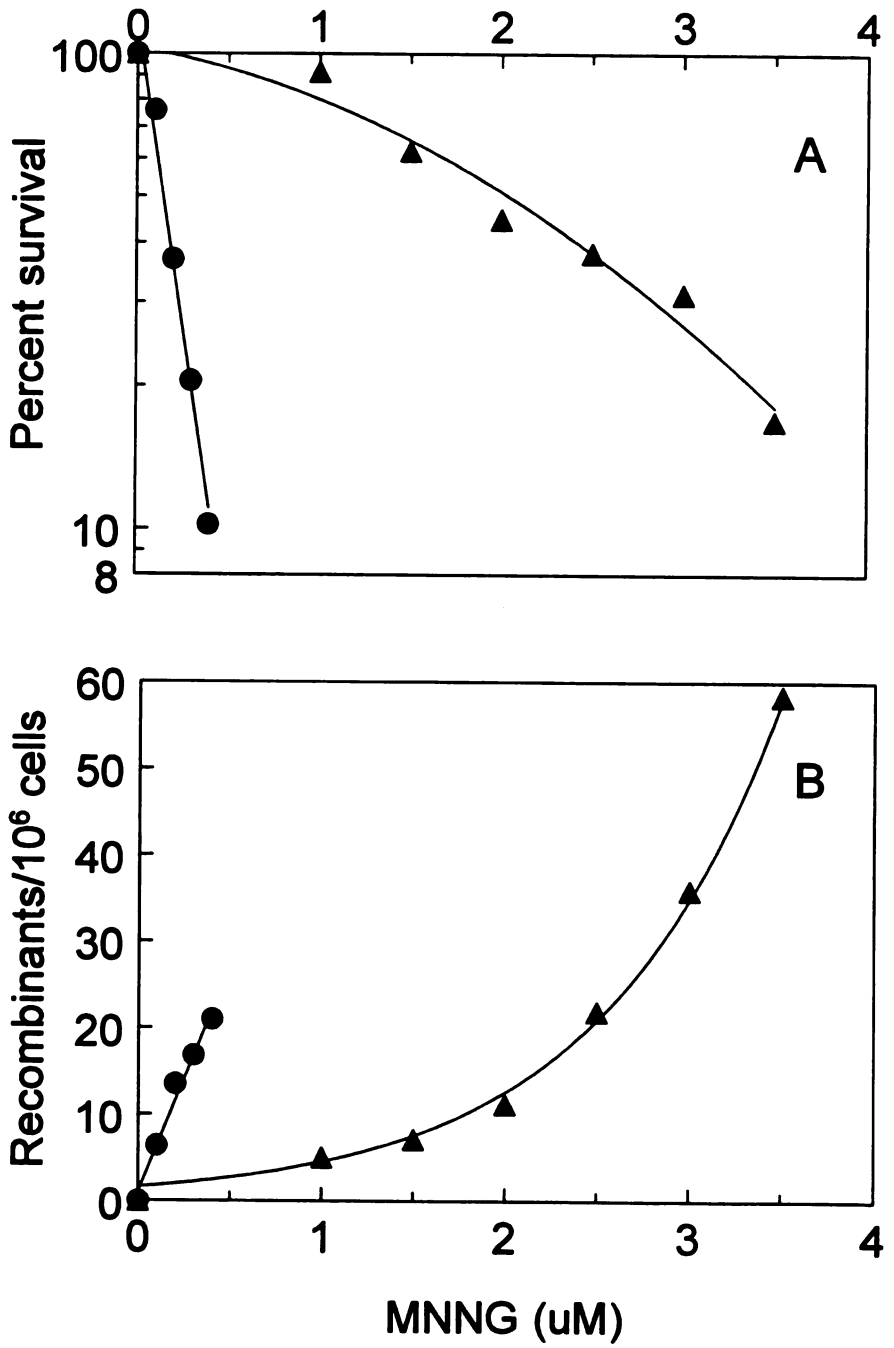
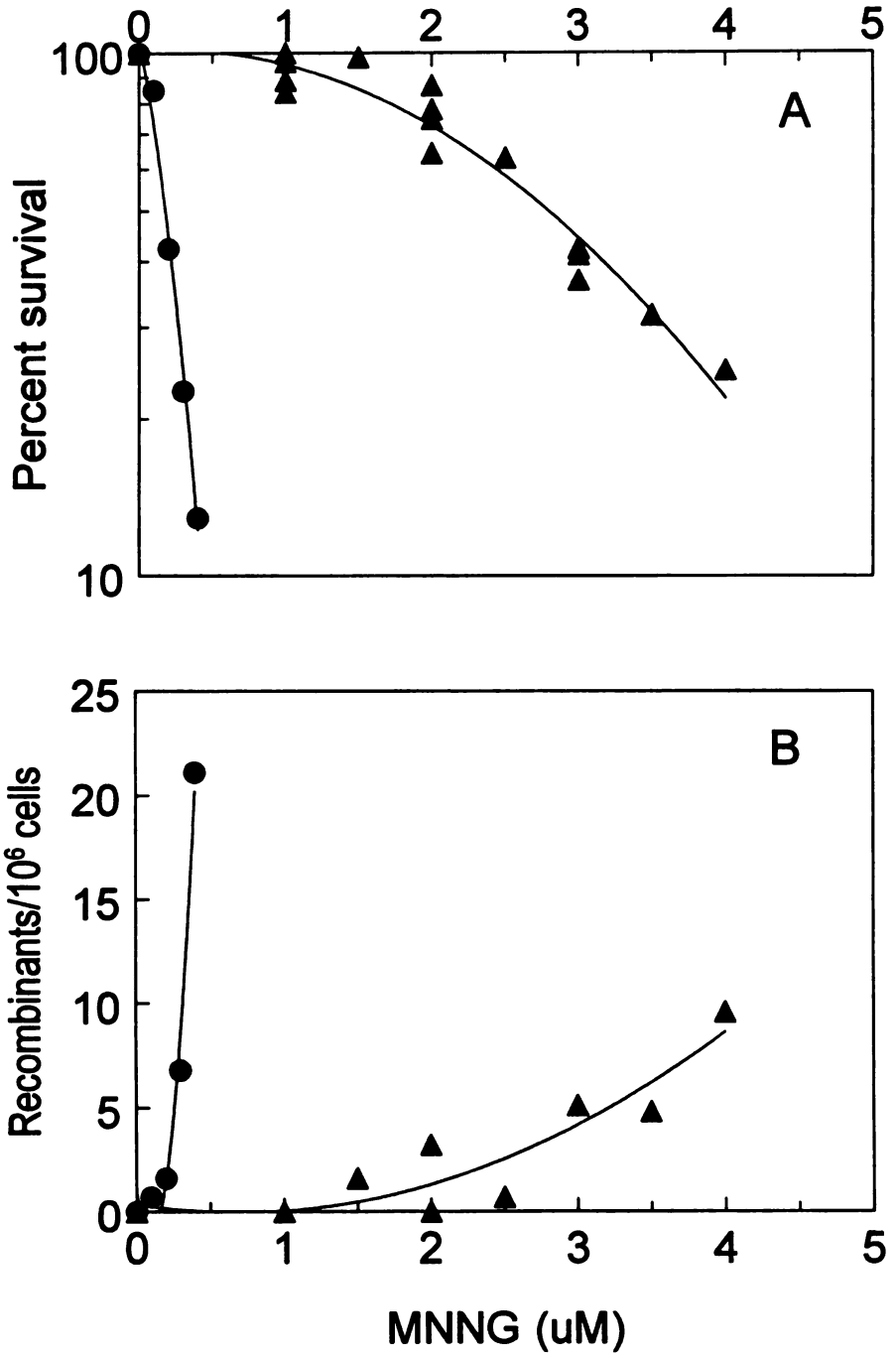

Percent survival
100
10
8
0
1
2
3
4
A
Recombinants/10^6 cells
60
50
40
30
20
10
0
B
MNNG (uM)

Figure 6. Cell killing (A) and induction of homologous recombination (B) as a function of the concentration of MNNG in cell strain MSU1.2-D10.4 with and without O^6-benzylguanine (25 μM). Circles, with O^6-benzylguanine; triangles, without O^6-benzylguanine. The background recombination frequencies per 10^6 cells in the two experiments without O^6-benzylguanine pretreatment were 0, and in the experiment with O^6-benzylguanine was 2.1. These values have been subtracted to give the induced frequencies. The concentration of hygromycin-B used was 50 U/ml. This concentration was the highest that could be used without a cytotoxic effect on recombinants.


0
1
2
3
4
5
100
10
Percent survival
A
25
20
15
10
5
0
Recombinants/10^6 cells
B
MNNG (uM)

genes results in a single wild-type copy of the *hyg* gene. PCR products amplified from the wild-type *hyg* gene using primer 1 and primer 2 are resistant to *Hin*d III digestion. A gene conversion event preserves the *hyg* gene duplication, resulting in one wild-type gene and one mutant gene. The copy of the *hyg* gene that is converted to wild-type can be determined from the *Hin*d III digestion pattern of the PCR products. If the *Sac* II mutant *hyg* gene is converted to wild-type, an 889-bp and a 278-bp fragment will be missing from the *Hin*d III digestion, whereas the absence of a 695-bp and a 472-bp fragment indicates that the *Pvu* I mutant *hyg* gene has been converted to wild-type. In addition, a double reciprocal exchange can generate a wild-type copy and a copy of the *hyg* gene containing two *Hin*d III sites. PCR products amplified from such a double mutant gene yield a 278-bp, a 417-bp and a 472-bp fragment after *Hin*d III digestion, along with the full length copy from the wild-type gene. Examples of some recombinants generated from cell strains MSU-1.2-D10.4 and MSU-1.2-E7.2 are shown in Figure 7. Some of the recombinants were also analyzed by Southern blotting using *Hin*d III digestion. The results were in agreement with those from the corresponding PCR experiments (data not shown). The types of recombination products induced by MNNG in cell strains MSU-1.2-E7.2 and MSU-1.2-D10.4 were compared with their respective spontaneous recombination products. The results (Table 2) showed that all but one of the events involved gene conversion. Within each cell strain, there was no significant difference between the products of spontaneous and MNNG-induced productive recombination events. In both spontaneous and MNNG-induced gene conversions, cell strain MSU-1.2-E7.2 showed a greater bias towards conversion

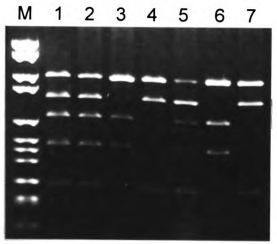


Figure 7. *Hind* III digestion patterns of PCR products amplified from recombinants. Lane M, DNA molecular marker VI from Boehringer Mannheim (pBR328-*Bgl* I DNA + pBR328-*Hinf* I DNA); Lane 1, plasmid pJH-1; Lane 2, MSU-1.2-D10.4 cells; Lane 3, a recombinant of *Sac* II gene conversion generated from MSU-1.2-D10.4 cells; Lane 4, a recombinant of *Pvu* I gene conversion generated from MSU-1.2-D10.4 cells; Lane 5. MSU-1.2-E7.2 cells; Lane 6, a recombinant of *Sac* II gene conversion generated from MSU-1.2-E7.2 cells; Lane 7, a recombinant of *Pvu* I gene conversion generated from MSU-1.2-E7.2 cells.

Table 2. Molecular characterization of independent spontaneous and MNNG-induced hyg^r recombinants from the two cell strains derived from MSU-1.2 cells.

Cell strain	No. of recombinants tested	Gene conversion		Reciprocal exchange	
		Sac II mutant	*Pvu* I mutant	Single	Double
MSU-1.2-D10.4					
Spontaneous [a]	6	2	4	0	0
MNNG-induced [b]					
No O^6-benzylguanine	18	6	12	0	0
With O^6-benzylguanine	6	3	3	0	0
Total	30	11 (36.7%)	19 (63.3%)	0	0
MSU-1.2-E7.2					
Spontaneous [a]	30	1	29	0	0
MNNG-induced [b]					
No O^6-benzylguanine	75	4	70	0	1
With O^6-benzylguanine	26	6	20	0	0
Total	131	11 (8.4%)	119 (90.8%)	0	1 (0.8%)

a: Each spontaneous recombinant was isolated independently from untreated control groups. b: Each MNNG-induced recombinant, with and without O^6-benzylguanine pretreatment, was isolated from independent populations exposed to different MNNG doses. Values in parentheses are percentages of total.

of the *Pvu* I mutant *hyg* gene than did cell strain MSU-1.2-D10.4.

