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Targets for and consequences of, radiation-induced chromosomal instability

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Targets for, and Consequences of, Radiation-Induced Chromosomal Instability

by

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DISSERTATION

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Preface

ACKNOWLEDGMENTS

My first year of graduate school was largely spent wondering what compelled this Chicago native to move all the way to the City by the Bay. In the next four, though, I found the answer. No, it wasn't merely avoidance of winter. Graduate school has been an incredible period of growth and development for me, both scientifically and personally, and moving to a new place and breaking some old ties was necessary for new, stronger ones to have been forged. This could not have happened, however, without the help of some very special people.

I begin with my advisor, Bill Morgan, who had no small influence on my enjoyment of graduate school. Bill has a great ability to see the big picture and think logically about experiments, qualities I hope to emulate as a scientist. More importantly, Bill was a friend who made sure I progressed steadily in my research and graduate school, and he genuinely looked out for my best interests. My labmates, Charlie Limoli, James "Corky" Corcoran, Andreas Hartmann, and Brian Ponnaiya, have provided unbridled camaraderie and have patiently listened to my wild ideas. I especially thank Charlie, who taught me tissue culture and cytogenetics when I first arrived, and has since provided guidance and constructive criticism. Morgan lab East, consisting of Ceecee, Angel, Jim, and Jeff, have showed me what little I know about molecular biology, as well as broadened my musical horizons. My orals committee, Dennis Deen, John Murnane, John Fike, Amy Kronenberg, and Dan Pinkel, gave much time and effort before, during, and especially after the exam to ensure that I was well prepared for my career in science. Dennis and John continued on my thesis committee, and I appreciate their support and input.

I was fortunate to have made many good friends at UCSF, especially in the biophysics program. Among bridge, intramural sports, skiing, hiking, cycling, sporting events, and tooling around San Francisco, it's amazing I had time left for research. I wouldn't have thrived without my friends Chris, Russ, Manish, Kevin, Karen, Sherry, Jennifer, Keith, Wade, and Sarah. Julie Ransom, part administrator, part den mom, has been an invaluable resource as well. My continued friendships with Lee, Archan, Dan, Brett, Jordan, Darrell, Adri, Ken, and Andy from the pre-graduate school years, all 22 of them, have continued to enrichen my life.

Last but certainly not least, the love of my parents, my brother Brian, my step-siblings, and my fiancé Debbie have been my bedrock without that I could not have blossomed. To paraphrase one of history's greatest scientists, Isaac Newton, I can see so far and grow so high only because my foundation is solid.

STATEMENT ON CO-AUTHORS AND PUBLISHED MATERIAL

This thesis contains material that is or will be published in the scientific literature.

Chapter 1 is an oveview on the current status of radiation-induced chromosomal instability. It serves as an introduction, and text is largely taken from two papers. One is titled *Perpetuating Radiation-Induced Chromosomal Instability*, **Radiation Oncology Investigations**, Vol. 5, pp. 124-128, 1996, authored by M. Kaplan, Charles L. Limoli, Ph.D. and William F. Morgan, Ph.D. This is a review article outlining our current views on perpetuating chromosomal instability. As primary author, his contribution to the paper was writing the manuscript, creating Figure 1, and organizing Figures 2-3. The second paper is titled *Genomic Instability Induced by Ionizing Radiation.*, **Radiation Research**, Vol. 146, pp. 247-58, 1996, authored by William F. Morgan, Ph.D., Joseph P. Day, Ph.D., M. Kaplan, Charles L. Limoli, Ph.D., and Eva M. McGhee, Ph.D. His contribution to this paper was writing the section on mismatch repair and contributing to the discussion on the biological significance of radiation-induced instability.

Chapter 2 contains the full text of the manuscript *The Nucleus is the Target for Radiation-Induced Chromosomal Instability*, submitted for publication, authored by M. Kaplan and William F. Morgan, Ph.D. This manuscript comprises the bulk of his thesis work. His contribution was to write the manuscript and perform all the experimental work.

Chapter 3 contains the full text of the manuscript *Differential Induction of Chromosomal Instability by DNA Strand Breaking Agents.*, **Cancer Research**, Vol. 57, pp. 4048-4056, 1997, written by Charles L. Limoli, Ph.D. M. Kaplan contributed to the work by preparing of genomic DNA and analyzing APRT mutations by the polymerase chain reaction (PCR). He was also actively involved in the collection of metaphase cells, fluorescence *in situ* hybridization, and cytogenetic analysis of individual clones to determine instability. John W. Phillips, Ph.D. assisted with electroporation and Gerald M. Adair, Ph.D. provided a cell line used in the study.

Chapter 4 contains the full text of the manuscript *Chromosomal Instability and its Relationship to Other Endpoints of Genomic Instability*, **Cancer Research**, Vol. 57, pp. 5557-5563, 1997, written by Charles L. Limoli, Ph.D. M. Kaplan was a co-author, and his contribution to the work included developing the techniques for the DNA mobility-shift assay, a Topo I unwinding assay, and doing the experimental work involved in the sister chromatid exchange assay. He also contributed to the experimental design, statistical analysis, and writing of the manuscript. James Corcoran assisted with experiments involving gene amplification and delayed mutation, and Mark Meyers and David A. Boothman, Ph.D. performed Western blot analyses.

William F. Morgan, Ph.D.

Chairman, Thesis Committee

Abstract

Targets for, and Consequences of, Radiation-Induced Chromosomal Instability

by Mark Isaac Kaplan

Chromosomal instability has been demonstrated in a human-hamster hybrid cell line, GM10115, after exposure to x-rays. Chromosomal instability in these cells is characterized by the appearance of novel chromosomal rearrangements multiple generations after exposure to ionizing radiation. To identify the cellular target(s) for radiation-induced chromosomal instability, cells were treated with ^{125}I-labeled compounds. Labeling cells with ^{125}I-iododeoxyuridine, which caused radiation damage to the DNA and associated nuclear structures, did induce chromosomal instability. While cell killing and first-division chromosomal rearrangements increased with increasing numbers of ^{125}I decays, the frequency of chromosomal instability was independent of dose. Incorporation of an ^{125}I-labeled protein, ^{125}I-succinyl-concanavalin A, into either the plasma membrane or the cytoplasm, failed to elicit chromosomal instability. These results show that radiation damage to the nucleus, and not to extranuclear regions, contributes to the induction of chromosomal instability.

To determine the role of DNA strand breaks as a molecular lesion responsible for initiating chromosomal instability, cells were treated with a variety of DNA strand breaking agents. Agents capable of producing complex DNA double strand breaks, including X-rays, Neocarzinostatin and bleomycin, were able to induce chromosomal instability. In contrast, double strand breaks produced by restriction endonucleases as well as DNA strand breaks produced by hydrogen peroxide failed to induce chromosomal instability. This demonstrates that the type of DNA breakage is important in the eventual manifestation of chromosomal instability.

In order to understand the relationship between chromosomal instability and other end points of genomic instability, chromosomally stable and unstable clones were analyzed for sister chromatid exchange, delayed reproductive cell death, delayed mutation, mismatch repair and delayed gene amplification. Although individual clones within each group were significantly different from unirradiated clones for many of the endpoints, there was no significant correlation between chromosomal instability and the phenotypes of sister chromatid exchange, delayed mutation, and mismatch repair. Delayed gene amplification weakly correlated chromosomal instability ($0.05<p<0.1$) and delayed reproductive cell death correlated strongly ($p<0.05$) with chromosomal instability. These data indicate that multiple pathways exist for inducing genomic instability in GM10115 cells after radiation exposure.

William F. Morgan, Ph.D.

Chairman, Thesis Committee

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Abbreviations Used

αMEM	alpha minimal essential medium
ANOVA	analysis of variance
APRT	adenine phosphoribosyltransferase
BLM	bleomycin
BBR	bridge-breakage-refusion
BrdU	bromodeoxyuridine
CAD	carbamoylphosphate synthetase, aspartate transcarbamylase, and dihydrooratase
CFA	colony forming assay
con A	concanavalin A
dch	decays per cell per hour
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DSB	double-strand break
FBS	fetal bovine serum
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
HC#4	human chromosome four
HPRT	hypoxanthine phosphoribosyltransferase
IdU	iododeoxyuridine
ITB	interstitial telomere band
LET	linear energy transfer
MMR	mismatch repair
NCS	neocarzinostatin

PALA	N-(phosphonacetyl)-L-aspartate
PBD	phosphate-buffered detergent
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RE	restriction endonuclease
SCE	sister chromatid exchange
SFM	serum-free medium
suc-con A	succinyl-concanavalin A

Chapter 1 Introduction

OVERVIEW

The interaction of ionizing radiation with biological systems produces physical, chemical, and biological effects over a vast range in timescales. An X-ray will traverse a mammalian cell in less than a picosecond. Chemical processes, including the fixation of radiation damage by oxygen or chemical repair from free radical scavengers, are completed within a second (105). Biological processes such as enzymatic repair (56), activation of gene expression (14), signal transduction pathways (31) and induction of cell cycle arrests (81) can occur within minutes to hours. These are "immediate" biological effects, operationally defined as end points measurable within the first cell cycle following irradiation. There are also longer term effects, called "delayed" effects, of radiation exposure, which can be manifested many cell generations after radiation exposure. Understanding the interrelationships among events happening during these disparate timescales is a central goal of radiobiology.

The immediate biological effects of ionizing radiation not only include transient changes like DNA repair, signal transduction and cell cycle perturbations, but permanent alterations such as mutations, chromosomal aberrations and compromised reproductive integrity. The cellular target for these genetic changes is, unsurprisingly, the cell nucleus (113, 114, 153). Of the various types of radiation damage to DNA, including DNA base damage, DNA-protein crosslinks, and single- and double-strand breaks (146), the most biologically significant radiation-induced lesion appears to be the DNA double-strand break (148). If misrepaired, double-strand breaks can lead to mutations and chromosomal aberrations (113, 115). A subset of these genetic changes will be incompatible with cell survival, resulting in a loss of reproductive integrity. Indeed, there is a one-to-one correlation between chromosomal aberrations seen in the first mitosis following irradiation and cell killing events (18).

While the immediate effects of ionizing radiation result in biological changes, delayed effects are characterized by increased rates of change. The delayed effects of ionizing radiation cataloged to date include increased rates of mutations (24), chromosomal instability (62, 84), gene amplification (48), microsatellite instability (37), delayed reproductive cell death (a decrease in plating efficiency, or the increase in the rate reproductive failure) (21) and transformation (67). It has been demonstrated that radiation induces genomic instability, broadly taken to describe an increased rate of acquisition of alterations in genomic DNA. As radiation has been long known to induce cancer (49, 127), and carcinogenesis requires multiple genetic alterations in normal cells (38, 76), an emerging paradigm is that genomic instability is an important mechanism for radiation-induced carcinogenesis (30).

The experiments chronicled in this thesis help to elucidate the mechanisms contributing to the phenomenon of chromosomal instability. In studies done to date by several groups, chromosomal instability was induced by irradiating the entire cell. This makes it difficult to make definitive statements on the location of the cellular target(s) responsible for chromosomal instability. Experiments described in Chapter 2 were undertaken to determine whether the target for radiation-induced chromosomal instability involved a nuclear target, an extranuclear target, or both. Likewise, experiments described in Chapter 3 were performed to determine whether DNA double-strand breaks are necessary for the induction of chromosomal instability. While various delayed effects of ionizing radiation have been described individually, the interrelationships among these disparate end points have not been investigated. Experiments in Chapter 4 were done to determine how the manifestation of chromosomal instability is related to other end points of genomic instability.

GENOMIC INSTABILITY AND CANCER

Genomic instability is characterized by the increased rate of acquisition of alterations in the mammalian genome. Genomic instability is necessarily an all embracing term which embodies a variety of genomic alterations, including chromosomal destabilization, gene amplification, and mutation. The loss of stability of the genome in tumor cells is becoming widely accepted as one of the most important aspects of cancer (27, 142). Nowell (104) has suggested that genomic instability provides the requisite genomic plasticity needed to drive the stepwise progression of genetic change required for the neoplastic phenotype.

The importance of genomic changes in tumor progression and their association with cancer underscore the importance of studying the mechanisms by which they arise. Multiple metabolic pathways govern the accurate duplication and distribution of DNA to progeny cells; other pathways maintain the integrity of the information encoded by DNA and regulate the expression of genes during growth and development (27). Together, these pathways may be regarded as genomic stability functions. For each of these functions, there is a normal baseline frequency at which errors occur, leading to spontaneous mutations and other genomic alterations. Genomic instability results in an increased frequency of mutations, gene amplification, and karyotypic alterations that can arise by radiation-induced interference with these pathways.

The relative genomic instability of cancer cells extends to molecular, biochemical, and morphological characteristics, but it is most visible cytogenetically (121, 137). Multiple chromosomal abnormalities have been described in a variety of human cancers (91). Patients afflicted with a range of leukemias and lymphomas (chronic myeloid leukemia, acute lymphoblastic leukemia, non-Hodgkin's lymphomas, and Burkitt's lymphoma, among others) show karyotypic abnormalities, many of which include distinct chromosome translocations (50, 91). Furthermore, the chromosome breakage syndromes xeroderma pigmentosum, ataxia telangiectasia, Bloom's syndrome, and Fanconi's anemia

are all characterized by increased incidence of cancers (50, 91). The association between chromosomal rearrangements and cancer suggests that chromosomal instability may underlie some fraction of those changes leading to cancer.

RADIATION-INDUCED GENOMIC INSTABILITY

There is a long history linking radiation exposure and the elevated incidence of cancer (49, 127), and the potential molecular mechanisms of radiation oncogenesis have recently been described (30). Not surprisingly, substantial interest has focused on the role of genomic instability in radiation carcinogenesis (69).

Cellular exposure to ionizing radiation results in a variety of directly and indirectly induced DNA lesions, including DNA base alterations, DNA-DNA and DNA-protein crosslinks, and single- and double-strand breaks (DSBs) (146). Cells react rapidly to irradiation, evoking a mammalian stress response that includes a plethora of biological responses, such as the initiation of signal transduction pathways (154), the activation of gene transcription (15), the repair of damaged DNA (56, 145, 147, 151), and cell cycle-specific growth arrest (81, 100). These early events are likely to be preconditions and determinants of the later fate of the irradiated cells (i.e. whether a cell will necrose, senesce, apoptose, or ultimately survive and proliferate). If a cell does survive, the initial biological response to the radiation-induced insult may influence whether the cell participates in normal differentiation, exhibits a limited life span, or proliferates and begins to acquire, via genomic instability, those characteristics associated with the neoplastic transformation of a normal cell to a cancerous cell. Alternatively, radiation may induce a subset of "lesions" that are not repaired, or are fixed as mutations, until many generations after initial exposure. However, this is unlikely, as the half-life of radiation-induced lesions is far shorter than the doubling time of mammalian cells (56).

Several delayed effects in the progeny of cells surviving acute exposure to X-rays have been described: variability in colony size (120, 136); a persistent reduction in plating

efficiency (heritable lethal mutations or delayed reproductive cell death) (21, 22, 135, 136); giant cell formation (40); cell fusion (9); lowered cell attachment ability (21); delayed mutation (24); transformation (67, 88); and delayed chromosomal instability (55, 61, 62, 84, 85, 123, 124).

RADIATION-INDUCED CHROMOSOMAL INSTABILITY

Chromosomal aberrations have long been visualized in the first mitosis following irradiation. More recently, it has been proposed radiation also induces chromosomal instability, as manifested by an increased rate of chromosomal alterations multiple generations after exposure. In a typical experiment, cells are irradiated, surviving cells grow into colonies, and the colonies may be grown for longer periods of time. The clonal descendants of individual cells are analyzed for chromosomal aberrations. Any aberration which is seen in every cell in the population is assumed to have been caused directly by radiation. However, there are also chromosomal aberrations which are only seen in a certain subpopulation of cells in the clonal population. Since in principle all cells descended from the same progenitor cell should have the same karyotype, these aberrations are assumed to have arisen at delayed times following radiation exposure. Figure 1.1 shows six metaphase cells from chromosomally unstable clones of a human-hamster hybrid cell line, GM10115. These cells contain a single copy of human chromsome four (yellow-green) in a background of hamster chromosomes (red).

Transmissible chromosomal instability was first demonstrated by Weissenborn and Streffer (155, 156) who irradiated one-cell mouse embryos with X-rays or neutrons and demonstrated that new aberrations were produced after the first postirradiation mitosis and expressed during the second and third mitoses (155). Pampfer and Streffer (108) also showed a significant increase in chromatid and chromosome fragments in cell cultures derived from fetal skin biopsies of mice x-irradiated at the zygote stage. Kadhim *et al.* (62) were the first to report that α-particles were also efficient inducers of delayed chromatid-

and chromosome-type aberrations in clonal descendants and nonclonal cultures of both mouse and human hematopoietic stem cells. Similar effects after x-irradiation either were not observed or were observed at greatly reduced frequencies (61, 62). Martins *et al.* demonstrated chromosomal instability in normal human fibroblasts after exposure to high-linear energy transfer (LET) particles as well (85). Other groups have reported delayed effects of exposure to low-LET radiation as manifested by cytogenetic aberrations (55, 84) in other cell systems. The dissimilarities involving the induction of chromosomal instability by radiations of differing LET may reflect the genetically predetermined susceptibility of specific cell types, or the specific assays used to measure chromosomal instability.

OTHER DELAYED EFFECTS OF IONIZING RADIATION

Delayed Reproductive Cell Death

The ultimate fate of the irradiated cell depends upon the extent to which the damaged genome is restored to its pre-irradiated condition. There is general agreement that cells survive irradiation when the progeny can form a colony of >50 cells However, an early, well-documented delayed effect of radiation exposure in the surviving progeny is the tendency toward reduced plating efficiency (136). Specifically, the progeny of irradiated cells show reduced clonogenic survival compared with unirradiated cells (21, 135, 136)

Gene Amplification

Gene amplification is a dynamic process by which specific gene regions are altered at the molecular and cytogenetic levels during cell propagation and drug selection. Amplified sequences are often detected in one of two types of chromosomal anomalies. These are paired acentric circular structures called double-minute chromosomes and expanded chromosomal regions (130). This phenomenon is frequently observed in cancer cells resistant to selective agents. For example, mammalian cells resistant to the

chemotherapeutic agent methotrexate usually contain extra copies of the dihydrofolate reductase gene (7). Similarly, cells resistant to N-(phosphonacetyl)-L-aspartate (PALA) have always shown amplification of the *CAD* gene (144). CAD is an acronym for the multifunctional protein that catalyzes the first three steps of uridine biosynthesis. Hahn and coworkers (48) demonstrated that ionizing radiation could significantly enhance the frequency of both methotrexate resistance and gene amplification in Chinese hamster ovary cells in culture.

Delayed Mutation

Ionizing radiation was first shown to be a mutagen more than 65 years ago (99). Most mutations induced directly by ionizing radiation are large deletions (126). Studies that took advantage of newly available fine-structure restriction fragment length polymorphisms of the Chinese hamster ovary hypoxanthine phosphoribosyl transferase (HPRT) gene indicated that >70% of x-ray-induced mutations were of the deletion type, with most showing total deletion of the gene (41, 102). In contrast, Little (74) has recently demonstrated that the mechanisms for delayed mutations (those arising at delayed times following X-ray exposure) are different. The great majority of delayed mutations appear to involve point mutations.

Delayed mutation has also been described in mice after whole-body γ-irradiation (133). Mammary tissue isolated from irradiated mice becomes neoplastic with time *in vitro* or *in vivo*, and multiple mutations in *p53* occur before acquisition of the neoplastic phenotype. However, these mutations in *p53* were not directly induced by radiation. Rather, *p53* mutations arose in the progeny of irradiated cells several generations following exposure (133).

RELATIONSHIP TO CHROMOSOMAL INSTABILITY

It is uncertain how these disparate delayed effects of radiation are related to chromosomal instability and each other. Chang and Little (24) have found correlations among delayed reproductive cell death, delayed mutations, and aneuploidy in CHO cells. One possibility is that one of these processes is causative of the others. For example, the manifestation of chromosomal instability can be a driving force for delayed reproductive cell death by creating novel chromosomal rearrangements which are incompatible with cell survival. Alternatively, delayed effects of radiation may be independent of one another. The potential interrelationships among various delayed effects of ionizing radiation and end points of genomic instability are investigated in Chapter 4.

DNA DOUBLE-STRAND BREAKS AS AN INITIATOR OF INSTABILITY

Although the nature of the molecular lesion that initiates genomic instability after cellular exposure to ionizing radiation is not known, two lines of evidence suggest that DSBs may be involved. Rat fibroblasts placed under anoxic conditions show induction of an endonuclease activity that cleaves DNA without specificity for sequence (139). Stoler and coworkers (139) speculate that this endonuclease activity underlies the known propensity of anoxic cells to undergo gene amplification, and that it may be associated with the break-related genomic instability of cancer cells. There is also evidence that DSBs, as well as the endogenous repair processes that recognize and rejoin DSBs, are associated with the phenotype of delayed reproductive cell death. Chang and Little (23) demonstrated that restriction endonuclease-induced DSBs led to reduced plating efficiency in the progeny of treated cells. This was not observed in mutant xrs5 cells deficient in DSB repair, suggesting that the inherent DSB repair capacity of the cell can modulate the integrity of the genome.

The notion that DNA DSBs constitute a molecular change capable of sending cells down the path toward genomic instability (23) raises a number of interesting questions.

DSBs are efficient at inducing chromosome aberrations (114), deletion mutations (41, 102, 115), and gene amplification (20), so it is reasonable to speculate that DSBs are involved in causing delayed chromosomal rearrangements. However, it is not obvious how DSBs could be manifested at delayed times. It is generally believed that both restriction endonuclease- and x-ray-induced DSBs are repaired rapidly (6, 56, 147). Cells with persistent unrepaired DSBs should trigger specific cell cycle checkpoints that abrogate further proliferation. For example, restriction endonuclease-induced DSBs have been shown to block cells in G_1 phase (103) through a process mediated by *p53*. Experiments described in Chapter 3 will investigate whether agents which produce DSBs are capable of initiating chromosomal instability.

TARGETS FOR RADIATION-INDUCED CHROMOSOMAL INSTABILITY

Delayed reproductive cell death (21) and delayed chromosomal instability (62, 84) are frequent events occurring in the progeny of irradiated cells. This indicates a large target size for the initiation of delayed chromosomal instability. Consequently, the target is probably not a single gene or gene family, and a single radiation-induced mutation is probably insufficient to induce genomic instability.

While the immediate biological effects of radiation, such as cell killing, chromosomal aberrations and mutations, are largely mediated by damage to a nuclear target, delayed effects may involve extranuclear targets. Delayed reproductive cell death can be initiated in cells by a variety of treatments, such as incubation with conditioned medium from irradiated cells or with cadmium salts, in the apparent absence of DNA damage (79, 134). Radiation-induced minisatellite instability can be enhanced by factors *in vivo* (110), and the survival of human epithelial cells after irradiation is affected by the status of gap junction intercellular communication (98). Moreover, damage to the plasma membrane of mouse embryos from treatment with Streptolysin-O can induce chromosomal aberrations at delayed times (158). These observations suggest extracellular signaling

molecules, acting through the plasma membrane, can affect the cellular response to radiation. It has also been observed that the frequency of radiation-induced chromosomal instability and the frequency of sister chromatid exchanges from high-LET particles are higher than the frequency of cells that had survived and received at least one particle through the nucleus (35, 62, 83, 101). This implicates damage to extra-nuclear targets as important for the manifestation of chromosomal instability and sister chromatid exchanges. Experiments performed in Chapter 2 will investigate whether damage to nuclear and/or extranuclear targets is sufficient to initiate chromosomal instabiliy.