Discussion

We compared MNNG-induced intrachromosomal homologous recombination in two sets of three human fibroblast cell strains that differ in their level of AGT activity. With each set, the cells with lower AGT activity were more sensitive to the induction of homologous recombination by MNNG. Since AGT removes methyl groups from the O^6 position of guanine, the inverse correlation between the frequency of MNNG-induced homologous recombination and the level of AGT activity implies that O^6-methylguanine is the DNA lesion that is principally responsible for MNNG-induced homologous recombination. To further investigate the role of O^6-methylguanine in homologous recombination, we compared MNNG-induced homologous recombination in two cell strains, MSU-1.2-E7.2 and MSU-1.2-D10.4, with and without the treatment with O^6-benzylguanine to deplete the cells of AGT activity. Pretreatment with O^6-benzylguanine greatly increased the sensitivity of the cells to the induction of homologous recombination by MNNG and also by *N*-methyl-*N*-nitrosourea, strongly supporting the hypothesis that O^6-methylguanine is the principal lesion responsible for the induction of homologous recombination by these methylating agents.

Previous studies (10, 11, 25) indicate that unrepaired UV-induced DNA damage, rather than the nucleotide excision repair process *per se*, stimulates homologous recombination induced by UV radiation. By using O^6-benzylguanine to deplete the AGT activity in human cells, we showed that AGT-mediated repair does not

stimulate homologous recombination, but rather, the unrepaired O^6-methylguanine lesion stimulates homologous recombination. One possible explanation for how O^6-methylguanine could do so is that during replication, it miscodes as adenine, generating a base mismatch. If mismatch repair were to recognize the O^6-methylguanine:T mismatch and cut the DNA in the vicinity of the error, this could generate single-stranded DNA that could then initiate recombination. Studies designed to test this hypothesis are underway.

Using the two MSU-1.2-derived cell strains, we found a strong bias towards gene conversion, rather than single reciprocal exchange events. This was also found in other studies with human (10, 11, 17) or mouse cell strains (26). The simplest explanation for the observed bias is an inability to recover the single reciprocal exchange products. In the MSU-1.2-derived cell strains, in a single reciprocal exchange, two mutant *hyg* genes recombine to generate one wild-type copy, and at the same time, the intervening *gpt* gene is eliminated. It is possible that the lack of the *gpt* gene enhancer in the recombinants generated by a single reciprocal exchange makes the *hyg* gene expression inadequate. However, other mechanisms could also explain the predominance of gene conversion that we observed in these human cell strains. It will be interesting to determine if gene conversion is the predominant recombination event between two endogenous alleles in human cells, and if so, to determine the mechanisms responsible.

We also observed a strong difference between cell strains MSU-1.2-D10.4 and MSU-1.2-E7.2 in the degree of gene conversion polarity, *i.e.*, the frequency with which one mutant *hyg* gene is converted to wild-type was compared to the other

(see Table 2). The reason for this bias is not obvious. There is always the possibility that gene conversions involving the *Sac* II mutant *hyg* gene in MSU-1.2-E7.2 cells are being selected against, either because the promoter for that gene is not intact or because the concentration of the selection agent is too high. However, neither possible explanation is likely because we were able to isolate such recombinants, and when tested, they were completely resistant to 100 U/ml of hygromycin-B, just like the MSU-1.2-E7.2 cells in which the *Pvu* I mutant *hyg* gene had been converted. Furthermore, there was no difference between the MSU-1.2-D10.4 cells and MSU-1.2-E7.2 cells in the size of the hyg^r colonies containing a wild-type copy of the *Sac* II mutant *hyg* gene or of the *Pvu* I mutant *hyg* gene, suggesting an equal degree of resistance to the selective agent. The reasons behind the polarity difference observed in these two cell strains remain to be determined.

Acknowledgments

We thank Dr. R. M. Liskay (Oregon Health Sciences University) for the plasmids, Dr A. E. Pegg (Pennsylvania State University) for O^6-benzylguanine and for measuring the level of AGT activity in the cell strains. This research was supported by DHHS Grant CA48066 from the National Cancer Institute.

Abbreviations

AGT: O^6-alkylguanine-DNA alkyltransferase; *Htk*: the Herpes simplex virus I thymidine kinase gene; *hyg*: the hygromycin phosphotransferase gene; MNNG: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

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CHAPTER 3

Mismatch repair is required for O^6-methylguanine-induced intrachromosomal homologous recombination in human fibroblasts

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Running Title: Futile mismatch repair induces homologous recombination

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Abstract

Zhang et al. (1996) showed previously that O^6-methylguanine is the lesion principally responsible for homologous recombination induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in human fibroblasts. To determine if mismatch repair of base mismatches, formed by misreplication of O^6-methylguanine, was causally involved in initiating this recombination, we generated several mismatch repair deficient human fibroblast cell strains from a mismatch repair proficient cell strain and compared them with the parental strain for frequency of homologous recombination induced by MNNG. The parental strain contains a substrate for detecting intrachromosomal homologous recombination integrated into its genome. Compared to the repair proficient parental cell strain, the derivative cell strains were highly resistant to MNNG cell killing and very hypermutable by MNNG. Mismatch repair activity assays showed that cell-free extracts from these cell strains were completely devoid of ability to repair a substrate containing a G:T mismatch. Comparative studies showed that frequency of homologous recombination induced by MNNG in these repair deficient cell strains was significantly lower than that seen with the parental cells. The lack of MNNG-induced homologous recombination in these strains is not the result of a general defect in the recombination process because the frequency of recombination induced in these cell strains by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[α]pyrene was equal to that seen in the parental cells. These results indicate that functional mismatch repair is required for MNNG-induced

homologous recombination, and suggest that futile repair of mismatches containing O^6-methylguanine induces homologous recombination.

Introduction

Certain rare genetic recombination events play an important role in carcinogenesis (Lasko et al., 1991; Sengstag, 1994). Mitotic recombination between homologous genes is one of the mechanisms responsible for the cells becoming homozygous for a particular recessive allele (Cavenee et al., 1983; James et al., 1989; Fearon and Vogelstein, 1990; Mulligan et al., 1990). Spontaneous or carcinogen-induced homologous recombination may also be responsible for many other genetic changes that have been implicated in the malignant transformation, such as chromosomal rearrangement, translocation, deletion, gene amplification, etc. (Sengstag, 1994). Therefore, it is important to understand the mechanisms of homologous recombination, especially carcinogen-induced recombination in human cells.

Methylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) can induce intrachromosomal homologous recombination in mammalian cells (Wang et al., 1988; Zhang et al., 1996). Zhang et al. (1996) showed that O^6-methylguanine (O^6-MeG) is the lesion principally responsible for this induction. O^6-MeG is repaired by O^6-alkylguanine-DNA alkyltransferase (AGT) which directly transfers the methyl group from O^6-MeG to an interior cysteine residue in its active site (Pegg and Byers, 1992). O^6-MeG can pair with T to form a mispair

(Swann, 1990). O^6-MeG:T and even O^6-MeG:C are recognized as mismatches by the mismatch repair system (Duckett et al., 1996).

Mismatch repair corrects base mismatches and certain insertion/deletion mismatches in human cells (Kolodner, 1996; Modrich and Lahue, 1996). Cells defective in mismatch repair exhibit a high mutation rate. This is evident in hereditary nonpolyposis colorectal cancer (HNPCC) tumors which are defective in one or other of the mismatch repair genes, e.g., *hMLH1, hMSH2, hPMS1* or *hPMS2* (Fishel et al., 1993; Leach et al., 1993; Bronner et al., 1994; Nicolaides et al., 1994; Papadopoulos et al., 1994). Mismatch repair has also been reported to be defective in approximately 10-15% of sporadic colon cancer cells (Ionov et al., 1993; Thibodeau et al., 1993) as well as in cells from other types of cancer. The mismatch repair deficient tumor-derived cells have been shown to exhibit a mutator phenotype, microsatellite instability, and tolerance to methylating agents (Bhattacharyya et al., 1994; Karran and Bignami, 1994; Boyer et al., 1995; Branch et al., 1995; Eshleman et al., 1995; Glaab and Tindall, 1997). Restoration of the mismatch repair activity by chromosome transfer or single gene transfection has been shown to correct mutator phenotype and microsatellite instability, and to reduce MNNG tolerance in cells from colon cancer and endometrial cancer. This suggests that defects in mismatch repair cause the mutator phenotype and the tolerance to the cytotoxic effect of methylating agents in those cancer cells (Koi et al., 1994; Umar et al., 1997; Risinger et al., 1998; Tindall et al., 1998).

It has been hypothesized that the mismatch repair system recognizes the O^6-MeG:T and O^6-MeG:C mismatches and removes a stretch of the DNA strand that contains the T or C. The resulting gap is filled in by DNA synthesis, but this again results in mismatches. The cytotoxicity of methylating agents in mammalian cells has been attributed to this futile turnover of newly synthesized DNA (Karran and Marinus, 1982; Goldmacher et al., 1986). Tolerance of mismatch repair deficient cells to methylating agents is considered to result from the lack of such abortive repair events (Karran and Bignami, 1994). Selection of mammalian cells in culture for resistance to the cytotoxic effect of MNNG or *N*-methyl-*N*-nitrosourea has led to identification of several methylation-tolerant cell lines. Each of these tolerant cell lines proved to be defective in mismatch repair and to exhibit abnormally high mutation frequencies, either spontaneous or induced by methylating agents (Goldmacher et al, 1986; Branch et al., 1993; Kat et al., 1993; Aquilina et al., 1995; Papadopoulos et al., 1995; Hampson et al., 1997; Ciotta et al., 1998).

Zhang et al. (1996) proposed that futile mismatch repair of mismatches containing O^6-MeG results in the persistent single-stranded DNA regions that can initiate recombination. To test this hypothesis, we mutagenized the parental cell strain, MSU-1.2-10A, which contains a recombination substrate for detecting intrachromosomal homologous recombination between two mutant genes coding for hygromycin resistance (Zhang et al., 1996) integrated into its genome, and selected the survivors for resistance to MNNG. Characterization of four independent cell strains highly resistant to the cytotoxic effect of MNNG showed

that they were defective in mismatch repair and hypermutable by MNNG. The induction of homologous recombination induced by MNNG was virtually eliminated in the first three of the four cell strains and totally absent in the fourth. This latter strain was shown to have lost integrity of the recombination substrate. The lack of MNNG-induced recombination in the other three strains is not the result of a general defect in the recombination process because all three strains exhibited normal frequencies of recombination, both spontaneous and induced by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[α]pyrene (BPDE).

Materials and Methods

Cell strains and culture conditions

A human fibroblast cell strain, MSU-1.2-E7.2 (Zhang et al., 1996) which contains two mutated hygromycin phosphotransferase (*hyg*) gene, was transfected with a plasmid ptTA carrying a gene coding for a tetracycline-responsive fusion transcriptional activator and a *hisD* gene (H.Z., V.M.M., and J.J.M., in preparation). A cell strain, designated MSU-1.2-10A, was isolated to express the tetracycline-responsive transcriptional activator. MSU-1.2-10A cells were repeatedly treated with high doses of MNNG and subsequently selected for MNNG-resistant clones. Four cell strains were generated in this way to have defects in mismatch repair. Cells were routinely cultured in McM medium (Ryan et al., 1987) containing 10% (v/v) supplemented calf serum (Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin and 1 μg/ml hydrocortisone (culture medium).

Treatment with MNNG or BPDE

Treatment with MNNG (Pfaltz and Bauer, Flushing, NY) or BPDE (Chemsyn Science Laboratories, Lenexa, KS) was carried out following the same procedure as described (Zhang et al., 1996). In brief, cells were plated at 10^4 per cm^2. After 14-16 hours, cells were treated with carcinogens for 1 hour.

Assay for cytotoxicity

The cytotoxic effect of the treatment was determined from the relative colony-forming ability of the treated cells compared to the untreated control as described (Zhang et al., 1996). Briefly, after carcinogen treatment, cells were trypsinized and plated at cloning densities.

Depletion of AGT activity

O^6-Benzylguanine (25 μM) was used to deplete the AGT activity as described (Zhang et al., 1996).

Assay of mutation of the hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) gene

After treatment with carcinogens, one set of cells from each dose was maintained in exponential growth to allow expression of resistance to 6-thioguanine. After 8 days, $1x10^6$ cells from the control and each treated population were selected for resistance to 6-thioguanine as described (Maher and McCormick, 1996). The frequency was corrected for cloning efficiency at time of selection.

Assay for homologous recombination

Sufficient numbers of target cells (at least $2x10^6$) were used for each dose. Cells were selected for hygromycin resistance (hyg^r) in culture medium containing 100

U/ml hygromycin-B (Calbiochem) as described (Zhang et al., 1996). The frequency of recombination was determined from the number of hyg^r colonies divided by the number of viable cells as determined from the accompanying cytotoxicity assay. The frequency of induced recombination was determined by subtracting the background frequency observed in the untreated control population from the total frequency (Zhang et al., 1996).

Assay for the types of recombination

The *Hyg* gene was amplified from hyg^r clones using PCR (Zhang et al., 1996). The amplified PCR products were digested by *Hin*d III, and the types of the recombination were determined according to the *Hin*d III digestion pattern.

***In vitro* mismatch repair assay**

The cytoplasmic cell-free extracts were prepared as described (Li and Kelly, 1985). The protein concentrations ranged from 5 to 10 μg/μl. The efficiency of repairing mismatches by the cell-free extracts was tested as described by Marra et al. (1998). In brief, M13mp2 heteroduplexes containing a G:T mispair were incubated with 50 μg of cell-free extract (Thomas et al., 1991). The DNA was then purified, electroporated into a *mutS* strain of *Escherichia coli*, and plated to score plaques as described (Thomas et al., 1991). If no repair occurred, a high percentage of mixed plaques containing both blue and colorless progeny was observed. Reduction in the percentage of mixed plaques and a concomitant increase in single-color plaques were indicative of repair. Repair efficiency (%) is presented as 1-(% mixed plaques in extract-treated sample) / (% mixed plaques in extract-untreated sample).

Results

Isolation of MNNG-tolerant cell strains

Cell strain MSU-1.2-E7.2 contains a recombination substrate which has been previously shown to generate gene conversion products (Zhang et al., 1996). It was transfected with plasmid ptTA which carries the tetracycline-responsive transcriptional activator. A cell strain, expressing this transfected transcriptional activator, was identified and designated MSU-1.2-10A. To isolate cells resistant to the cytotoxic effect of methylating agents, $6x10^6$ MSU-1.2-10A cells were plated into four 150-mm diameter dishes, and exposed to O^6-benzylguanine (25 μM) for 2h to inactivate AGT, and then treated for 1h with1 μM MNNG. After four days, the surviving cells were exposed to 1.5 μM MNNG under the same condition. This regime was repeated two more times using 4 μM MNNG. The majority of the cells detached from the dishes, and the surviving cells were allowed to form colonies. A total of 54 clones were isolated, and 43 clones were expanded into cell strains.

We treated these 43 cell strains with 6 μM of MNNG in combination with O^6-benzylguanine (25 μM) pretreatment, and assayed them for the frequency of 6-thioguanine resistant cells. Four cell strains that were very hypermutable by MNNG and were derived from independent MNNG treatment were selected for further studies. These cell strains were subsequently subcloned once and designated MSU-1.2-A12.2, MSU-1.2-B7.7, MSU-1.2-C14.6 and MSU-1.2-D1.1. Subsequent testing showed that MSU-1.2-B7.7 cells had lost the integrity of their recombination substrate (the mutant *hyg* genes), and therefore, hygromycin

resistant recombinants could not be generated by any means in this strain (data not shown). Although this strain exhibits a phenotype similar to the other three strains, i.e., it is highly resistant to MNNG cell killing, very hypermutable by MNNG, and unable to repair G:T mismatches (data not shown), we have not included it in this report.

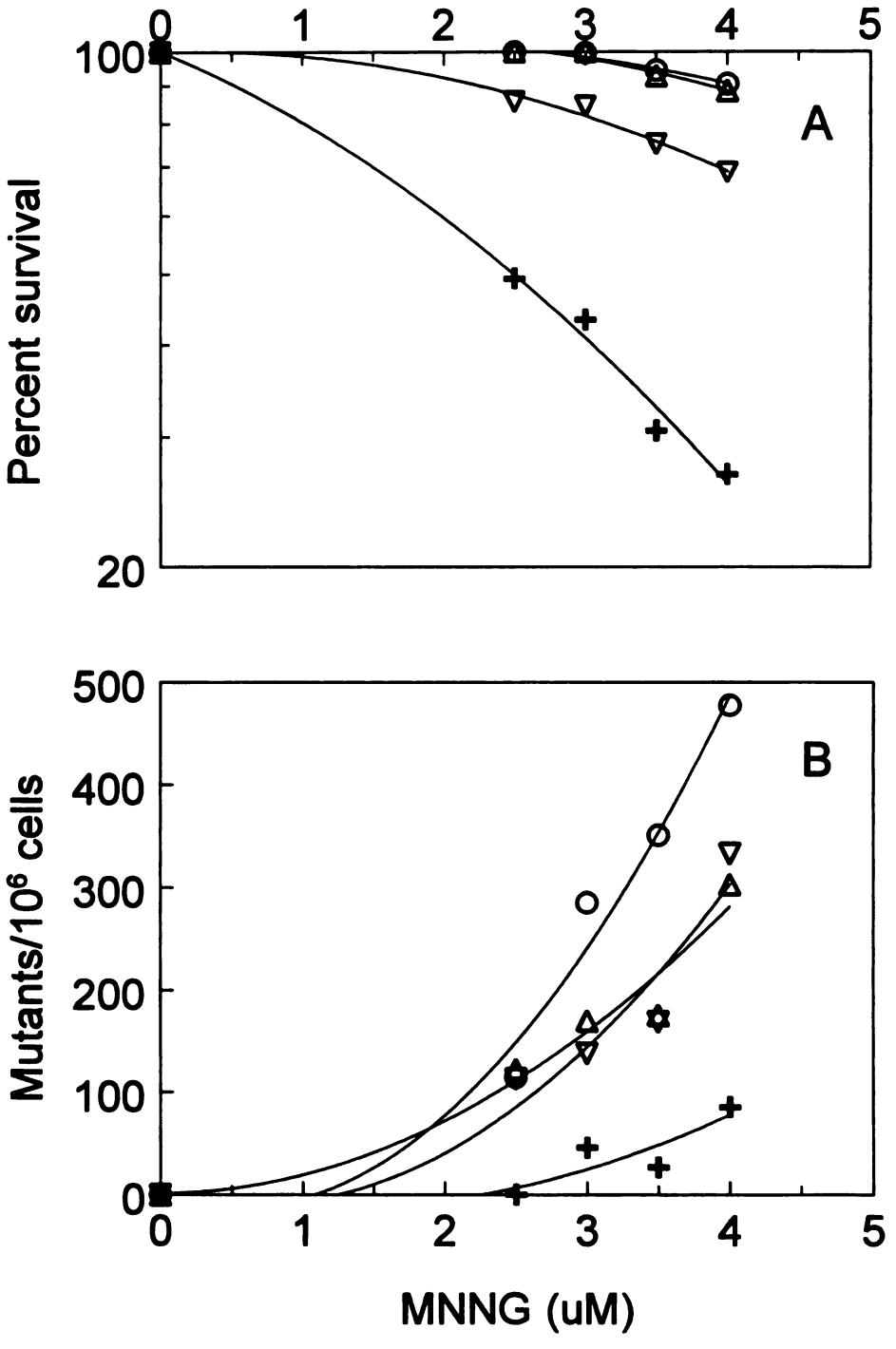
Characterization of the mismatch repair deficient cell strains

As shown in Figure 1A, three cell strains, MSU-1.2-A12.2, MSU-1.2-C14.6 and MSU-1.2-D1.1, were highly resistant to MNNG cell killing, compared to their parental cell strain MSU-1.2-10A. Since MSU-1.2-E7.2 cells, from which all these cell strains were derived, have a high level of AGT (Zhang et al., 1996), we used O^6-bezylguanine to deplete the cells of AGT activity and analyzed the cytotoxic effect of MNNG. All three cell strains were very resistant to the cytotoxic effect of MNNG (Fig. 2). The survival of these cells was virtually the same as when O^6-benzylguanine was not used, indicating that the observed tolerance of these three cell strain to MNNG was not the result of their removing methyl groups from O^6-MeG by AGT.

As shown in Figure 1B, these three tolerant cell strains were very hypermutable by MNNG as compared to their parental cell strain. These phenotypes are consistent with those reported for mismatch repair deficient cells.

We analyzed the mismatch repair capacity of these cell strains using an *in vitro* assay (Thomas et al., 1991). Cell-free extracts were prepared and tested for their ability to correct a heteroduplex containing a G:T mismatch. As shown in Figure 3, the parental MSU-1.2-10A cells were proficient in repairing the G:T

Figure 1. Cell killing (A) and mutation frequency at *HPRT* locus (B) as a function of the concentration of MNNG. +, MSU-1.2-10A cells; O, MSU-1.2-A12.2 cells; ∇, MSU-1.2-C14.6; Δ, MSU-1.2-D1.1 cells. The background mutation frequencies have been subtracted to give the induced mutation frequencies.


0
1
2
3
4
5
100
20
Percent survival
A
500
400
300
200
100
0
Mutants/10^6 cells
B
MNNG (uM)

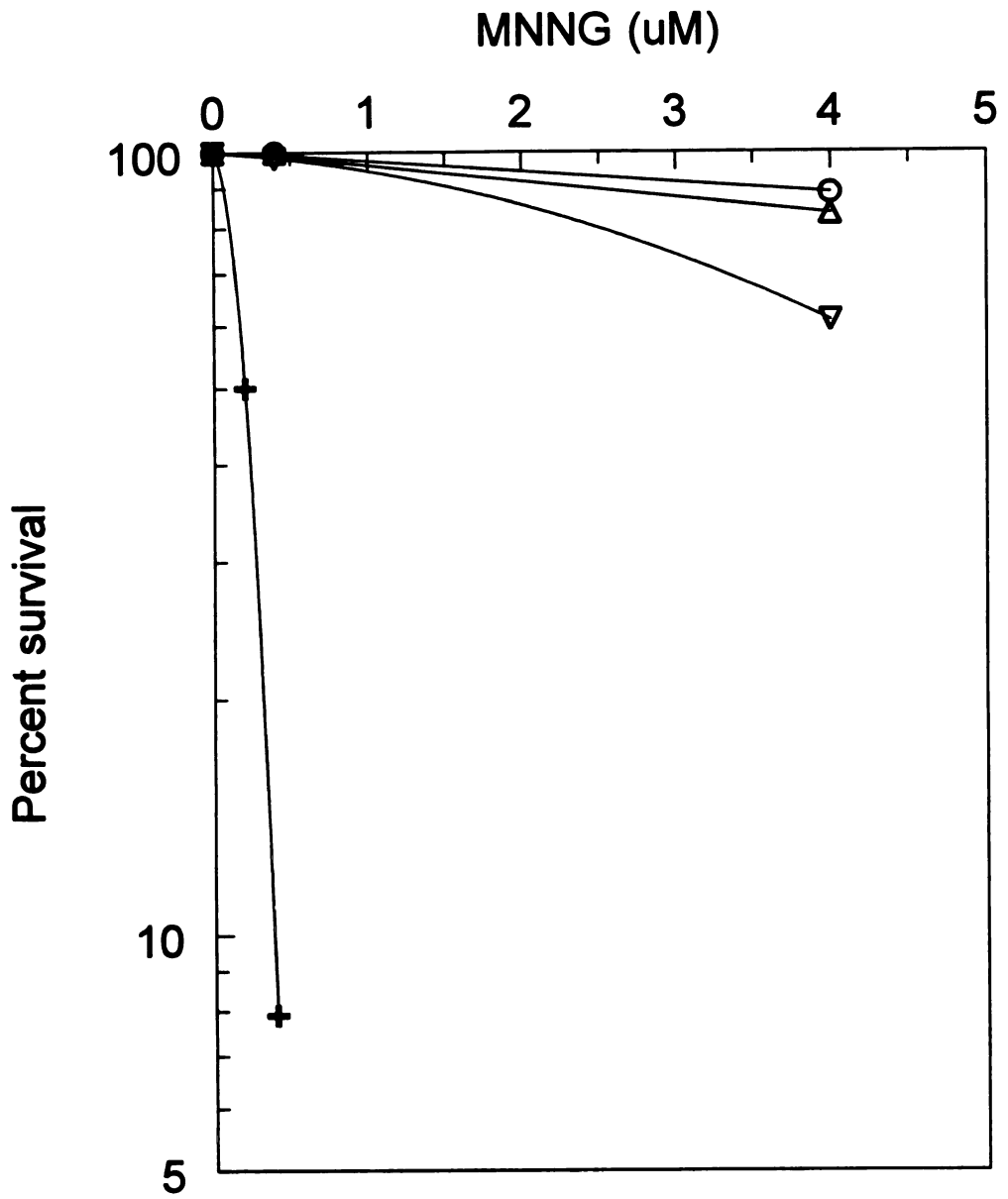


Figure 2. Cytotoxicity of MNNG in cells pretreated with O^6-benzylguanine (25 μM) to deplete AGT activity. +, MSU-1.2-10A cells; O, MSU-1.2-A12.2 cells; ∇, MSU-1.2-C14.6; Δ, MSU-1.2-D1.1 cells.