PERPETUATING CHROMOSOMAL INSTABILITY

Once radiation damage has initiated chromosomal instability in a particular cell, there must be mechanisms by which this phenotype is maintained in subsequent generations. These mechanisms are termed perpetuating events. If double-strand breaks are initiators for chromosomal instability, then dicentric chromosomes may serve as a perpetuating event. In normal cells, the formation of dicentric chromosomes is generally a lethal event (19), and the frequency of dicentric chromosomes declines during thc first few divisions following ionizing radiation (85). Hence, it is of interest that several groups have observed dicentric chromosomes 15-30 generations after exposure to ionizing radiation (85, 93). It is improbable that these dicentrics seen at delayed times are a direct consequence of radiation; rather, it appears that they arise *de novo* during the clonal expansion of irradiated cells.

The potential fate of a dicentric chromosome is shown in Figure 1.2. If both centromeres of the dicentric chromosome are recognized by the mitotic spindle apparatus, then 50% of the time the two centromeres on the same sister chromatid will be pulled to the same pole (Figure 1.2A). Each daughter will inherit a dicentric chromosome, and the cycle will repeat itself the next cell division. Also, there is a 50% probability that the two centromeres will be pulled towards opposite poles during mitosis (Figure 1.2B). This will

cause the dicentric chromosome to "bridge" at anaphase. Assuming the connection between the spindle and the centromere is maintained, the chromosome can break at a point between the two centromeres during chromosomal segregation (86). Consequently, two broken chromatids are passed to each daughter cell, which can have markedly different fates depending on how they are rejoined. The broken chromatids can heal, forming stable ends, fuse with other broken chromosomes, forming novel dicentrics, or replicate, then fuse, forming sister unions. Either of these later outcomes can lead to dicentric chromosomes in future cell cycles, thereby establishing repeated cycles of bridge-breakage-refusion (BBR). There is substantial evidence that BBR cycles are a major mechanism of gene amplification in Chinese hamster ovary cells (80) and there is evidence that BBR cycles contribute to chromosomal instability in multiple cell types as well (45, 93)

INTERSTITIAL REPEAT SEQUENCES CAN DRIVE BBR

Cytogenetic rearrangements in chromosomally unstable cells, including dicentrics, do not necessarily occur at random. GM10115 cells contain 14 cytogenetically visible blocks of telomere repeat-like sequences (TTAGGG), or interstitial telomere bands (ITBs) (Figure 1.3A). Recombination juxtaposing hamster and human chromatin at an ITB is seen as a "three color junction" using two color fluorescence *in situ* hybridization (FISH), where the one probe (red) hybridizes to the human chromosome, a second probe (green) hybridizes to ITBs, and the hamster chromosomes are counterstained with DAPI (blue) (Figure 1.3B). After exposing cells to X-rays, these three color junctions are observed 4-5 fold more frequently than expected from a random formation and association of broken ends (32). Taking chromosomal damage from x-rays to be random, recombination must occur preferentially at these ITBs. It is unclear whether ITBs are unique hotspots, or if any tandem repeat sequence is generally hyperrecombinogenic. Alternatively, there may be a difference between heterochromatin and euchromatin in terms of repair or recombination frequencies. Non-random recombination has also been seen in chromosomes 1, 13, and

16 in human fibroblasts at delayed times after exposure to heavy ions (85). There is, however, also evidence for random chromosome breakage in chromosomally unstable cells (6). Sites of increased chromosome recombination, like ITBs, can increase the rate of chromosomal rearrangements, thereby perpetuating chromosomal instability. Moreover, if these rearrangements create dicentrics, then ITBs could induce cycles of BBR as well.

GENETIC AND EPIGENETIC FACTORS

It is likely that the perpetuation of chromosomal instability will have some component of genetic control; i.e. the nature of the instability phenotype will depend on cell type. For example, while chromosomal rearrangements seen in GM10115 cells consist mostly of the chromosome type (71, 84), chromatid aberrations are seen frequently in other systems (62, 116). Also, the genetic controls responsible for chromosomal instability have been linked to the genetic controls responsible for tumor formation. After γ-irradiation of primary mouse mammary epithelial cells derived from mice either normal or hypersensitive to radiation-induced mammary cancer, only the cells derived from the hypersensitive mice manifested chromosomal instability (116).

Epigenetic factors, too, can play a role in genomic instability. Mouse C3H/10T$^1/_2$ cells, after *in vitro* transformation with X-rays, showed far higher frequencies of genomic rearrangements after forming tumors in C3H mice than simply being passaged *in vitro* (110). These experiments further support the idea that genomic instability, and in particular chromosomal instability, is important in carcinogenesis.

EXPERIMENTAL PLAN FOR THIS THESIS

The goal of this thesis is to better determine the target(s) for and the biological consequences of chromosomal instability. To investigate whether the chromosomal instability is initiated by damage to either a nuclear and/or an extranuclear target, ^{125}I-

labeled compounds were incorporated into specific regions of the cell. ^{125}I, a radioactive isotope of iodine, releases 93% of its decay energy within a 40 nm sphere (53, 128). Hence, localizing ^{125}I-labeled compounds within the cell will result in irradiation of selective subcellular regions. Treating cells with ^{125}I-iododeoxyuridine (^{125}IdU) will irradiate the DNA and associated nuclear structures, while treating cells with an ^{125}I-succinyl-concanavalin A (^{125}I-suc-con A), a lectin, will irradiate extranuclear regions. We then investigated whether clones surviving these treatments manifested chromosomal instability. These experiments are described in Chapter 2.

We tested the hypothesis that DSBs could initiate chromosomal instability. ^{125}IdU, when incorporated into DNA, causes an average of one DSB per decay (26). Therefore, experiments with ^{125}I-iododeoxyuridine described in Chapter 2 also test the hypothesis that ^{125}I-iododeoxyuridine-induced DSBs are sufficient for the induction of chromosomal instability. In order to determine whether the quality of the DNA strand break affected the initiation of chromosomal instability, cells were treated with a variety of DNA strand breaking agents: X-rays, restriction endonucleases (REs), hydrogen peroxide, and the radiomimetic drugs Neocarzinostatin (NCS) and bleomycin (BLM). The damage produced by these agents ranges from single strand breaks (hydrogen peroxide), to simple DSBs (REs) which do not have accompanying base damage, to complex DSBs (X-rays, NCS, BLM) which may have chemically modified 3' and/or 5' termini. We then asked whether chromosomal instability was observed in clones surviving these treatments. These experiments are described in Chapter 3.

We tested the hypothesis that there were relationships between chromosomal instability and other manifestations of genomic instability. As a result of experiments performed in Chapter 3, we generated many stable and unstable clones which had all been exposed to 10 Gy of X-rays. A battery of both stable and unstable clones were analyzed for a variety of other end points of genomic instability: delayed mutations, delayed

reproductive cell death, sister chromatid exchange, gene amplification, and the presence of functional mismatch repair. We then asked whether there was a significant difference between the chromosomally stable and unstable clones at each of these end points. These experiments are described in Chapter 4.

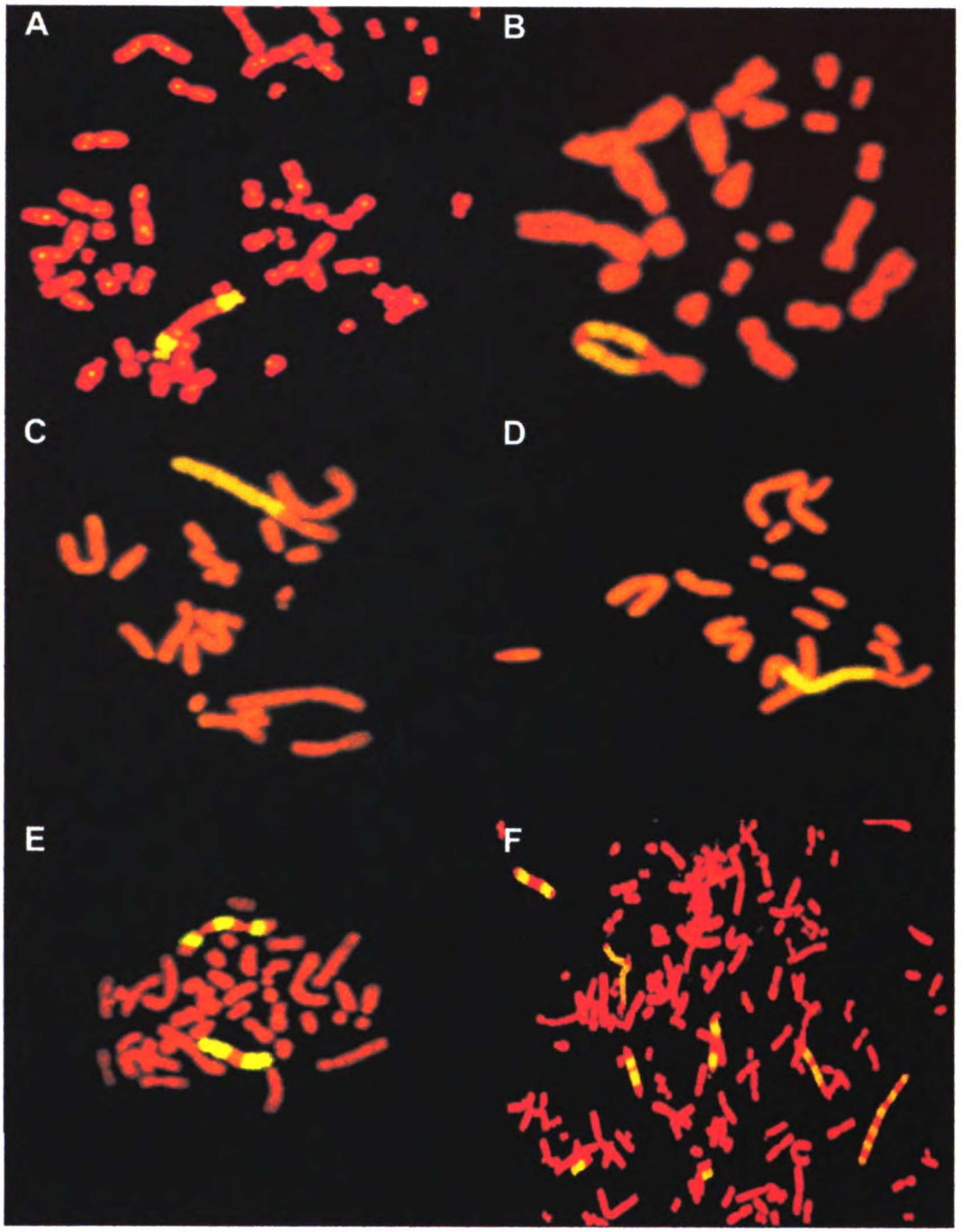

Figure 1.1. Metaphase chromosomes from chromosomally unstable GM10115 cells that contain rearrangements involving human (yellow) and hamster (red/orange) chromosomes. The rearranged chromosomes involve a symmetrical dicentric (A), a sister union (B), amplification of the human chromatin in one band (C,D), a symmetrical dicentric and amplification of multiple bands (E), and an amplification of multiple bands on multiple chromosomes in a giant cell (F).

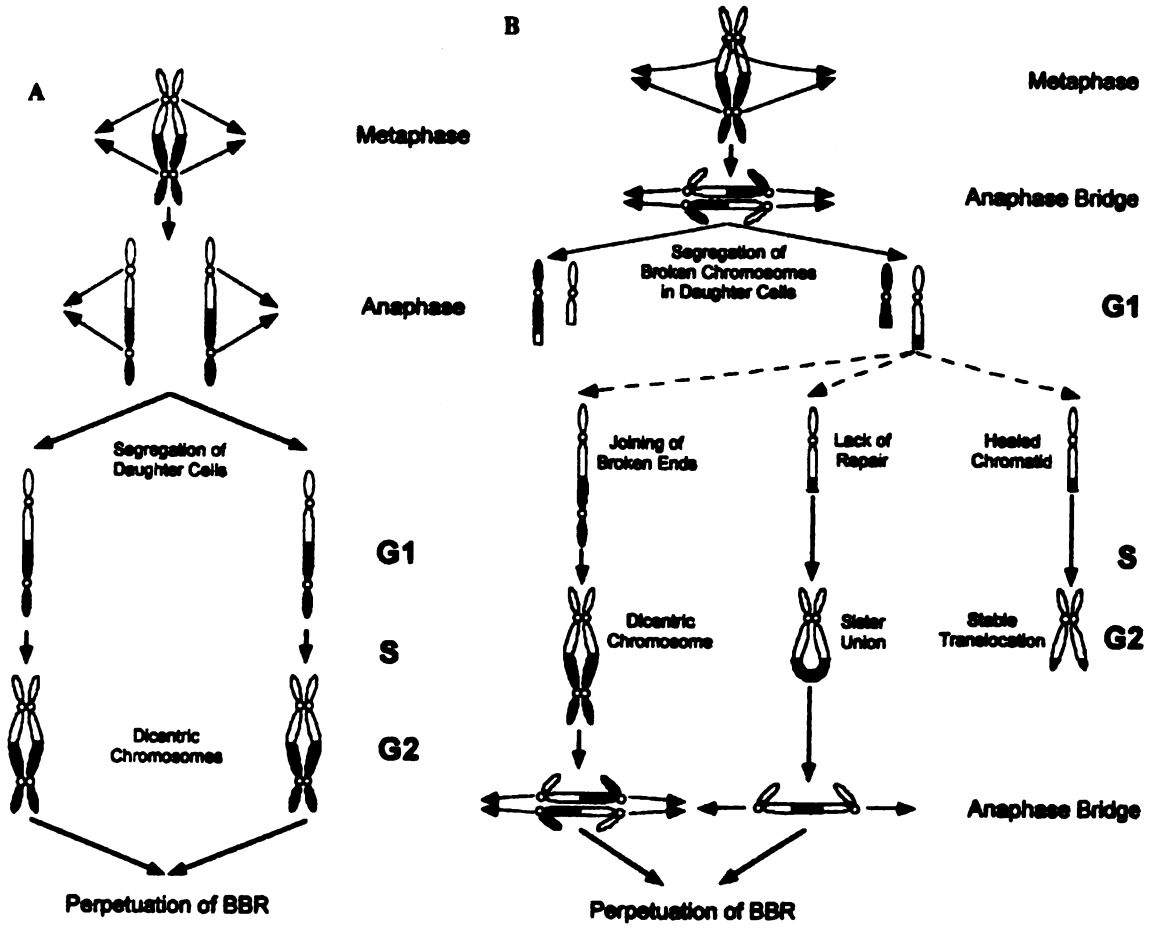

Figure 1.2. Possible fates of a dicentric chromosome at mitosis. A) Both centromeres on the same chromatid are attached to the same spindle pole. Each daughter cell will receive a dicentric chromatid, and, at the next mitosis, each daughter begins the bridge-breakage-refusion cycle anew. B) Centromeres on the same chromatid are attached to opposite poles. this creates an anaphase bridge, thereby breaking the chromosomes at a point between the centromeres. the possible fates of the broken chromosomes include 1) a new dicentric, 2) a sister union after replication, and 3) formation of a stable translocation. Outcomes 1 and 2 perpetuate BBR.

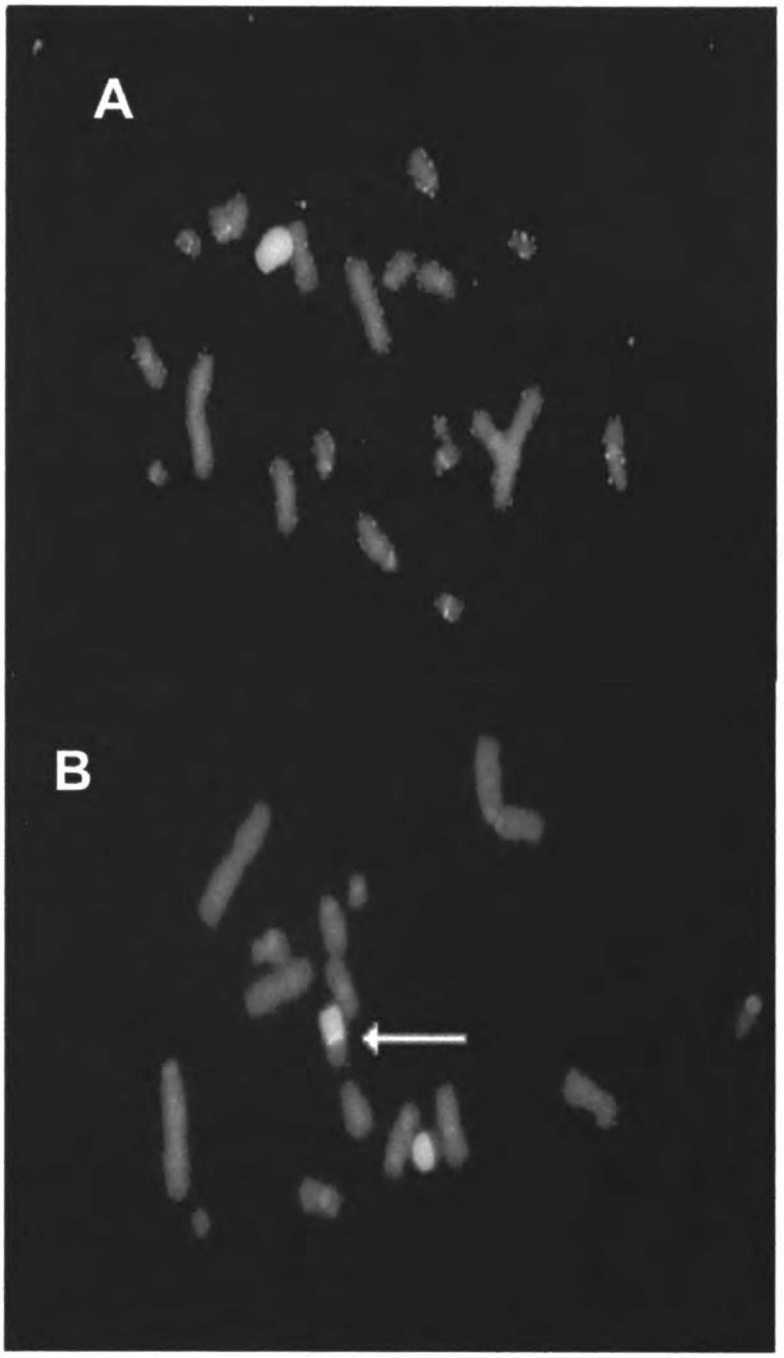

Figure 1.3. Three-color junctions in GM10115 cells. A) GM10115 metaphase chromosomes showing the human chromosome (red), hamster chromosomes (blue), and ITBs (green). B) Metaphase chromosomes from unstable GM10115 cells showing a three-color junction.

A
B

Chapter 2 The Nucleus is the Target for Radiation-Induced Chromosomal Instability

SUMMARY

We have previously described chromosomal instability in GM10115 human-hamster hybrid cells after exposure to X-rays. Chromosomal instability in these cells is characterized by the appearance of novel chromosomal rearrangements multiple generations after exposure to ionizing radiation. To identify the cellular target(s) for radiation-induced chromosomal instability, cells were treated with ^{125}I-labeled compounds and frozen. Radioactive decays from ^{125}I cause radiation damage to the cell primarily at the site of its decay, and freezing cells allows damage to accumulate in the absence of other cellular processes. We found that ^{125}IdU, which incorporates into the DNA, caused chromosomal instability. While cell killing and first-division chromosomal rearrangements increased with increasing numbers of ^{125}I decays, the frequency of chromosomal instability was independent of dose. Chromosomal instability could also be induced from incorporation of ^{125}IdU without freezing cells to accumulate decays. This indicates that DNA double-strand breaks in frozen cells resulting from ^{125}I decays failed to lead to instability. Incorporation of an ^{125}I-labeled protein (^{125}I-suc-con A), which was internalized into the cell and/or bound to the plasma membrane, neither caused chromosomal instability nor potentiated ^{125}IdU-induced chromosomal instability. These results show the target for radiation-induced chromosomal instability in these cells is the nucleus. However, radiation-induced double-strand breaks are not sufficient for the manifestation of chromosomal instability.

INTRODUCTION

The phenotype of cancer includes a host of alterations from the normal state. Genomic instability, broadly taken to describe the increased rate of change to the genome,

may be a driving force for the multiple genetic alterations necessary for carcinogenesis (76, 104). Ways in which genomic instability can be manifested include gene amplification, increased mutation rates, microsatellite instability, and chromosomal instability (93). Understanding how these disparate processes individually and collectively contribute to the carcinogenic phenotype remains a daunting task. An important first step is to identify both specific genes and carcinogenic agents, such as radiation, which can induce particular endpoints of genomic instability in model systems.

Ionizing radiation can induce delayed chromosomal instability, phenotypically characterized as a population of clonal cells showing multiple, distinct chromosomal alterations at delayed times following radiation exposure (45, 55, 62, 83-85, 116). Many mechanisms have been proposed for how radiation exposure can give rise to novel chromosomal rearrangements multiple generations post-irradiation, including BBR cycles (63), recombination involving hyperrecombinogenic sites such as interstitial telomere-like repeat sequence (32), activation of signal transduction pathways (93) and oxidative stress (29). Indeed, there are likely to be multiple pathways for initiating (72) and perpetuating (63) genomic instability, and the relative contribution of different pathways may depend on the cell's genetic background (61, 116).

Chromosomal instability, like other delayed effects of ionizing radiation such as delayed reproductive cell death (21), increased mutation frequency (24) and transformation (67), is a frequent event occurring in the progeny of irradiated cells (62, 84). This indicates a large target size for the initiation of delayed chromosomal instability. Consequently, the target is probably not a single gene or gene family, and a single radiation-induced mutation is probably insufficient to induce genomic instability. While directly-induced effects of radiation, such as cell killing, chromosomal aberrations, and mutations, are thought to involve damage to a nuclear target, delayed chromosomal instability may involve extranuclear targets. It has been observed that the frequency of radiation-induced chromosomal instability by high-LET particles is higher than the

frequency of cells that had survived and received at least one particle through the nucleus (62, 83).

In studies to date on radiation-induced chromosomal instability, all cellular regions were indiscriminately irradiated, thereby making the determination of precise cellular targets for radiation-induced chromosomal instability impossible. To positively identify cellular targets, one approach is to assay for chromosomal instability after specific subcellular regions have been irradiated. Here, we treated cells with different ^{125}I-labeled compounds and then froze the cells to accumulate damage from ^{125}I decays in the absence of other cellular processes, specifically, repair and growth. ^{125}I releases 93% of its decay energy within 40 nm of the decay site (53, 128), so localization of ^{125}I-labeled compounds within the cell can in turn cause radiation damage to specific intracellular regions. Cells were treated with ^{125}IdU to selectively irradiate the DNA and associated nuclear structures, and with an ^{125}I-labeled lectiin (^{125}I-suc-con A) to selectively irradiate the cytoplasm and/or the plasma membrane. We then investigated whether these treatments could induce delayed chromosomal instability.