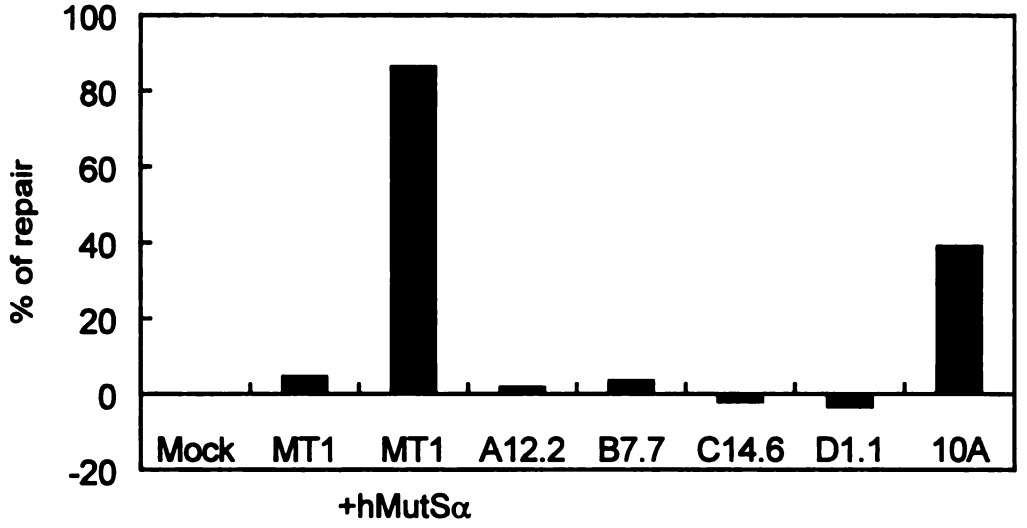


Figure 3. *In vitro* mismatch repair activity assay. The repair reaction was carried out as described in Materials and Methods using a substrate containing a G:T mismatch. Cell-free extract from MT1 cells, which have mutations in both alleles of their *hMSH6* gene, was used as a negative control. Cell-free extract from MT1 cells supplemented with hMutSα was used as a positive control.

mismatch, whereas extracts from MSU-1.2-A12.2, MSU-1.2-C14.6 and MSU-1.2-D1.1 were completely devoid of ability to repair the G:T mismatch, as was MSU-1.2-B7.7. Extract from MT1, a cell strain known to be defective in both alleles of the *hMSH6* gene (Kat et al., 1993; Pappadopoulos et al., 1995), served as a negative control. Extract from MT1 complemented by purified recombinant hMutSα complex (0.1 μg), which consists of hMSH2 and hMSH6 proteins, was used as the positive control. These data clearly indicated that cell strains MSU-1.2-A12.2, MSU-1.2-B7.7, MSU-1.2-C14.6 and MSU-1.2-D1.1 are deficient in mismatch repair. We also determined the *p53* gene statues in these cell strains, and they were all wild type (data not shown).

MNNG-induced homologous recombination

To determine the frequency of recombination induced by MNNG, the three cell strains and the parental strain were treated with MNNG as described in Materials and Methods. As shown in Figure 4, MNNG induced homologous recombination in a dose dependent manner in the mismatch repair proficient parental MSU-1.2-10A cells. The induction of homologous recombination by MNNG in the three mismatch repair deficient cell strains was greatly reduced, suggesting that the functional mismatch repair is required for MNNG-induced recombination.

Using our intrachromosomal homologous recombination system, we have demonstrated previously that the majority of recombination products are the result of gene conversion between the two mutated copies of the *hyg* gene (Zhang et al., 1996). As shown in Table 1, all the recombinants, either

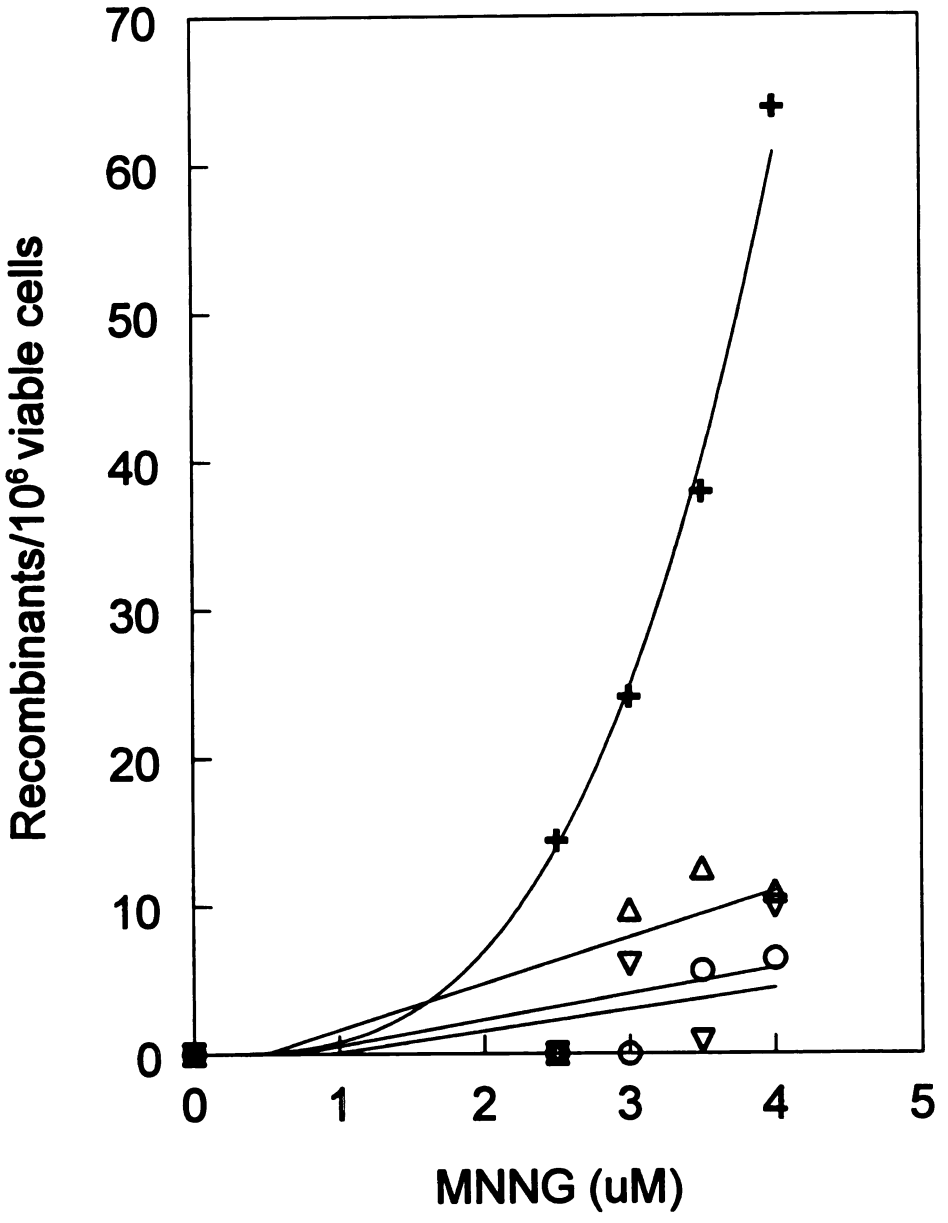


Figure 4. Cell killing (A) and the induction of homologous recombination (B) as a function of concentration of MNNG. +, MSU-1.2-10A cells; O, MSU-1.2-A12.2 cells; ∇, MSU-1.2-C14.6; Δ, MSU-1.2-D1.1 cells. The background recombination frequencies have been subtracted to give the induced mutation frequencies.

Table 1. Characterization of types of recombination products.

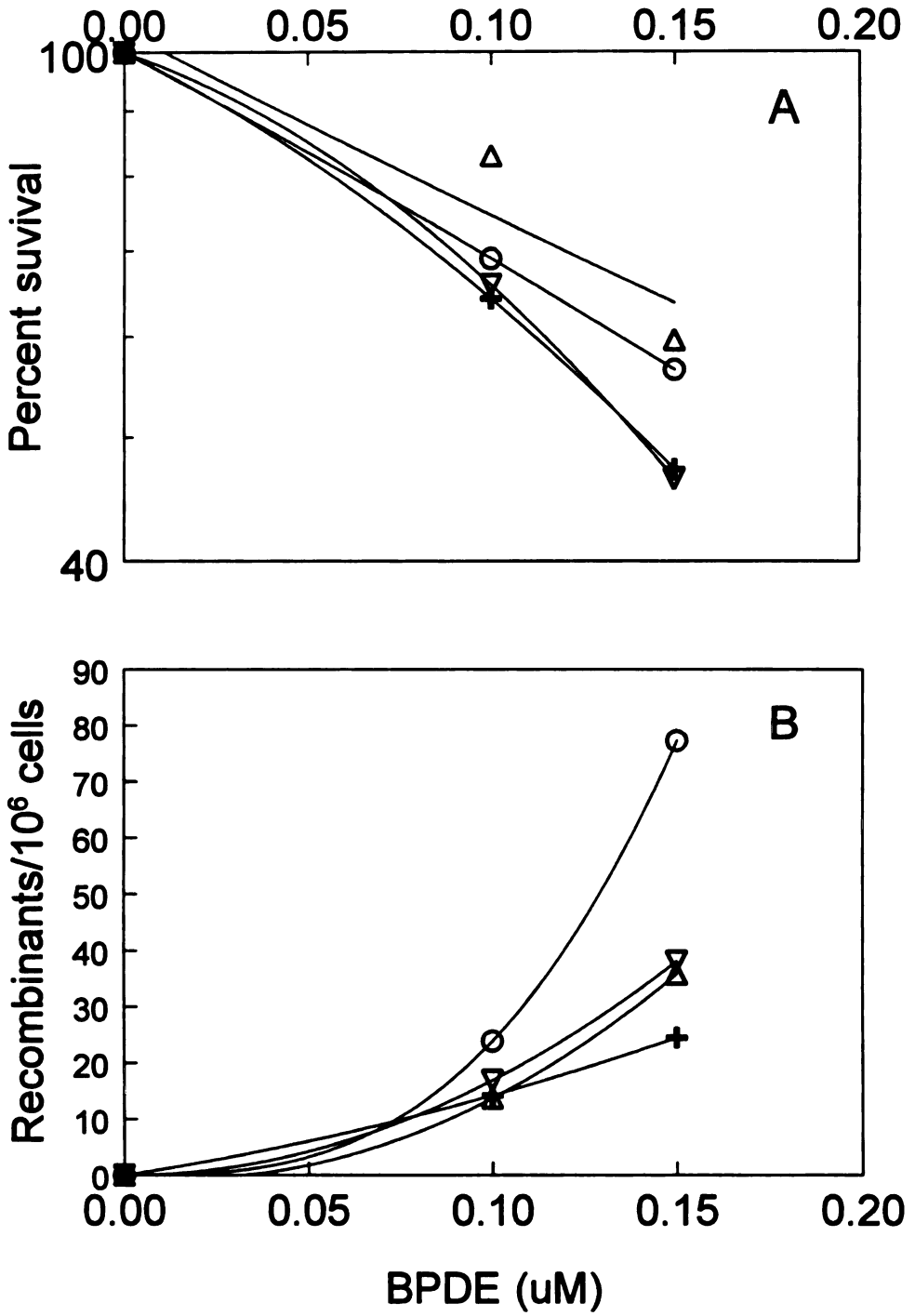
Cell strain	spontaneous		MNNG-induced		BPDE-induced	
	gene conversion	reciprocal exchange	gene conversion	reciprocal exchange	gene conversion	reciprocal exchange
MSU-1.2-10A	10	0	20	0	23	0
MSU-1.2-A12.2	2	0	6	0	8	0
MSU-1.2-C14.6	1	0	10	0	11	0
MSU-1.2-D1.1	1	0	12	0	19	0

spontaneously-derived or MNNG-induced, in the three mismatch repair deficient cell strains or the mismatch repair proficient parental strain, were the product of gene conversion. It has been suggested that gene conversion results from mismatch repair of the heteroduplex generated during the recombination process (Holliday, 1964; Meselson and Radding, 1975; Detloff et al., 1992; Alani et al., 1994). With our recombination system, the heteroduplex is formed by DNA containing and lacking the 10-base *Hin*d III linker, producing an insertion/deletion mismatch. It is possible that the highly reduced frequency of recombination observed in Figure 4 with the mismatch repair deficient cells is the result of their inability to repair these insertion/deletion mismatches, rather than as we hypothesize, their lack of ability to recognize the MNNG-induced mismatches and initiate recombination.

BPDE-induced homologous recombination

To address this possibility, we chose to induce homologous recombination in these cell strains using BPDE. BPDE forms multi-ringed adducts on guanine in DNA, and these bulky adducts interfere with DNA replication (Chary and Lloyd, 1995). Interference with replication folk progression has been suggested to result in temporal single-stranded DNA regions that can initiate the homologous recombination process (Bhattacharyya et al., 1990; Tsujimura et al., 1990). As shown in Figure 5A, the three mismatch repair deficient cell strains were not more sensitive to the cytotoxic effect of BPDE than the mismatch repair proficient parental strain. What is more important, the frequency of homologous recombination induced by BPDE in these three mismatch repair deficient cell

Figure 5. Cell killing (A) and the induction of homologous recombination (B) as a function of concentration of BPDE. +, MSU-1.2-10A cells; O, MSU-1.2-A12.2 cells; ∇, MSU-1.2-C14.6; Δ, MSU-1.2-D1.1 cells. The background recombination frequencies have been subtracted to give the induced recombination frequencies.