MATERIALS AND METHODS

Cell Culture

GM10115 human-hamster hybrid cells (CHO cells with a single copy of human chromosome four) were obtained from the Human Genetic Mutant Cell Repository (line HHW416; Institute for Medical Research, Camden, N.J.). Cells were maintained as monolayers in tissue culture flasks (Corning) in a humidified atmosphere of 5% CO_2 in air at 34°C. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin, 100 μg/ml of streptomycin, 2 mM L-glutamine, 0.2 mM L-proline, 1.5 μg/ml amphotericin B (GIBCO) and 1.5 μg/ml deoxycholic acid (GIBCO). In some cases, dialyzed FBS was used in place of FBS.

DNA labeling

Four hundred thousand cells were seeded into either 75 or 150 cm^2 tissue culture flasks in medium containing dialyzed FBS. Either two (for 75 cm^2 flasks) or three (for 150 cm^2 flasks) days later, the growth medium was replaced with new medium containing dialyzed FBS, 0.010-0.015 μCi/ml ^{125}IdU (ICN, specific activity=2000 Ci/mmol), and 10^{-6} M thymidine as a cold carrier. After 33 hours (approximately 1.5 population doublings), cells were rinsed twice with medium containing dialyzed FBS and incubated for another two hours in medium containing dialyzed FBS and 10^{-5} M thymidine. Cells were still in exponential growth at the end of this labeling period. Cells were harvested by trypsinization. Control cells were treated identically except no ^{125}IdU was added.

Determination of ^{125}I incorporation

After labeling, cell number was determined using a Coulter counter. ^{125}I incorporation per cell was determined using a Packard Model 5002 Cobra Auto-Gamma gamma counting machine. The dch_O (decays/cell/hour of ^{125}I, at the time of measurement) ranged from 0.5-0.9 for labeling with ^{125}IdU. Precipitating ^{125}IdU labeled cells with cold 10% trichloroacetic acid onto nitrocellulose filters demonstrated over 95% of the radioactivity was incorporated into genomic DNA.

Dose accumulation.

^{125}I decays were accumulated by freezing the cells according to the method of Hofer et al. (54). Briefly, cells were suspended in cryoprotective medium (15% FBS and 10% dimethylsulfoxide (DMSO) in DMEM) and placed in cryovials (Corning). The cryovials were immersed in a room temperature ethanol bath. The ethanol bath was placed in a -70°C Revco freezer for one hour, and subsequently the cryovials were transferred to storage in liquid nitrogen until the desired number of ^{125}I decays had accrued.

DNA labeling in absence of cell freezing

Labeling cells without freezing to accumulate decays was performed as described above, except that three different concentrations of ^{125}IdU were used. Cells were labeled with "low" (0.01 μCi/ml), "medium" (0.10 μCi/ml), or "high" (0.20 μCi/ml) levels of ^{125}IdU. Determination of ^{125}I incorporation was performed as described above.

X-irradiation of frozen cells

Cells in exponential growth were harvested by trypsinization, resuspended in cryoprotective medium, and frozen as described above. For irradiation, cryovials were removed from liquid nitrogen and placed into a dry ice/ethanol bath. Cells were irradiated with x-rays (dose rate=2.5 Gy/min) using a Philips RT100 X-ray machine (250-kV peak, 15 mA; half-value layer 1.0 mm Cu) while in the bath, and after irradiation cells were stored at -70°C for one hour. There was no evidence that cells had thawed during this procedure.

Plasma membrane labeling

Succinyl-concanavalin A (suc-con A) is a non-aggregating form of concanavalin A (con A) obtained by treating con A with succinic anhydride (46). We found that with GM10115 cells, as in other cell types (46), suc-con A causes minimal aggregation (data not shown). Suc-con A was purchased from Sigma and labeled by Dupont New England Nuclear with ^{125}I by a modified chloramine T technique (specific activity=96 μCi/μg).

Cells in exponential growth were harvested with versene (150 mM NaCl, 40 mM Tris, pH 7.5, 1 mM EDTA) because trypsinization has been shown to increase cellular agglutinability by lectins (82). Cells were rinsed once in 4°C serum free media (SFM) or phosphate buffered saline (PBS) and incubated in 10-40 μCi/ml of ^{125}I-suc-con A in either SFM or PBS for 90-120 minutes at 4°C. After labeling, cells were rinsed three times in cold SFM. Determination of ^{125}I incorporation and cell freezing were performed as

described above, except that the ethanol bath was initially at 4°C. The dch_O ranged from 4-41. Autoradiography (Amersham LM-1 emulsion) was performed to determine the percentage of labeled cells, and fluorescence imaging of cells labeled with fluorescein isothiocyanate (FITC) conjugated suc-con A (Sigma) was performed to confirm the localization of suc-con A after this treatment. Control cells were treated identically, except suc-con A was added instead of ^{125}I-suc-con A.

Cytoplasm labeling

Con A can be internalized into the cytoplasm by incubating cells with con A at 37°C (106). Labeling the cytoplasm with ^{125}I-suc-con A was accomplished in a manner similar to that for labeling the plasma membrane, except cells were incubated at 37°C rather than 4°C. Rinses with SFM or PBS were performed at ambient temperature instead of at 4°C. The dch_O ranged from 2-10. Autoradiography and FITC conjugated suc-con A labeling were also performed.

Combined labeling

Labeling of both DNA and the cytoplasm and/or plasma membrane was done by first labeling the DNA and subsequently labeling the plasma membrane or cytoplasm by the aforementioned protocols.

Calculating decays occurring in frozen cells

If dch_O is the decay rate (decays/cell/hour) measured before the cells are frozen, then while the cells are frozen the decay rate drops by the equation $dch_T = (dch_O) * e^{-\lambda T}$, where dch_T is the decays/cell/hour for cells which have been in liquid nitrogen for T hours, and $\lambda = \ln 2/(^{125}\text{I half-life}) = .693/(59.4 \text{ days}) = 4.86 * 10^{-4} \text{ hours}^{-1}$. Hence, the total number of decays occurring while the cells are frozen equals

$$\int_0^T dch_o * e^{-\lambda t}\, dt = (-1/\lambda) * dch_o * e^{-\lambda t}\Big|_{t=0}^{t=T} = 2058 * dch_o * (1-e^{-0.000486T})$$

Cell Survival

Cell survival was determined by the colony forming assay (CFA). Any frozen cells were rapidly thawed in a 37°C water bath, added to nine ml of growth medium, pelleted, and resuspended in fresh medium. Cells were plated in triplicate at two different concentrations in 100 mm Petri dishes (Falcon): a low concentration to yield ~20 colonies/dish to facilitate selection of colonies for clonal analysis of chromosomal instability (see below) and a higher concentration to yield ~80-100 colonies/dish to decrease the standard deviation for the surviving fraction. The dishes were incubated at 34°C for 12-14 days. Five to ten colonies per plate were removed from the low concentration dishes with a sterile trypsin-soaked cotton swab, and each colony was expanded individually in a 25 cm^2 flask. All dishes were fixed and stained with 0.1% crystal violet in 25% ethanol. Control cells for ^{125}IdU experiments had plating efficiencies between 80-90%, and control cells for ^{125}I-suc-con A experiments had plating efficiencies between 50-75%, irrespective of time spent in liquid nitrogen. Cells not used for the CFA were added to a 75 cm^2 flask, and metaphase cells were collected the following day for the analysis of directly induced chromosomal rearrangements.

Collection of metaphase cells

Metaphase cells, from both clonally expanded and non-clonal populations, were accumulated by a three hour incubation with colcemid ($2x10^{-7}$ M) and collected after shaking the flask to dislodge the mitotic cells. Cells were subsequently swollen in a hypotonic 0.075 M KCl solution for 15 minutes at 37°C, dehydrated in methanol, and fixed in a three to one mixture of methanol to acetic acid. Mitotic cells were dropped onto

glass microscope slides and dried at ambient temperature for at least two days before storage at -20°C.

Fluorescence in situ hybridization (FISH)

Cytogenetic analysis of rearrangements seen at the first mitosis and at delayed times following irradiation involved FISH with a biotinylated probe to human chromosome four (HC#4). The generation of this probe has been described previously (84). Briefly, bluescript plasmids containing HC#4 DNA sequences were nick translated with a nucleotide mix including biotinylated dUTP (GIBCO) for one hour at 16°C. Products were purified from unincorporated nucleotides with a Nuctrap purification column (Stratagene).

Glass slides with metaphase chromosomes were washed twice in 2x SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) for 15 minutes and dehydrated with two minute washes in 70, 90, then 100% ethanol. After drying on a slide warmer, slides were incubated in 70% formamide/2x SSC for three minutes at 70°C to denature the chromosomes, and then dehydrated again with the same ethanol series. After drying at ambient temperature, 35 µl of hybridization mix (70% formamide, 15% dextran sulfate, 2x SSC, and 50 ng labeled probe) was added to each slide. The slides were covered with a glass coverslip, sealed with rubber cement, and incubated for two to five days in a humidified incubator at 37°C.

After hybridization, slides were rinsed three times in phosphate-buffered detergent (PBD: 0.1 M sodium phosphate, pH 8.0, 0.5% NP-40 detergent), once briefly and twice for five minutes. To detect the biotinylated probe, slides were incubated with 40 µl of FITC conjugtated avidin (Oncor) for 15 minutes while covered with plastic coverslips, and slides were washed again three times with PBD. To amplify the signal, slides were incubated for 15 minutes with 40 µl of an anti-avidin antibody (Oncor), washed three times in PBD and incubated with 40 µl of FITC-avidin a second time. After three final washes in

PBD, 18 μl of propidium iodide in Antifade (Oncor) was added to counterstain the chromosomes, and the slides were covered with a glass coverslip.

Cytogenetic analysis

After FISH, metaphase chromosomes were visualized with a Zeiss Axioskop microscope equipped with a dual-band pass FITC/Texas Red filter set. With this system, hamster chromosomes appear red, and HC#4 appears yellow-green. We scored chromosomal rearrangements between HC#4 and hamster chromosomes according to the system of Tucker et al. (143). We operationally define a clone as chromosomally unstable if, after scoring 200 metaphase cells, we find three or more abnormal subpopulations comprising at least 5% of the total metaphases; one subpopulation represents all cells with the same chromosomal rearrangement of HC#4.

RESULTS

DNA labeling and cell freezing

To investigate the hypothesis that irradiating the nucleus could induce delayed chromosomal instability, ^{125}IdU was incorporated into the DNA and the cells were frozen to accumulate ^{125}I decays. Figure 2.1 shows the survival of ^{125}IdU labeled cells that had been exposed to different numbers of ^{125}I decays by keeping the cells frozen for various lengths of time (two days to seven weeks). Cell survival was normalized to that of cells which were labeled with ^{125}IdU and kept in liquid nitrogen for one hour. In agreement with previous studies in CHO cells (54), we found ^{125}IdU incorporated into the DNA was cytotoxic. The best fit of a single exponential curve through the data yields a D_O of 40 decays.

As another end point of ^{125}IdU induced damage, chromosomal rearrangements involving HC#4 were scored at the first mitosis following thawing (Figure 2.2). For ease of analysis, cells were placed into groups exposed to approximately 50, 100, 200, and 300

decays of ^{125}I. There was a dose-response between the number of decays and the percentage of chromosome rearrangements observed at the first mitosis following thawing.