0.00
0.05
0.10
0.15
0.20
100
40
A
Percent suvival
90
80
70
60
50
40
30
20
10
0
B
Recombinants/10^6 cells
BPDE (uM)

strains was not reduced. The frequency was somewhat higher than or equal to that seen in the repair proficient parental strain (Fig. 5B). The BPDE-induced recombination products from these three mismatch deficient cell strains, as well as the mismatch repair proficient strain, all resulted from gene conversion (Table 1). The defect of mismatch repair in these three cell strains, did not interfere with the BPDE-induced or spontaneous homologous recombination. These data support the hypothesis that the lack of MNNG-induced homologous recombination in these cell strains is not the result of a general defect in the recombination process, e.g., interference with repair of the insertion/deletion mismatches generated in Holliday recombination intermediates, but rather results from futile mismatch repair of the O^6-MeG:T and O^6-MeG:C mismatches.

Discussion

Aberrant homologous recombination plays an important role in carcinogenesis (Sengstag, 1994). Homologous recombination can be induced by many carcinogens in mammalian cells. X-ray-induced DNA strand breaks provide a likely explanation for the induction of homologous recombination by X-rays (Resnick, 1976). UV radiation and chemical carcinogens, such as polycyclic aromatic carcinogens and cross-linking agents, generate bulky DNA adducts, which can interfere with DNA replication, causing a delay in DNA replication fork progression (Reardon et al., 1990; Basu et al., 1993; Chary and Lloyd, 1995). Using a large series of nucleotide excision repair deficient cell lines, Maher and colleagues found that unexcised DNA damage caused by such agents, rather

than the excision repair process itself, is responsible for the induction of homologous recombination by these carcinogens. They proposed that unrepaired DNA lesions block DNA replication, leading to the generation of discontinuities, and the resulting single-stranded DNA regions initiate homologous recombination (Bhattacharyya et al., 1990; Tsujimura et al., 1990).

Alkylating agents such as MNNG can also induce homologous recombination in mammalian cells (Wang et al., 1988), and Zhang et al. (1996) showed that O^6-MeG is the lesion that is principally responsible for this induction. It is also the lesion responsible for the cytotoxic and mutagenic effect of alkylating agents (Domoradzki et al., 1984; 1985; Lukash et al., 1991). Although human polymerase β has been shown to replicate synthetic template containing O^6-MeG with less efficiency (Reha-Krantz et al., 1996; Singh et al., 1996), the existence of alkylating agent-tolerant cells indicates that chromosomes containing large quantities of O^6-MeG are nevertheless completely replicated. It has been proposed that mismatch repair proteins recognize base pairs containing O^6-MeG in DNA as mismatches and produce long excision tracks in the strand that lacks the O^6-MeG. If the O^6-MeG remains, replication of the single-stranded regions will once again result in generation of O^6-MeG:T mismatches. The resulting O^6-MeG:T mispair will again be recognized by mismatch repair proteins, and the process of excision followed by replication will be repeated. These futile cycles of excision and synthesis are predicted to produce persistent discontinuities in the DNA. The persistent discontinuities have been hypothesized to contribute to the

cytotoxic effect of O^6-MeG (Karran and Marinus, 1982; Goldmacher et al., 1986; Karran and Bignami, 1994).

We hypothesized that these persistent discontinuities generated by the futile cycles of excision and synthesis of mismatch containing O^6-MeG initiate homologous recombination, and that removal of methyl groups from O^6-MeG by AGT diminishes the generation of persistent discontinuities and decreases frequency of MNNG-induced recombination (Zhang et al., 1996). This predicts that cells unable to initiate mismatch repair of O^6-MeG-induced mismatches would be unable to undergo homologous recombination induced by MNNG. By selecting methylating agent-tolerant cells, we have been able to isolate cell strains that are also hypermutable by methylating agents. The resistance to the cytotoxic effect of MNNG did not result from increased AGT capacity, and therefore, represented increased ability to withstand the presence of O^6-MeG in DNA. This ability was correlated with the loss of mismatch repair in these cell strains. Taken together, the data in Figure 3 and 4 support our hypothesis that functional mismatch repair is required for MNNG-induced homologous recombination in human cells. The defective mismatch repair shown in Figure 3 for these methylation tolerant cells is correlated with their greatly decreased ability to carry out homologous recombination induced by methylating agents. This lack of homologous recombination induced by MNNG was not the result of a defect in the general recombination process, since these mismatch repair deficient cells were capable of carrying out spontaneous or BPDE-induced homologous recombination. Although the derivation of these cell strains, i.e.,

repeated mutagenesis, makes it very likely that they each contain mutations in several genes, in addition to their common loss of mismatch repair, it is highly unlikely that all three cell strains have concomitant defects in genes that are also involved in other steps of MNNG-induced recombination.

Mismatch repair of mismatches within the heteroduplex recombination intermediate has been proposed as one model to explain gene conversion (Holliday, 1964; Meselson and Radding, 1975). Yeast mutants with decrease in gene conversion were found to harbor mutations in mismatch repair genes *PMS1*, *MSH2* or *MLH1* (Kramer et al., 1989; Reenan and Kolodner, 1992; Alani et al., 1994; Prolla et al., 1994). Another mechanism for gene conversion that does not require mismatch repair is through repair of double strand breaks (Szostak et al., 1983). Ciotta et al. (1998) isolated two HeLa cells defective in *hMSH6* and *hPMS2*, respectively. Both cell strains are able to undergo spontaneous recombination between two mutant *hyg* genes containing a 10-bp *Hin*d III linker. We observed gene conversion products in our cells regardless of their ability of repairing G:T mismatches. Our data as well as those of Ciotta et al. suggest that either human cells frequently use double strand break repair to initiate gene conversion, or the defects in *hMSH6* or *hPMS2* do not interfere with repair of 10-base insertion/deletions. The *hMSH6* gene is known to mainly involve in repair of base mismatches and 1-base insertion/deletion mismatches. However, *hPMS2* is involved in repairing all types of mismatches (Kolodner, 1996; Modrich and Lahue, 1996). It is not yet clear whether 10 base insertion/deletions are efficiently repaired by the mismatch repair system in

humans. One possibility is that there is an as yet unidentified protein having a functional redundancy with hPMS2 that can repair these longer insertion/deletions. Further studies are needed to address these questions.

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CHAPTER 4

Evidence of induced expression of a potential senescence gene in an immortal human fibroblast cell strain, leading to cellular senescence

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Running Title: Expression of a potential senescence gene in human cells

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Abstract

We transfected a plasmid carrying an antisense *hMSH6* cDNA under the control of a tetracycline-regulatory enhancer-promoter into an immortal human fibroblast cell strain that expresses a tetracycline-responsive transcriptional activator. From 74 independent transfectants, we serendipitously identified one clone, which exhibited the characteristics of senescent cells after tetracycline was removed from the medium, i.e., the rate of growth slowed significantly, and the cells underwent morphological changes, becoming flattened, enlarged and granular. Such cells also stained blue for senescence-associated β-galactosidase (SA-β-gal). After tetracycline was returned to the medium, virtually the whole population of senescent-looking cells resumed a normal growth rate, took on a normal appearance, and no longer stained blue for SA-β-gal. We hypothesize that in the cells from this clone, the tetracycline-regulatory enhancer-promoter was inserted into a site close to a silenced senescence gene, and that withdrawal of the tetracycline from the medium allowed activation of this enhancer-promoter, which reactivated the silenced gene. When tetracycline is present in the culture medium, the tetracycline-regulatory enhancer-promoter is inactive, and therefore the cells would exhibit an immortal phenotype. We repeated the experiment and identified this time from 350 independent transfectants one clone that gave the same response, i.e., entered senescence shortly after tetracycline was removed from the medium. These results strongly support the hypothesis that expression of a potential senescence gene or genes is being induced in these two cell strains when tetracycline is withdrawn, and that this rapidly leads to cellular

senescence. We determined that the inserted tetracycline-regulatory enhancer-promoter in the first cell strain is located on the short arm of one Chromosome 1 (1p31.3-33). The sequence of genomic DNA flanking the inserted tetracycline-regulatory promoter-enhancer in that cell strain was determined and found not to share sequence homology with any known human genomic sequence. Screening a human P1 genomic library using the genomic DNA fragment obtained from chromosomal walking yielded five P1 clones for future study.

Introduction

Normal somatic mammalian cells in culture can replicate for only a limited number of times before entering a nonproliferating stage called cellular or replicative senescence (Hayflick and Moorhead, 1961; Hayflick, 1965). Senescent cells are arrested in G1 phase of the cell cycle (Sherwood et al., 1988; Cristofalo et al., 1989). The cells do not die after entering senescence, and if weekly changes of medium are carried out, they can remain viable for years (Matsumura et al., 1979). In contrast, cells derived from many human tumors exhibit infinite life span in culture (escape from senescence) (Bruland et al., 1985; Leibovitz, 1986). Therefore, cellular senescence is often considered to be a tumor suppression mechanism (Sager, 1991; Smith and Pereira-Smith, 1996). Considering that multiple mutations are required in multistep carcinogenesis (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996), immortalization, the process by which cells escape from senescence, would confer on cells

enough time to acquire the necessary mutations. This suggests that immortalization may be an early and common event in neoplasia.

It is now generally accepted that cellular senescence is genetically programmed (Vojta and Barrett, 1995). This program becomes activated at the end of a normal cell's proliferative life span, causing the characteristic morphological changes and growth arrest. Somatic cell fusions between normal (mortal) and immortal cells have most often led to hybrid cells with limited proliferative capacity (Pereira-Smith and Smith, 1981; 1983; Koi and Barrett, 1986; Ryan et al., 1994), suggesting that cellular senescence is a dominant phenotype. Pereira-Smith and Smith (1988) used this method to assign 40 different immortal human cell lines to four complementation groups (termed complementation groups A, B, C, and D), indicating that at least four genes or gene pathways control cellular senescence. In theory, immortal cells of different complementation groups carry different genetic alterations, and these defects can be complemented when a cell from one complementation group is fused with a cell from another complementation group to form a hybrid.

Using microcell-mediated chromosome transfer in various immortal cell lines, the activity to undergo senescence has been mapped to a number of human chromosomes, including 1, 2, 3, 4, 6, 7, 11, 18, and X (Sugawara et al., 1990; Klein et al., 1991; Ning et al., 1991; Koi et al., 1993; Ogata et al., 1993; Hensler et al., 1994; Sandhu et al., 1994; Sasaki et al., 1994; Ohmura et al., 1995; Uejima et al., 1995; Horikawa, et al., 1998; Uejima et al., 1998). Chromosomes 1 (group C), 4 (group B), and 7 (group D) have been shown to induce senescence

in cell lines that have been assigned to the same complementation group, but not to have an effect on cell lines assigned to other groups (Ning et al., 1991; Ogata et al., 1993; Hensler et al., 1994). None of these putative senescence genes has yet been cloned, although it was reported at a meeting that a senescence gene on chromosome 4 had been cloned (Ehrenstein, 1998).

Recently, in the course of a study designed to turn off the expression of the *hMSH6* (*GTBP*) gene in an infinite life span cell strain derived from MSU-1.1 cells (Morgan et al., 1991) using a tetracycline-regulatory expression system to control the expression of antisense *hMSH6* cDNA, we made an interesting observation. The tetracycline regulatory expression system uses the tetracycline-responsive transcription activator (tTA) to activate the tetracycline regulatory enhancer-promoter (Gossen and Bujard, 1992; Shockett et al., 1995). The transcription activator, tTA, is a fusion protein combining the DNA binding domain of the tetracycline repressor (TetR) and the transactivation domain of VP16 (Gossen and Bujard, 1992; Shockett et al., 1995). The tetracycline regulatory enhancer-promoter combines seven copies of the enhancer element TetO (Deuachle et al., 1995; Rose and MacDonald, 1997) with the minimal CMV promoter (Gossen and Bujard, 1992; Shockett et al., 1995). When tTA binds to the TetO sequence, it activates transcription of the gene of interest. Tetracycline in the medium prevents tTA from binding to TetO, and thereby inhibits transcription (Gossen and Bujard, 1992; Shockett et al., 1995).

We transfected a vector containing a marker gene for resistance to puromycin along with an antisense *hMSH6* cDNA under the control of the tetracycline-

regulatory enhancer-promoter into an MSU-1.1-derived strain that expressed a transfected *tTA* gene, and selected a large number of transfectants for puromycin resistance. In the course of expanding these clones, we identified two transfectants whose senescence/immortal phenotype was controlled by the absence and presence of tetracycline in the medium. Our working hypothesis is that in these two cell strains, when tetracycline is removed from the medium, the activated tetracycline-regulatory enhancer-promoter induces the expression of a potential senescence gene which otherwise is silenced in the immortal cells. The presence of tetracycline in the medium prevents the reactivation of this putative senescence gene. For one cell strain, we have determined that the tetracycline regulatory enhancer-promoter is inserted into one chromosome 1 at 1p31.3-33. We hypothesize that a potential senescence gene is located in that region.

Materials and methods

Cell strain and culture condition

Cell strain MSU-1.2-E7.2 used in this study has been described previously (Zhang et al., 1996). It contains a transfected substrate for detecting intrachromosomal homologous recombination. Cells were routinely cultured in McM medium containing 10% (v/v) supplemented calf serum (Hyclone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin and 1 μg/ml hydrocortisone (Zhang et al., 1996).