Clones derived from cells exposed to decays from ^{125}IdU were expanded to approximately two million cells (about twenty generations) and analyzed for chromosomal instability. In total, 167 clones were investigated at six different numbers of decays (25, 50, 100, 200, 300, 400). We found chromosomal instability was induced at all six doses by ^{125}IdU at frequencies ranging from 3-9%. The cytogenetic characterization of these clones is summarized in Table 2.1A. Clones are classified as having zero subpopulations (no rearrangements detected in 200 metaphases), one or two subpopulations (rearrangements were detected, but the clone is still chromosomally stable according to our definition), or unstable if there were three or more subpopulations comprising $\geq$5% of the cells analyzed. It is noteworthy that there is no significant difference in the frequency of instability among all the doses (chi-square test, $p>0.5$), suggesting a lack of a dose-response for chromosomal instability induced by ^{125}IdU. No chromosomal instability was observed in 20 control clones, nor has chromosomal instability ever been observed in any of at least 150 unirradiated control clones over several years.

When colonies were picked for these experiments, we noted the size of the colony as either large or small relative to other colonies on the same dish. We found no correlation between the initial size of the colony and the eventual onset of chromosomal instability (data not shown).

DNA labeling in the absence of cell freezing

Since increasing numbers of ^{125}IdU decays in frozen cells do not significantly increase the frequency of chromosomal instability, the chromosomally unstable clones we observed may have resulted from ^{125}IdU decays that occurred during either the 33 hour labeling period or the post-thawing period. Furthermore, we hypothesized increasing the number of decays that occurred during the labeling period would increase the frequency of

instability. Hence, we labeled cells with three different levels of ^{125}IdU: 0.010 μCi/ml ("low"), equivalent to the level used in the initial experiments, 0.10 μCi/ml ("medium"), and 0.20 μCi/ml ("high"). The cells were not frozen to accumulate decays. Increasing the level of ^{125}IdU did increase the amount of ^{125}IdU incorporated (the dch_O) but not in direct proportion.

Figure 2.3 shows the cell kill resulting from this treatment. Higher levels of incorporated ^{125}IdU resulted in higher levels of cell kill. The best fit of a single exponential curve through the data yields a D_O of 1.6 dch_O, which is similar to that found by Schneiderman and Schneiderman (131). Figure 2.4 shows the frequency of directly induced chromosomal aberrations in labeled, unfrozen cells. Again, there is a dose-response for the induction of rearrangements involving HC#4 in the first mitosis following labeling. Figure 2.4 also shows the data from Figure 2.2 (directly induced chromosomal aberrations in labeled, frozen cells); the two x-axes in Figure 2.4 were scaled so equivalent distances represented equivalent levels of cell kill (1.6 dch_O = 40 decays). Hence, ^{125}IdU produces similar levels of chromosomal rearrangements in both frozen and log phase cells at equivalent levels of cell killing.

Table 2.1B shows the cytogenetic data from clones expanded from cells treated with low, medium, and high levels of ^{125}IdU. The frequency of chromosomal instability was 3% (1/37), 8% (2/26), and 0% (0/32) in cells after low, medium, and high levels of labeling, respectively. Thus, the frequency of instability induced by low levels of labeling can account for the instability seen in cells which were frozen to accumulate ^{125}I decays. Higher levels of ^{125}IdU incorporation from medium and high levels of labeling did not yield significantly higher frequencies of chromosomal instability (chi-square test, $0.25>p>0.1$).

X-irradiation of frozen cells

A possible reason decays from ^{125}IdU occurring while the cells were frozen did not contribute to chromosomal instability is that frozen cells are resistant to chromosomal instability. Since it has been determined that X-rays can induce chromosomal instability in GM10115 cells (71, 84), we irradiated cells with 25 Gy of X-rays while cells were frozen. This resulted in a mean surviving fraction of 0.0030 (SD=0.002). This is approximately the same level of cell killing as from 10 Gy to cells in media at ambient temperature (0.00127) (84). The enhancement of cell survival under freezing conditions is presumably from the combination of 10% DMSO in the cryoprotective medium and the fact that the cells were frozen. The percentage of rearrangements in first-division metaphases involving HC#4 was 40% (174/440).

We found chromosomal instability in 5% (2/40) of the clones exposed to 25 Gy of X-rays while frozen (Table 2.1C). While frozen cells are not completely refractory to radiation-induced chromosomal instability, the frequency of chromosomal instability is significantly lower than the 33% (50/152) (71) seen with 10 Gy of X-rays to cells in medium (z-test, $p<0.001$)

Plasma membrane and cytoplasm labeling

To test the hypothesis that irradiating an extranuclear target could induce chromosomal instability, we incorporated ^{125}I-suc-con A into cells at either 4°C or 37°C in order to irradiate either the plasma membrane or both the plasma membrane and cytoplasm, respectively. In addition to measuring uptake of ^{125}I-suc-con A with a gamma counter, we used autoradiography to determine the percentage of cells that were labeled with ^{125}I-suc-con A. We found all cells treated with ^{125}I-suc-con A at both temperatures were labeled (200/200 for both conditions, Figure 2.5A). Treating cells with suc-con A at 4°C will result in exclusive labeling of the plasma membrane, while labeling cells at 37°C will cause suc-con A to be incorporated into the cytoplasm via endocytosis (106). The

intracellular localization of suc-con A was verified by labeling cells with FITC-suc-con A at either 4°C or 37°C. We found cells labeled at 4°C showed primarily labeling of the plasma membrane, as evidenced by the fine halos of light encircling the cells (Figure 2.5B). In contrast, cells treated with FITC-suc-con A at 37°C were labeled both on plasma membrane and in the cytoplasm, as evidenced by a strong intracellular signal in addition to the halos around the cells (Figure 2.5C).

As with ^{125}IdU labeled cells, cells labeled with ^{125}I-suc-con A were frozen to accumulate decays. Cells labeled at 4°C were subjected to a range of doses, from 100 to 8500 decays, the approximate dose equivalent of 2.6 to 221 Gy to the plasma membrane (153). Cells labeled at 37°C were exposed to 4645 decays. No cell killing was observed at any of the doses used, consistent with previous studies showing the plasma membrane and cytoplasm are resistant to cell killing by Auger emitters (52, 153). Only background levels of directly-induced chromosomal rearrangements were found in ^{125}I-suc-con A labeled cells: three rearrangements involving HC#4 in 1914 metaphases analyzed. This frequency is comparable to that seen in unirradiated cells (84).

We investigated chromosomal instability in plasma membrane labeled clones. None of the 75 clones labeled at 4°C nor any of the 31 clones labeled at 37°C were found to be chromosomally unstable (Tables 2.1D,E).

DNA and either plasma membrane or cytoplasm labeling

Although extranuclear ^{125}I decays in and of themselves failed to induce chromosomal instability, we tested the hypothesis that irradiating both extranuclear and nuclear targets can yield higher frequencies of chromosomal instability over irradiation of nuclear targets alone. Cells were first labeled with ^{125}IdU and then with ^{125}I-suc-con A, either at 4°C and at 37°C. We investigated chromosomal instability in cells that had sustained 202 decays resulting from incorporated ^{125}IdU and either 2447 decays from ^{125}I-suc-con A incorporated at 4°C or 930 decays from ^{125}I-suc-con A incorporated at

37°C. We investigated cells sustaining approximately 200 decays to the nucleus because that dose would result in significant biological effects, such as cell killing (see Figure 2.1).

The surviving fractions of ^{125}IdU/^{125}I-suc-con A cells were 0.025 for DNA/plasma membrane labeling and 0.029 for DNA/cytoplasmic labeling. These levels of cell killing are comparable to that seen with ^{125}IdU alone; hence, non-nuclear decays do not appear to enhance cell killing. The frequency of chromosomal instability after these treatments was 4% (1/26) for ^{125}IdU/^{125}I-suc-con A labeling at 4°C and 0% (0/25) for labeling at 37°C (Tables 2.1F,G). As with cell killing, decays occurring from just the ^{125}IdU can account for chromosomal instability in these dually labeled clones. Extranuclear damage from ^{125}I-suc-con A does not augment the frequency of chromosomal instability.

DISCUSSION

Chromosomal instability has been seen in a variety of mammalian cell lines after treatment with ionizing radiation. Irradiation with X-rays or with high linear energy transfer (LET) particles, however, indiscriminately damages the entire cell. Consequently, it has proved difficult from these studies to elucidate the nature of the cellular target responsible for chromosomal instability. Treating cells with ^{125}I-labeled compounds assures that the majority of radiation damage from ^{125}I decays to the cell will be localized to sites of ^{125}I incorporation. Labeling cells with ^{125}IdU will selectively damage the DNA and any associated nuclear structures. Labeling cells with ^{125}I-suc-con A will damage either the plasma membrane or the plasma membrane and cytoplasm, depending on whether labeling occurs at 4°C or 37°C. Damage from ^{125}I decays was often accumulated in the absence of other cellular processes by freezing the cells. This procedure mimics an acute radiation exposure, for when the cells are thawed the cells must contend with all the damage at once.

Our data argues strongly that radiation damage to the nucleus, and not to the plasma membrane nor the cytoplasm, is responsible for chromosomal instability in GM10115 cells. This is consistent with results from our laboratory showing that incorporation of bromodeoxyuridine (BrdU) into DNA can increase the frequency of chromosomal instability after X-rays (C. L. Limoli and co-workers, manuscript in preparation). Similarly, work by Grosovsky *et al.* has shown that incorporation of 6-thioguanine in TK6 cells increases the frequency of complex chromosomal rearrangements seen at delayed times following x-irradiation (45). Moreover, chromosomal instability in GM10115 cells is induced after either a photolysis treatment of BrdU, Hoechst 33258, and UV-A light, or after exposure to the radiomimetic drugs NCS or BLM (71, 73). Both of these treatments primarily, if not exclusively, damage DNA.

There were two intriguing aspects of ^{125}IdU induced chromosomal instability. One was that ^{125}IdU was not a strong inducer of chromosomal instability. When incorporated into DNA, decays from ^{125}IdU produce damage reminiscent of that produced by high LET radiation (53) and lead to DNA DSBs (57). Previous work in our laboratory had demonstrated that agents capable of producing complex DNA DSBs could cause chromosomal instability in GM10115 cells (71). Moreover, chromosomal instability in hematopoetic stem cells is induced by high LET radiation but not low LET radiation (62). Therefore, we reasoned that ^{125}IdU would be an effective inducer of chromosomal instability, possibly more effective than X-rays. In fact, ^{125}IdU induced chromosomal instability in fewer than 10% of clones at all doses investigated. This contrasts with the 33% frequency of instability observed after 10 Gy of X-rays, which produces the same level of cell killing as about 300 decays of ^{125}IdU. The other intriguing aspect was that cell kill and directly-induced chromosomal rearrangements from ^{125}IdU showed clear dose-responses, yet there was no significant increase in the induction of chromosomal instability over a 16-fold range in the number of decays (25 to 400). Although the lack of a dose response for delayed mutations after ionizing radiation has been reported (75), other

delayed effects of radiation do show a dose response, notably chromosomal instability in GM10115 cells (73 and C.L. Limoli and co-workers, manuscript in preparation) and transformation in vitro (66).

Our data indicate that ^{125}IdU-induced double-strand breaks that occur while the cells are frozen do not contribute to chromosomal instability because chromosomal instability can be induced at a similar frequency by labeling with ^{125}IdU in the absence of freezing cells. The fact that chromosomal instability can be induced by x-irradiation in frozen cells, but at a significantly lower frequency than in non-frozen cells, argues that frozen cells, for whatever reason, have an increased resistance to radiation-induced chromosomal instability. Another possible explanation for why accumulating decays from ^{125}IdU does not induce chromosomal instability is that the DNA lesion for radiation-induced chromosomal instability is distinct from the lesion involved in cell killing and chromosomal rearrangements. This is consistent with our results that restriction enzymes, when electroporated into cells, and hydrogen peroxide cause DNA DSBs, cell killing and chromosomal rearrangements, yet do not induce chromosomal instability (71). As there is evidence for a second, non-DNA target for both radiation-induced cell killing (53) and cell cycle delays (132), there may be a second nuclear target for the induction of chromosomal instability as well.

There is evidence that radiation-induced chromosomal instability can involve a non-nuclear target (62, 83). However, this evidence is indirect, relying on the fact that the frequency of chromosomal instability is greater than the number of cells hit with a nuclear traversal, as calculated from the Poisson distribution. Our results with ^{125}I-suc-con A demonstrated that damage to a non-nuclear target alone does not induce chromosomal instability in our cell system. Our results showing cells labeled with both ^{125}IdU/^{125}I-suc-con A have a similar frequency of chromosomal instability as do cells labeled with ^{125}IdU alone further argues that non-nuclear targets do not contribute to chromosomal instability. An important caveat with the ^{125}I-suc-con A experiments is that there may be

extranuclear targets involved in chromosomal instability that we failed to damage by labeling at either 4°C or at 37°C. Other methods of selectively irradiating subcellular regions, such as placing α-particles through selected cellular regions, nucleus or cytoplasm, will help answer this question.

While the target for radiation-induced chromosomal instability is the nucleus, DNA DSBs seem necessary but not sufficient for the induction of chromosomal instability. Clearly, a full understanding of not only the complexity of DSBs but also the genomic context of the DSB will be necessary to understand how chromosome stability is compromised by ionizing radiation.

Table 2.1: Cytogenetic data from all clones scored. A) Cells labeled with ^{125}IdU and frozen to accumulate decays. B) Cells labeled with different levels of ^{125}IdU. C) Cells exposed to 25 Gy of X-rays while frozen D) Cells labeled with ^{125}I-suc-con A at 4°C. E) Cells labeled with ^{125}I-suc-con A at 37°C. F) Cells labeled with both ^{125}IdU and ^{125}I-suc-con A at 4°C. G) Cells labeled with both ^{125}IdU and ^{125}I-suc-con A at 37°C.

	# decays or dose	Total # of clones	# clones with 0 subpopulations[a]	# clones with 1-2 subpopulations[b]	# clones with ≥3 subpopulations[c]
A	25	25	18 (75%)[d]	5 (20%)	2 (5%)
	50	25	18 (75%)	5 (20%)	2 (5%)
	100	38	33 (87%)	4 (10%)	1 (3%)
	200	27	19 (70%)	7 (26%)	1 (4%)
	300	30	20 (67%)	8 (26%)	2 (7%)
	400	22	15 (68%)	5 (23%)	2 (9%)
B	Low	37	28 (76%)	8 (21%)	1 (3%)
	Medium	26	16 (62%)	8 (30%)	2 (8%)
	High	34	22	12	0
C	25 Gy	40	19 (47.5%)	19 (47.5%)	2 (5%)

[a] stable, no rearrangements detected in 200 metaphases

[b] rearrangements were detected, but the clone is still chromosomally stable

[c] chromosomally unstable

[d] percentages are given when chromosomally unstable clones were found

Table 2.1 (continued)

	# decays or dose	Total # of clones	# clones with 0 subpopulations[a]	# clones with 1-2 subpopulations[b]	# clones with ≥3 subpopulations[c]
D	101	20	18	2	0
	292	8	7	1	0
	531	12	11	1	0
	980	4	3	1	0
	1099	10	10	0	0
	4048	15	14	1	0
	8509	6	5	1	0
E	4645	35	28	7	0
F	202 + 2447	26	21 (81%)	4 (15%)	1 (4%)
G	202 + 930	25	18	7	0

[a]stable, no rearrangements detected in 200 metaphases

[b]rearrangements were detected, but the clone is still chromosomally stable

[c]chromosomally unstable

[d]percentages are given when chromosomally unstable clones were found

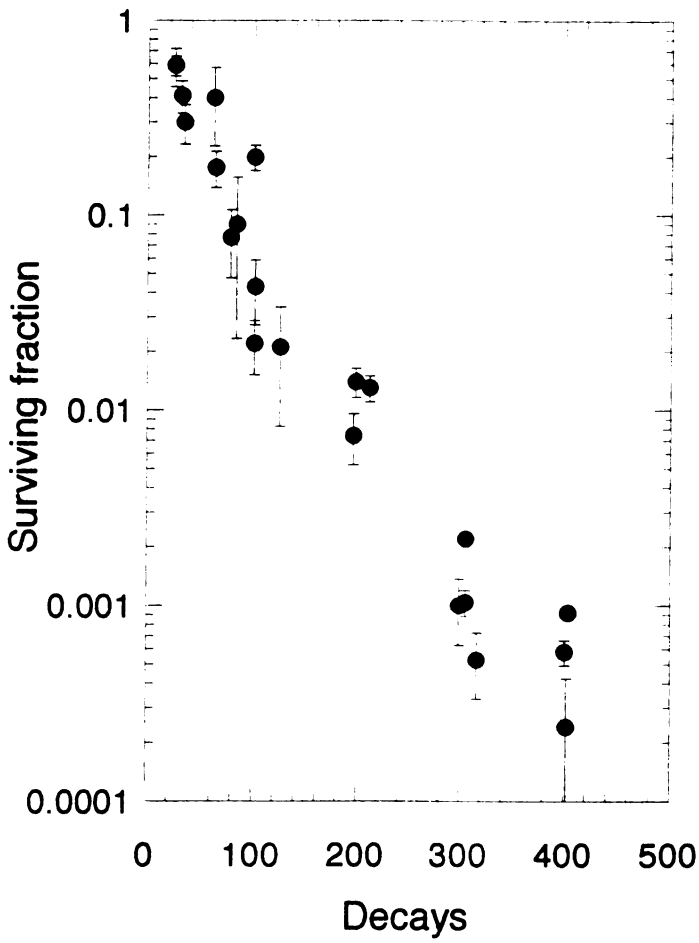

Figure 2.1. Surviving fraction of GM10115 cells after labeling with ^{125}IdU and freezing the cells. Survival is normalized for cells which were labeled and frozen for one hour. Data are taken from four independent experiments. Error bars show +/- standard deviation.

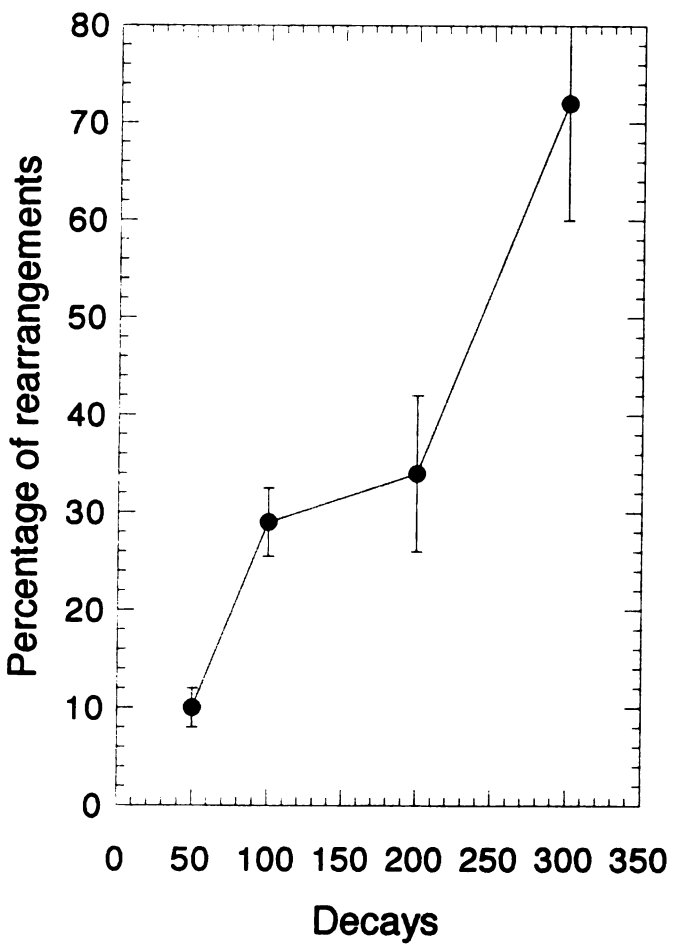

Figure 2.2. Percentage of chromosomal rearrangements involving HC#4 in the first mitosis following damage from ^{125}IdU versus the number of decays. Error bars show the 95% confidence interval.

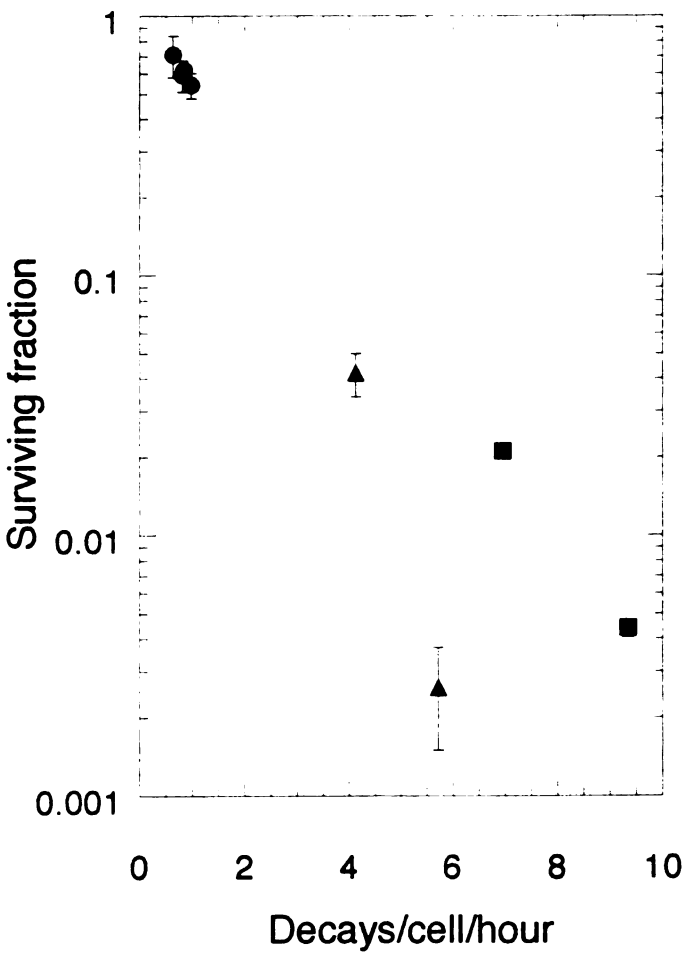

Figure 2.3. Surviving fraction of GM10115 cells labeled with different levels of ^{125}IdU -- low (●), medium (▲), high (■) -- against the decays/cell/hour. Each point represents an individual experiment. Error bars show +/- standard deviation.