Plasmids

Plasmid pTet-tTA (GibcoBRL) was linearized at a *Not* I site and filled in by the Klenow fragment. A 6.0 kb DNA fragment containing a *hisD* gene was isolated from *BamH* I and *EcoR* I digested pMSV-hisD (Hartman and Mulligan, 1988) and filled in by the Klenow fragment. This fragment was ligated to the linearized pTet-tTA, and the resulting plasmid is designated ptTA (Fig. 1A). A 2.3 kb fragment containing the tetracycline-regulatory enhancer-promoter and SV40 polyadenylation signal was isolated after digestion of pTet-Splice (GibcoBRL) with *Not* I and *Xho* I. pBabe-Puro (Morgenstern and Land, 1990) was digested with *Pst* I and *Sal* I to generate a 3.6 kb fragment containing the ampicillin resistance gene and the puromycin resistance gene. The 2.3 kb and 3.6 kb fragments were filled in by the Klenow fragment and ligated together, and the resulting plasmid was designated pTet-Puro. A 3.9 kb partial cDNA of *hMSH6* containing part of exon1 and complete exons 2 to 10, was reverse-transcribed and amplified using expand long template PCR system (Boehringer Mannheim). The primers used in amplification were: VM-100: 5'-CAAGGCGAAGAAC-CTCAACGGAGGG-3' and VM-103: 5'-ACAGTTGACCTTTCACTAGCCAGGC-3'. This 3.9 kb cDNA fragment was inserted into pTet-Puro at the *Sal* I site in the antisense orientation to give plasmid pTet-GTBP (Fig. 1B).

DNA transfection

Cells were transfected using lipofectAMINE according to the manufacturer's (GibcoBRL) recommendations. Two days later, cells were selected for drug resistance. For the *hisD* marker, the McM medium lacked histidine, but was

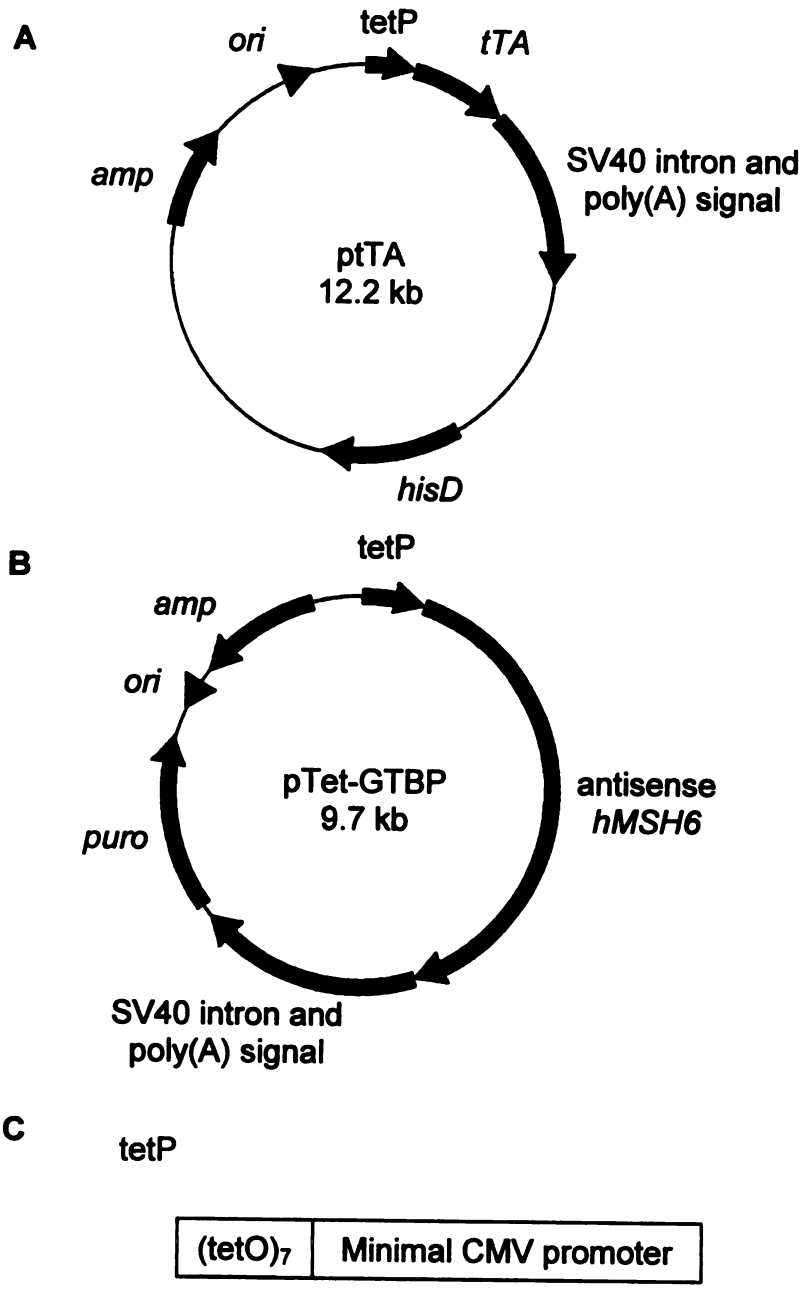


Figure 1. Diagrams of plasmids. (A). ptTA. (B). pTet-GTBP. (C). Tetracycline-regulatory enhancer-promoter (tetP).

supplemented with 1 μM histidinol (Sigma). For the *puro* marker, the McM medium contained 0.5 μg/ml puromycin (Sigma). After 2 wk, drug-resistant colonies were isolated and propagated in selective medium.

Northern analysis

Total RNA was purified using RNAZol B (TEL-TEST, Texas), according to the manufacturer's recommendations. 15 μg of total RNA was subjected to Northern analysis as described (Qing et al., 1997). DNA probes were labeled with [α-^{32}P]dCTP by the random primed labeling method (Freinberg and Vogelstein, 1983). Variation in RNA loading was evaluated by probing with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA as control.

Flow cytometry analysis

Cells were cultured with and without tetracycline (1 μg/ml) in the medium. At different time points, cells were trypsinized, suspended in heat-inactivated fetal bovine serum and PBS, and fixed in 50% ethanol. 1-2 x 10^6 fixed cells were washed twice with 1% bovine serum albumin in PBS, treated with RNase A (1 μg/ml) and stained with propidium iodide (50 μg/ml) at 37°C for 1 hour or room temperature for overnight. Cell-cycle distribution was analyzed using FACS vantage (Becton Dickinson).

Senescence-associated β-galactosidase staining

Cells were stained for senescence-associated β-galactosidase (SA-β-gal) activity at pH 6.0 as described (Dimri et al., 1995).

Fluorescence *in situ* hybridization (FISH)

FISH analysis was carried out in the Cytogenetic/FISH core facility in the Huntsman Cancer Institute at the University of Utah. Human metaphase chromosome spreads were prepared by standard procedures (Marchilli, 1980). The plasmid pTet-GTBP was nick-translation-labeled with biotin. *In situ* hybridization to metaphase chromosomes was performed according to the procedure described by Pinkel et al. (1986). The hybridization signal was detected with Cy3-conjugated streptavidin (Jackson Immuno Research) and counterstained with DAPI. The Vysis program (SmartCapture) was used to visualize metaphases and convert the DAPI image into a black and white G-banded image, facilitating band localization.

Chromosomal walking

The universal genomewalker kit (Clontech) was used in chromosomal walking. In brief, genomic DNA was digested with restriction enzymes *Dra* I, *EcoR* V, *Pvu* II, *Sca* I and *Stu* I, respectively. Digested DNA was ligated to genomewalker adapter to generate five genomewalker "libraries". Two-round PCR amplification was performed on these "libraries" with gene-specific primers and adapter primers, using expand long template PCR system (Boehringer Mannheim). The gene specific primers used in the chromosomal walking were: VM-176: 5'-TGAGAAGTCACAACTGGTGGGGGCAGCA-3', which anneals part of exon 1 and part of exon 2 of the *hMSH6* gene; VM-177: 5'-GCTACCGATCTCCG-CAGCCCTCCGTTGA-3', which anneals to exon 1 of the *hMSH6* gene; VM-186: 5'-GAAGAGTTCTTGCAGCTCGGTGACC-3', which anneals to the puromycin

resistance gene; and VM-200: 5'-AGGCGCACCGTGGGCTTGTACTCGGTCA-3', which anneals to the puromycin resistance gene.

DNA sequencing

PCR products from chromosomal walking were purified using Qiaquick gel extraction kit (Qiagen) and sequenced using a thermo sequenase cycle sequencing kit (Amersham Life Science) with ^{32}P end-labeled primers.

Genomic library screening

PCR products were gel-purified using a Qiaquick gel extraction kit (Qiagen) and were sent to Genome Systems Inc. (St Louis, MO) for hybridization screening of a human P1 genomic library.

Results

For a study of the mechanisms of recombination induced by methylating agents, we were using antisense to turn off the expression of the *hMSH6* (*GTBP*) gene in an immortal cell strain, designated MSU-1.2-E7.2. To do so, we used a tetracycline-regulatory expression system to control the expression of a partial antisense *hMSH6* cDNA. The parental cell strain for MSU-1.2-E7.2 is MSU-1.2, a cloned derivative of MSU-1.1 cells that had been selected for ability to form large clones in the absence of growth factors. Cell strain MSU-1.1 is a near diploid, karyotypically stable infinite life span human fibroblast cell strain which was generated in this laboratory (Morgan et al., 1994) from a diploid human fibroblast cell line, LG1, transfected with a plasmid carrying a *v-myc* oncogene and the gene for resistance to geneticin. A geneticin-resistant clone expressing the *v-myc*

gene was passaged to the end of its life span in culture, but an infinite life span cell strain with a diploid karyotype arose spontaneously. From these diploid cells, designated MSU-1.0, a spontaneous variant strain with a growth advantage arose. This cell strain, designated MSU-1.1, has a stable karyotype with 45 chromosomes including two unique marker chromosomes (Morgan et al., 1991). Cell strain MSU-1.2 has a stable karyotype identical to that of MSU-1.1 cells but with two additional marker chromosomes (J. J. M., unpublished data).

Transfection with ptTA

Plasmid ptTA contains a fusion gene *tTA*, combining the DNA binding domain of tetracycline repressor (TetR) with the transactivation domain of VP16 under the control of tetracycline regulatory enhancer-promoter (Shockett et al., 1995). The tetracycline regulatory enhancer-promoter, as shown in Figure 1C, combines seven copies of the enhancer element TetO with the minimal CMV promoter (Gossen and Bujard, 1992; Shockett et al., 1995). The expression of *tTA* is self-transactivated, and is inhibited in the presence of tetracycline. We transfected this plasmid into MSU-1.2-E7.2 cells and isolated individual stable, histidinol resistant transfectants. The transfectants were cultured for five days in the absence or presence of 1 μg/ml tetracycline in the culture medium. Total RNA was extracted and subjected to Northern analysis using *tTA* cDNA as the probe. From 12 transfectants tested, 10 showed *tTA* expression in the absence of tetracycline. Tetracycline in the medium turned off *tTA* expression to undetectable level in eight transfectants. One of these clones, cell strain MSU-1.2-10A, was selected for further studies (Fig. 2).

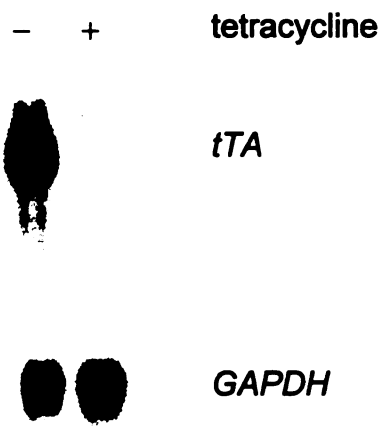


Figure 2. Northern analysis of expression of *tTA* in MSU-1.2-10A cells cultured in the medium with and without tetracycline (1 μg/ml). *GAPDH* was probed as a loading control.

Transfection with pTet-GTBP

MSU-1.2-10A cells were transfected with pTet-GTBP which contains a partial antisense *hMSH6* cDNA under the control of tetracycline-regulatory enhancer-promoter (Fig. 1B). Stable, puromycin-resistant transfectants were isolated individually, cultured in the absence or presence of 1 μg/ml tetracycline, and cell lysates from these cells were prepared and tested for their hMSH6 protein level by Western analysis using an hMSH6 polyclonal antibody. During this cell culture, we observed an interesting phenomenon. From total 74 independent transfectants, one, designated MSU-1.2-18A, grew extremely slowly in the absence of tetracycline, even though the rate of growth of this clone was normal in the presence of tetracycline. The entire population of slow-growing cells was shown to resume normal growth rate after tetracycline was returned to the medium (Fig. 3A). The parental MSU-1.2-10A cells and other transfectants grew equally well whether tetracycline was present in the medium or not (Fig. 3B).

We used flow cytometry to analyze the cell-cycle distribution of this cell strain in the absence or presence of tetracycline. No apoptotic population was seen under either culture conditions. We found that when tetracycline was withdrawn from the medium there were significantly more cells in G0/G1 phase and fewer cells in S phase of the cell cycle than that when tetracycline was present in the medium (Table 1). This suggests a growth arrest in G0/G1 phase when tetracycline is withdrawn.

The senescence-associated β-galactosidase (SA-β-gal) activity at pH6.0 is a good biomarker for cellular senescence. This activity is only detected in

Figure 3. Growth curves of cells cultured with or without tetracycline in the medium. (A). MSU-1.2-18A cells with tetracycline (crosses), without tetracycline (open circles), and tetracycline being returned to cells without tetracycline in the medium on day 4 (open triangles). (B). MSU-1.2-10A cells with tetracycline (crosses) and without tetracycline (open circles).

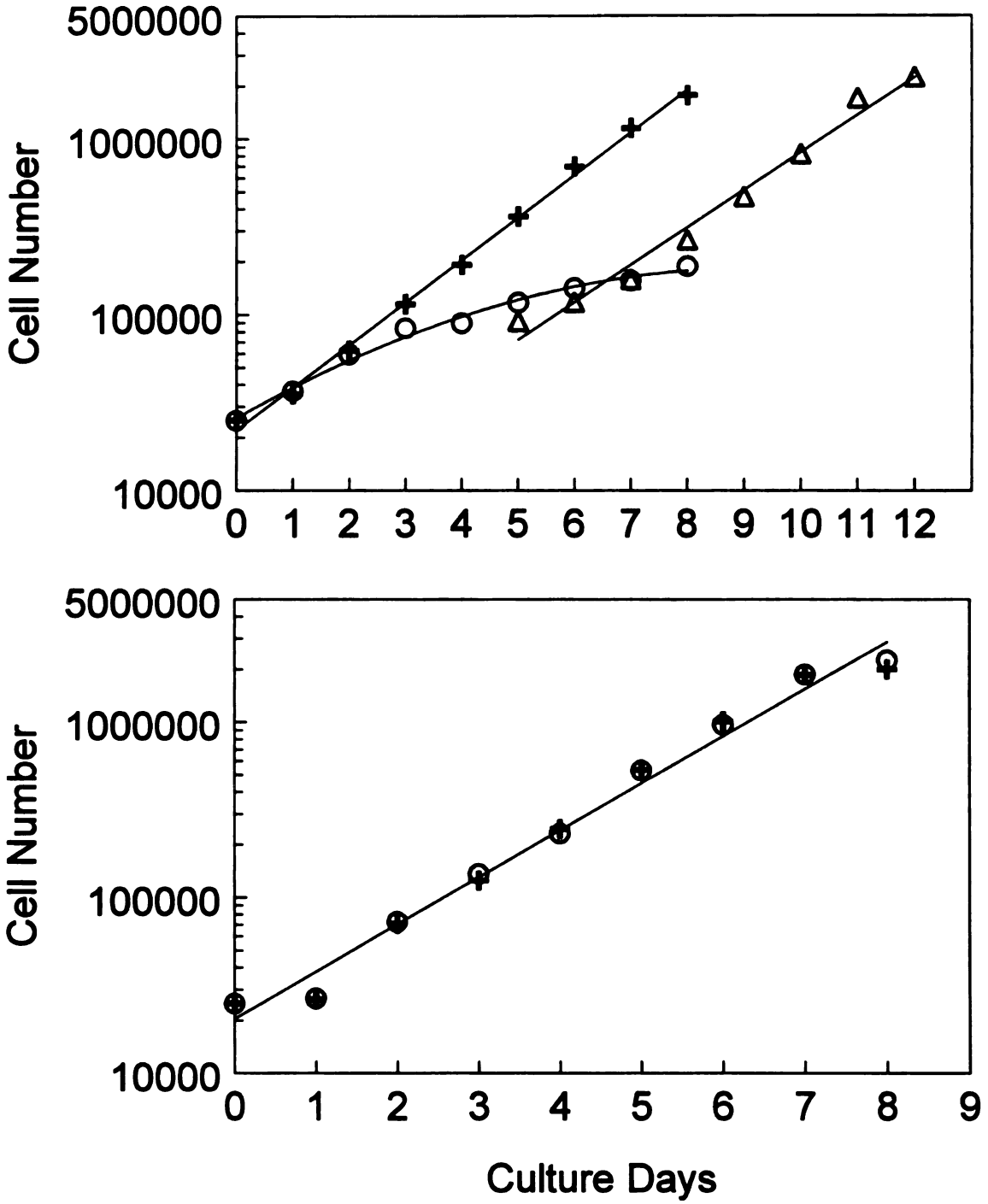

5000000
1000000
100000
10000
Cell Number
0 1 2 3 4 5 6 7 8 9 10 11 12
5000000
1000000
100000
10000
Cell Number
0 1 2 3 4 5 6 7 8 9
Culture Days

Table 1. Cell-cycle distribution of MSU-1.2-18A cells cultured for various length of time in medium with or without tetracycline.

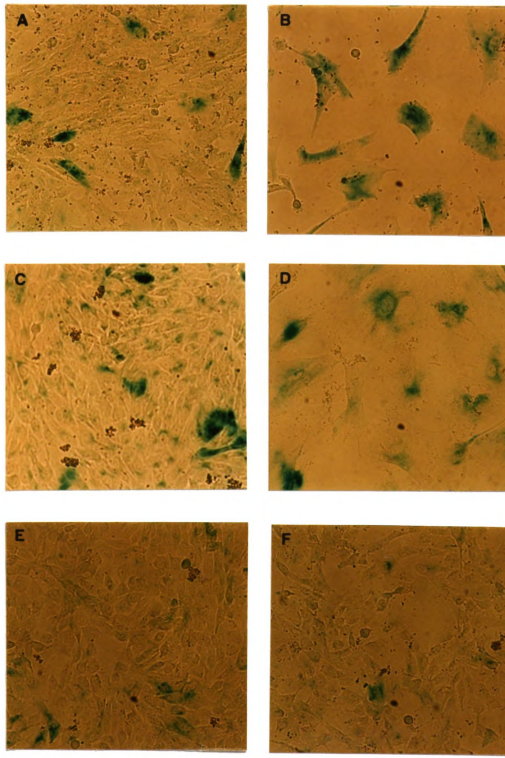
Phase of cell cycle	Tetracycline status[a]	Days in culture 1	2	3	4	5	6	7
G0/G1	+	57.0	69.3	48.3	60.4	51.7	57.4	49.3
	–	58.2	79.6	75.4	74.4	72.5	78.8	64.2
S	+	31.9	19.5	34.1	22.6	24.4	34.0	31.0
	–	23.1	10.9	14.2	14.5	15.9	10.5	19.5
G2	+	11.0	11.2	17.6	17.0	23.8	8.6	19.7
	–	18.7	9.4	10.4	11.0	11.6	10.7	16.3

a: +, with 1μg/ml tetracycline in medium; –, without tetracycline in medium.

senescent cells, but not in immortal cells, presenescent cells, or quiescent cells (Dimri et al., 1995). Equal numbers of MSU-1.2-18A cells were plated into culture dishes in the presence of tetracycline. Tetracycline was removed from half of the dishes on the next day, and cells were allowed to grow for 7 days in the absence or presence of tetracycline, with refreshing the medium every other day. Cells growing in the presence of tetracycline almost reached confluence, while the cell density in the dishes without tetracycline was very low. As shown in Figure 4A, when tetracycline was not present in the medium, every single cell stained blue, and the cells also underwent morphological changes, becoming flattened, enlarged and granular. These cells were senescent. In contrast, when tetracycline was maintained in the medium, most of the cells did not stain blue (Fig. 4B), and they continued to display the normal spindle-shaped morphology, with a few exceptions. These exceptions stained blue and became flattened and enlarged, indicating that they were senescent. The parental cells, MSU-1.2-10A showed no change from their infinite life span phenotypes in either the absence or presence of tetracycline in the medium (Fig. 4E and 4F).

The primary difference between MSU-1.2-18A cells cultured in the absence of tetracycline compared to when it was included in the medium was the induced expression of *tTA* and *hMSH6* antisense mRNA. It is highly unlikely that the phenotypes we observed in MSU-1.2-18A cells are the results of the induced expression of *tTA* since the parental cell strain from which it is derived, MSU-1.2-10A, has this gene integrated at the same site, and it did not exhibit the observed changes. Although the level of expression of hMSH6 protein in MSU-1.2-18A

Figure 4. Senescence-associated β-galactosidase activity (pH 6.0) staining. (A). MSU-1.2-18A cell with tetracycline; (B). MSU-1.2-18A cells without tetracycline; (C). MSU-1.2-A9D cells with tetracycline; (D). MSU-1.2-A9D cells without tetracycline; (E). MSU-1.2-10A cell with tetracycline; (F). MSU-1.2-10A cell without tetracycline. Photographs were taken at the same magnification.


A
B
C
D
E
F

cells decreased when tetracycline was withdrawn (date not shown), it is also unlikely that the striking phenotypes were caused merely by the induced expression of *hMSH6* antisense RNA, since the same expression level of the *hMSH6* antisense RNA was found in many other transfectants, but they did not show altered rates of growth. Although it is possible that the decreased hMSH6 protein level in MSU-1.2-18A cells due to the expression of antisense *hMSH6* RNA led to these phenotypes, *hMSH6* homozygous knock-out mice are viable, and show no apparent growth defect (Edelmann et al., 1997).

Second transfection with pTet-GTBP to repeat the result

To see if we could obtain another conditionally senescing cell strain using the same approach, we again transfected parental MSU-1.2-10A cells with pTet-GTBP, and isolated 350 individual puromycin resistant transfectants. When these were cultured in the absence and presence of 1 μg/ml tetracycline, we observed a similar slow-growing phenomena when tetracycline was not present in the medium in one transfectant. When this cell strain, designated MSU-1.2-A9D, was stained for SA-β-gal, it showed a result similar to that of MSU-1.2-18A. The same culture and staining procedure were used as described early. As shown in Figure 4C, when tetracycline was not present in the medium, cells from this clone had the characteristics of senescent cells, stained blue, became flattened and enlarged. When tetracycline was maintained in the medium, most of the cells did not stain blue, and they continued to display the spindle-shaped morphology, with a few exceptions (Fig. 4D). We also observed the difference in the cell density between these two culture conditions.

It is possible that in these two cell strains, the tetracycline-regulatory enhancer-promoter was inserted into a site on chromosomes adjacent to a silenced senescence gene. We hypothesize that the induced expression of this potential senescence gene by tTA transactivation leads these cells to senescence. Tetracycline inhibits the tTA transactivation of the tetracycline-regulatory enhancer-promoter, therefore the silenced senescence gene remains silent.

Chromosomal localization of the transfected pTet-GTBP

To determine the location of the inserted pTet-GTBP in chromosomes and the number of insertions, metaphase chromosomes were prepared from MSU-1.2-18A cells, and hybridized to labeled pTet-GTBP. Fluorescent signals were observed in 18 of 20 metaphases analyzed. Two metaphases gave inconsistent probe hybridization. Among 16 metaphases giving consistent probe hybridization, the probe localized only on the short arm of one chromosome 1, in the region 1p31.3-1p33, in 10 metaphases. In 2 metaphases, the probe localized only to the short arm of one chromosome 1, in the region 1p12. The probe localized to two regions, 1p12 and 1p31.3-33 of one chromosome 1 in 2 metaphases, and in 2 other metaphases, the probe localized to regions 1p12 and 1p31.3-33 of one chromosome 1 and region 2p16 of one chromosome 2. Total 14 metaphases showed consistent hybridization signal at 1p31.3-33 of one chromosome 1. The band localization was confirmed on G-banded chromosomes. An example is shown in Figure 5.

Figure 5. Fluorescence *in situ* hybridization of MSU-1.2-18A cells with pTet-GTBP as the probe.

Since *hMSH6* (*GTBP*) gene is mapped to a region of chromosome 2p15-16 (Acharya et al., 1996), we think that the hybridization signal on 2p16 in 2 metaphases reflected that of hybridization with *hMSH6* gene. The signal on 1p12 may come from hybridization of pTet-GTBP with other DNA fragments which had been previously transfected into these cells and is also present in pTet-GTBP. The consistent hybridization signal was only on one chromosome 1 in the region 1p31.3-33. Signal on only one chromosome 1 is also consistent with the transfection nature. Most likely there is only one insertion site for plasmid pTet-GTBP in MSU-1.2-18A cells, and it is on 1p31.3-33.

Chromosomal walking

To obtain the flanking genomic DNA sequences of the inserted plasmid, we used chromosomal walking from the unique sequence of pTet-GTBP, the puromycin resistance gene and the junction sequence between each exon of the antisense *hMSH6* cDNA.

Starting from the puromycin resistance gene, two primers, VM-186 and VM-200, were used in the chromosomal walking. Both primers anneal to the puromycin resistance gene. The direction of walking was towards the promoter of the puromycin resistance gene. Two DNA fragments which contained non-plasmid sequences were obtained from the *Pvu* II and *Sca* I genomewalker 'libraries" in MSU-1.2-18A cells. These two fragments were designated 18A-*Pvu* II and 18A-*Sca* I. One DNA fragment containing non-plasmid sequences was obtained from the *Pvu* II genomewalker "library" in MSU-1.2-A9D cells, and it was named A9D-*Pvu* II. Sequencing of these three fragments indicated that 18A-

Pvu II and 18A-*Sca* I were identical except that 18A-*Sca* I was 228 bp longer, and the sequence of A9D-*Pvu* II was different from those of 18A-*Sca* I/18A-*Pvu* II as expected.

Starting from the *hMSH6* cDNA, two primers, VM-176 and VM-177, were used to walk towards polyadenylation signal sequences. VM-176 anneals to the junction between exon 1 and exon 2 of the *hMSH6* gene. VM-177 anneals to exon 1 of the *hMSH6* gene. We did not obtained DNA fragment containing non-plasmid sequences from MSU-1.2-A9D cells. One DNA fragment containing non-vector sequences was obtained from the *Sca* I genomewalker 'library" in MSU-1.2-18A cells, which was designated 18A-2D. Sequencing analysis of 18A-2D revealed unique sequence that is different from those of 18A-*Sca* I/18A-*Pvu* II and A9D-*Pvu* II.

We also performed chromosomal walking starting from the puromycin resistance gene towards its polyadenylation signal sequences, and from the junction of exon 9 and exon 10 of the *hMSH6* cDNA towards the tetracycline-regulatory enhancer-promoter. We did not obtain DNA fragments containing non-plasmid sequences in these experiments.

PCR amplification of human genomic DNA using primers that anneal to 18A-2D and 18A-*Sca* I/18A-*Pvu* II respectively generated a DNA fragment whose sequence is identical to those of 18A-2D and 18A-*Sca* I/18A-*Pvu* II, suggesting they are on the same chromosome adjacent to each other. Database search indicated that there was no significant sequence homology of 18A-2D, 18A-*Sca* I/18A-*Pvu* II and A9D-*Pvu* II with any deposited human genomic sequence.

Human genomic library screening

We designed two primers from 18A-2D sequence, and walked further along the chromosome. The extended 18A-2D sequences were obtained and searched in the database. No significant sequence homology with known sequence was found. We amplified a 1.2 kb DNA fragment, confirmed by sequencing that it contained the extended 18A-2D sequence, and sent it to Genome System Inc. for hybridization screening of a human P1 genomic library. Five positive P1 clones hybridizing to 18A-2D were obtained. DNA was prepared from each of these P1 clones. We determined that a DNA fragment containing part of 18A-2D and 18A-*Sca* I/18A-*Pvu* II can amplified from these P1 DNA as expected, also determined that the A9D-*Pvu* II sequence could not be amplified from these P1 DNA (Fig. 6), indicating that the two integration sites differ. This is consistent with the fact that MSU-1.2-18A and MSU-1.2-A9D cells were derived independently, and pTet-GTBP would not be expected to be inserted into the same site.

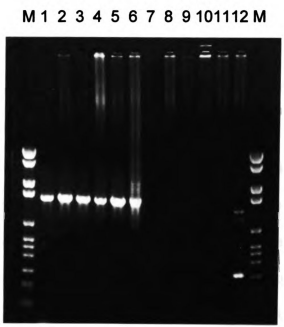


Figure 6. PCR amplification using DNA from P1 clones. Lane M, DNA molecular marker from Boehringer Mannheim (pBR328-*Bgl* I DNA + pBR328-*Hinf* I DNA). Lane 1 through 6, PCR amplification of a DNA fragment containing part of 18A-2D and 18A-*Sca* I/18A-*Pvu* II from DNA of P1 clone #1 through #5 and genomic DNA of MSU-1.2-10A cells. Lane 7 through 12, PCR amplification of a DNA fragment containing the A9D-*Pvu* II sequence from DNA of P1 clone #1 through #5 and genomic DNA of MSU-1.2-10A cells.

Discussion

We serendipitously generated two cell strains whose senescence/immortal phenotypes can be controlled. When tetracycline was present in the medium, these cells maintained their immortal phenotypes, i.e., grew fast and did not stain blue for SA-β-gal at pH6.0. When tetracycline was withdrawn from the medium, these cells grew very slowly and underwent morphological changes, becoming flattened, enlarged and granular typical of senescence. They stained blue for SA-β-gal. These phenotypical changes are not the results of the insertion of transfected plasmids since all the cells have the same insertion whether tetracycline is present in the medium or not. These phenotypes cannot have resulted from the induced expression of *tTA* because the parental cell MSU-1.2-10A did not exhibit this phenomenon. In two transfection experiments with pTet-GTBP, many transfectants including MSU-1.2-18A and MSU-1.2-A9D, exhibited the induced expression of antisense *hMSH6* RNA. Therefore, it is unlikely that the induced expression of antisense *hMSH6* RNA *per se* caused these phenotypes in these two cell strains. Another possible explanation is that the decreased hMSH6 protein levels in these two cell strains, resulting from the induced expression of antisense *hMSH6* RNA when tetracycline was removed from the medium caused the phenotype. In our two cell strains, the hMSH6 protein level was decreased when tetracycline was absent, but was still more than 50% of that when tetracycline was present (data not shown). We can not rule out this possibility. However, hMSH6 gene knockout transgenic mice are viable with no apparent imparity in growth (Edelmann et al., 1997), suggesting

that hMSH6 gene is not likely to be involved in senescence/immortalization. We are convinced that induced expression by the tetracycline regulatory expression system of a potential senescence gene(s) in these two cell strains led to cellular senescence. To our knowledge, these two cell strains are the first examples of cells in which the senescence/immortal phenotypes can be rapidly controlled. These cell strains provide a unique experimental tool for studying cellular senescence.

Tetracycline-regulatory expression system is a powerful tool to tightly regulate gene expression in mammalian cells (Gossen and Bujard, 1992; Shockett et al., 1995). The TetO sequence used in this system is an enhancer element (Deuachle et al., 1995; Rose and MacDonald, 1997). It is possible to utilize this system to control gene expression from a relatively long distance and bidirectionally. Senescence genes are silenced in immortal cells. If the TetO is inserted into a site in a chromosome near a silenced senescence gene, the expression of that gene might be turned on by this system. Because of the dominant nature of senescence genes, induced expression of a senescence gene from one allele should be sufficient to induce cellular senescence. This approach does not require the previous knowledge of the chromosomal locations of the potential senescence genes, and provides a way to narrow the chromosome regions where potential senescence genes locate.

The frequency of generating these two cell strains was very high, 1/74 and 1/350 respectively. It is possible that the cell strain MSU-1.2 has lost the expression of several senescence genes, and restored expression of any one of

them leads to cellular senescence. Several chromosomes have been implicated to carry senescence genes, including chromosomes 1, 2, 3, 4, 6, 7, 11, 18 and X (Sugawara et al., 1990; Klein et al., 1991; Ning et al., 1991; Koi et al., 1993; Ogata et al., 1993; Hensler et al., 1994; Sandhu et al., 1994; Sasaki et al., 1994; Ohmura et al., 1995; Uejima et al., 1995; Horikawa, et al., 1998; Uejima et al., 1998). Some of these chromosomes may even carry more than one senescence gene (Vojta et a., 1996; Banga et al., 1997; Morelli et al., 1997). The possibility of a plasmid inserting into a specific chromosome arm is 1/92. Since either one of the two chromosomes would have the same effect of cellular senescence, this possibility increases to 1/46. In addition, the TetO enhancer can function in a relatively long distance. These combinations may help to explain the high frequency at which we obtained these two cell strains, but they are not sufficient. The mystery remains unsolved.

In cell strain MSU-1.2-18A, the transfected pTet-GTBP is inserted into one chromosome 1 in the region of 1p31.3-33. Chromosome 1 has been shown to carry senescence genes (Sugawara et al., 1990). In fact, two loci on the long arm of chromosome 1 are considered to carry two potential senescence genes (Vojta et al., 1996). Chromosome 1p31.3-33 has been found to be deleted in many human cancers, including neuroblastoma (Gilbert et al., 1982; Avigad et al., 1997; Bieche et al., 1998), carcinoma of pancreas (Ding et al., 1992), male germ cell tumors (Mathew et al., 1994), breast cancer (Hoggard et al., 1995; Nagai et al., 1995), colorectal adenocarcinoma (Bardi et al., 1995; Ogunbiyi et al., 1997), endometrial cancer (Arlt et al., 1996), hepatocelluar carcinoma (Kaplanski et al.,

1997), T cell acute lymphoblastic leukemia (Iolascon et al., 1997), meningioma (Sulman et al., 1998), liver cancer (Parada et al., 1998), non-small cell lung cancer (Gasparian et al, 1998). These results suggest that there is at least one tumor susceptibility gene located in that region. Senescence genes are considered to be tumor suppressor genes. It is possible that the tumor suppressor gene (or genes) located in 1p31.3-33 acts as a senescence gene.

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APPENDIX

To understand the molecular mechanisms of cellular senescence, and to assess the significance of cellular senescence in carcinogenesis and aging, an important step is to identify the genes that control cellular senescence. For future reference, I discuss here the possible paths to the discovery and characterization of the potential senescence gene described in Chapter 4.

I conclude in Chapter 4 that there is a potential senescence gene in at least one of the five P1 genomic clones. To clone this senescence gene, it is important to determine which P1 clones contain it. To do so, DNA from each of the five P1 clones will be labeled with α-^{32}P-dCTP using random-primed labeling so that labeled DNA is of various lengths. The labeled P1 DNA will then be used as probe in Northern analysis to analyze the gene expression patterns in cells that are either senescent or immortal, such as cell strain MSU-1.2-18A cultured in the absence or presence of tetracycline, later passage cultures of LG1 cells, and MSU-1.1 cell strain. A true senescence gene should be expressed only in senescent cells, but not in immortal cells. If such desired expression patterns are identified, the corresponding P1 DNA will be used for further studies.

Direct sequencing of large genomic clones that cover a particular region on a chromosome has been used successfully to identify genes located within these genomic clones. The sequencing data can be analyzed using the GRAIL II program (Uberbacher and Mural, 1991; Xu et al., 1994) or the BCM Genefinder program (Solovyev et al., 1994) to identify exons. cDNA can then be obtained

after screening a relevant cDNA library using the identified exons as probe. The disadvantage of this approach is that it requires a large amount of sequencing.

These large genomic DNA can be used as probe to screen a cDNA library to identify genes residing in that genomic DNA. Usually this approach can be expected to yield a substantial number of cDNA clones. Although characterization of a large number of cDNA clones can be difficult, if all the alternative approaches discussed below fail, such direct screening using DNA from P1 clones is a good choice.

Alternatively smaller DNA fragments can be isolated from the P1 DNA digested by appropriate restriction endonucleases, and used as probe to continue the examination of gene expression patterns. Use of this method could narrow the region containing the senescence gene, which can then be studied using the direct sequencing approach as described above. These smaller DNA fragments can also be used directly as probe to screen an appropriate cDNA library as described above.

The mammalian genome is made of exons dispersed along very variable lengths of DNA. CpG islands constitute a distinctive fraction of the genome and contain a high frequency of the dinucleotide CpG. The method of identification of CpG islands has been successfully used to identify cDNA since many genes are associated with CpG islands at their transcription start sites (Lindsay and Bird, 1987; Antequera and Bird, 1993). Large genomic DNA from P1 clones, for instance, can be digested with a suitable restriction endonuclease to give DNA fragments that are readily separated on agarose gel. These digested fragments

can then be further digested using restriction endonucleases *Bss*H II, *Eag* I or *Sac* II. The recognition sequences of these three enzymes are comprised only of G and C and contain two CpGs. If a DNA fragment contains CpG islands, it would be expected to be digested by *Bss*H II, *Eag* I or *Sac* II. The CpG island-containing DNA fragment than can be used as probe to screen a relevant cDNA library to identify the corresponding cDNA. This approach is relatively simple. However, not all human genes contain CpG islands. Therefore, other methods will be required if CpG islands are not found in a particular genomic clone.

Several other methods are also available for identification of protein-coding regions in a large genomic clone. For example, cDNA selection (Parimoo er al., 1991; Akiyama et al., 1997) and exon trapping (Buckler et al., 1991; Church et al., 1994) have been successfully used to isolate desired cDNA. However, these methods are technically challenging and require a lot of experience.

A candidate senescence gene can be expected to fulfill the following criteria: 1). It will induce growth arrest (senescence) following expression in a proliferating cell, with cell viability being preserved; 2). It will be down-regulated or mutated in immortal cells; and 3). If mutated or down-regulated in normal cells, it will induce immortalization. Once the gene of interest has been cloned, experiments can be designed to examine its characteristics using these criteria.

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