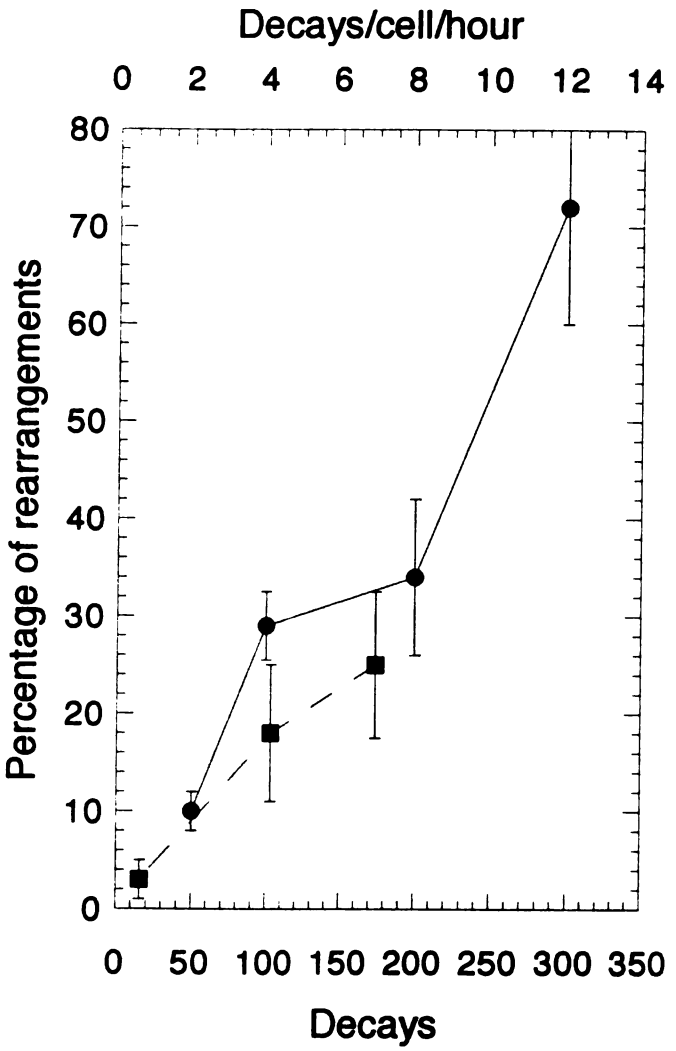

Figure 2.4. Percentage of chromosomal rearrangements involving HC#4 in the first mitosis following damage from ^{125}IdU versus either the number of decays for cells accumulating decays while frozen (●), or for the decays/cell/hour for cells labeled with different levels of ^{125}IdU but not frozen (■). The two x-axes were aligned so equal distances from the origin represent equivalent levels of cell killing. Error bars show the 95% confidence interval.

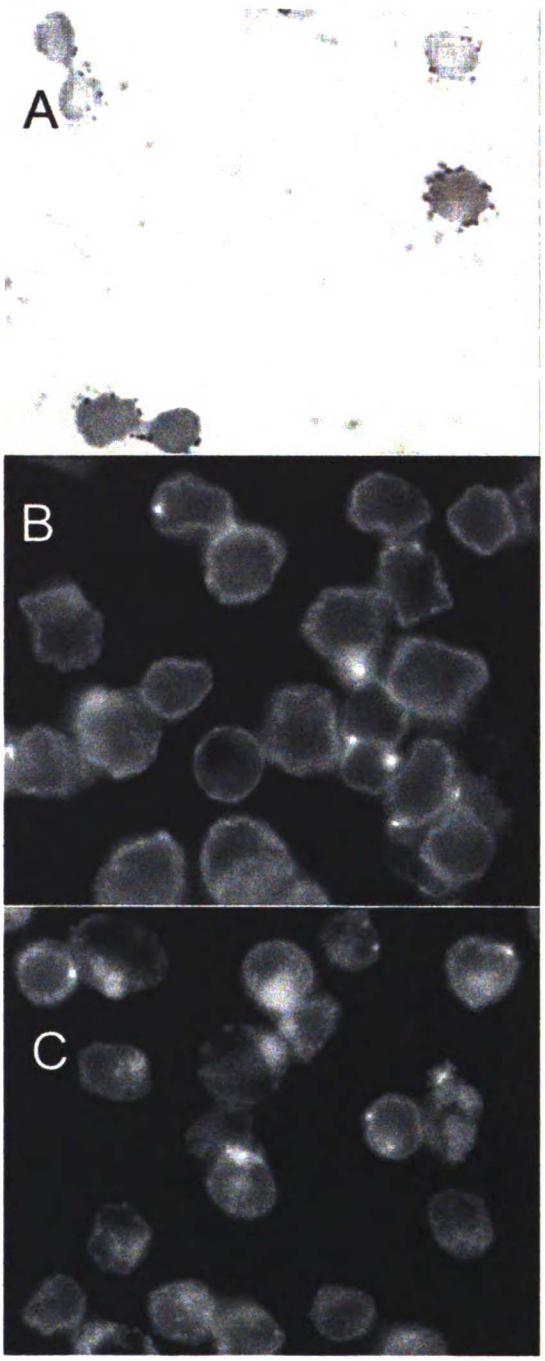

Figure 2.5. Labeling of GM10115 cells with suc-con A. A) Autoradiography of cells labeled with ^{125}I-suc-con A at 37^{o}C, frozen, and thawed. Cells are stained with Giemsa. B) Cells labeled with FITC-suc-con A at 4^{o}C, frozen, and thawed. C) Cells labeled with FITC-suc-con A at 37^{o}C.

Chapter 3 Differential Induction of Chromosomal Instability by DNA Strand-breaking Agents

SUMMARY

To investigate the role of DNA strand breakage as the molecular lesion responsible for initiating chromosomal instability, five different strand breaking agents, bleomycin, neocarzinostatin, hydrogen peroxide, restriction endonucleases and ionizing radiation, were examined for their capacity to induce delayed chromosomal instability. These studies utilized GM10115 human-hamster hybrid cells that contain one copy of human chromosome four in a background of 20-24 hamster chromosomes. Chromosomal instability was investigated using fluorescence *in situ* hybridization to visualize chromosomal rearrangements involving the human chromosome. Rearrangements are detected multiple generations post-treatment, in clonal populations derived from single progenitor cells surviving treatment of the specified DNA damaging agents. Clastogenic and cytotoxic activities of all agents were tested by examining chromosome aberration yields in first division metaphases and by clonogenic survival assays. Analysis of over 250 individual clones representing over 50,000 metaphases demonstrates that when compared at comparable levels of cell kill, ionizing radiation, BLM and NCS are equally effective at eliciting delayed genomic instability. These observations document for the first time the persistent destabilization of chromosomes following chemical treatment. In contrast, the analysis of nearly 300 clones and 60,000 metaphases, involving treatment with four different restriction endonucleases and/or hydrogen peroxide, did not show any delayed chromosomal instability. These data indicate that DNA strand breakage per se does not necessarily lead to chromosomal instability but that the complexity or quality of DNA strand breaks are important in initiating this phenotype.

INTRODUCTION

The propensity of cancer cells to show multiple mutations and karyotypic abnormalities underscores the concept that multistep carcinogenesis progresses by the accumulation of discrete genetic alterations. Central to understanding the cause and effect relationships between the many endpoints associated with neoplasia is a knowledge of how this genetic change occurs and what proliferative advantages are bestowed on the cell. Accumulating evidence suggests that genomic instability may provide the driving force behind the genetic plasticity characteristic of cancer cells (76, 104, 133). Genomic instability is an all embracing term that includes such changes as gene mutation, gene amplification, chromosomal destabilization, and cellular transformation (93). Substantial effort aimed at studying cancer progression has focused on characterizing specific treatments capable of inducing the same endpoints observed in cancer cells, and on developing systems capable of accurately measuring the resultant changes. Successful application of these approaches have established that specific genetic lesions can lead to mutations, many of which result from gross chromosomal change. Indeed, the preponderance of chromosome rearrangements identified in neoplasms serves to emphasize the importance of studying processes contributing to the formation of chromosomal rearrangements (91, 137).

Cells exposed to ionizing radiation can become genomically unstable as manifested by dynamic chromosomal destabilization. This genomic instability can be observed cytogenetically as chromosome aberrations and rearrangements multiple generations post-irradiation (55, 60, 62, 84, 85, 124). The consequences of damage-induced-genomic instability includes delayed mutation, cell fusion, transformation, delayed reproductive cell death and gene amplification (reviewed in 93). The majority of DNA damaging agents produce genetic lesions which are rapidly repaired and seldomly persist for more than a few hours (146, 151). This makes it difficult to reconcile how a transient exposure to a noxious agent can result in such prolonged and persistent destabilization of the genome.

Nonetheless, exposure to ionizing radiation can initiate the processes associated with genomic instability which can ultimately contribute toward an increased rate of acquisition of alterations in the genome (93).

The capacity of ionizing radiation to compromise genomic stability in the progeny of GM10115 cells surviving acute exposure to ionizing radiation provides a unique model system with which to study the initiation and progression of genome destabilization (84). Emerging views concerning the underlying mechanisms behind genomic instability must identify the critical intracellular target(s) and potential molecular interactions capable of initiating this process (72, 93). The molecular mechanisms initiating genomic instability must account for the long-lived and dynamic nature of chromosomal destabilization. We report an investigation involving five agents known to produce specific types of DNA strand breaks, with different chemical termini, i.e., BLM, NCS, hydrogen peroxide, restriction endonucleases and ionizing radiation, and how these modulate the induction of delayed genomic instability. The data presented entails the analysis of over 550 individual clones representing the scoring and classification of over 100,000 metaphases. The variable induction of delayed genomic instability found after treatment of cells with each of these five agents is discussed in regard to the specific types of DNA damage produced.

MATERIALS AND METHODS

Cell culture

Two genetically distinct subclones of the human/hamster hybrid cell line GM10115 were used for these studies. GM10115 cells contain a single copy of human chromosome 4 in a background of 20-24 hamster chromosomes. GM10115$^{+/+}$ cells contain two copies of the adenine phosphoribosyltransferase (*APRT*) gene and GM10115$^{+/o}$ cells are hemizygous at the *APRT* locus. Both cell lines were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U of penicillin, 100 µg/ml of streptomycin, and

0.2 mM L-proline. Cells were maintained at 34°C in humidified incubators containing 5% CO_2 in air.

Isolation and characterization of GM10115$^{+/o}$ cells

Spontaneous *APRT*$^{+/o}$ (hemizygous) GM10115 sublines, which have lost one copy of the Chinese hamster *APRT* gene, were selected on the basis of their resistance to intermediate levels of 8-aza-adenine (1, 17, 141). GM10115$^{+/+}$ cells were subcloned to derive a series of independent, clonally derived cell populations. Expanded clonal populations were then plated at 10^4 cells per 100 mm tissue culture dish with selective medium containing 8 μg/ml of 8-aza-adenine. The medium was replaced with fresh selection medium every four days. Single clones surviving selection were then picked, grown to mass culture and characterized by Southern blot analysis to determine whether they were heterozygous or hemizygous for the *APRT* locus.

Restriction enzyme treatment of cells and selection of APRT mutants

The hemizygous GM10115$^{+/o}$ cells were generated specifically for the detection of *APRT* mutants following treatment with REs. RE cleavage and subsequent misrepair at the unique recognition site within the endogenous *APRT* gene generates *APRT* mutations and provides evidence that an enzymatically active endonuclease entered and produced DNA DSBs within a given cell (19). Mutations were induced by treatment of these *APRT*$^{+/o}$ cells with one of four restriction enzymes obtained from Boehringer Mannheim (Indianapolis, IN): *Bsa*HI (GPu↓CGPyC; 100 U/electroporation, 10 U/μl), *Pvu*II (CAG↓CTG; 25 U/electroporation, 10 U/μl), *Stu*I (AGG↓CCT; 10 and 15 U/electroporation, 10 U/μl), or *Eco*RV (GAT↓ATC; 300 and 450 U/electroporation, 50 U/μl). Each RE cleaves only once

within one of the 5 exons of the endogenous *APRT* gene, and was introduced separately by electroporation into cells as described previously (115).

APRT$^{-/o}$ cells were selected by plating 2-3x10^5 RE treated cells into 100 mm dishes with 10 ml of selective medium containing 80 μg/ml of 8-aza-adenine. Two to three weeks of growth were generally required before *APRT* mutant colonies reached a size sufficient for subsequent counting, isolation and expansion. Isolation of mutant colonies and subsequent characterization of the *APRT* mutation at the molecular level allow us to determine whether an endonuclease entered the cell and induced the observed mutation.

Analysis of APRT mutations

Genomic DNA was isolated and purified from expanded *APRT* mutant clones by a "salting out" procedure (90). PCR amplification across specific regions of the *APRT* gene was carried out by selecting primer pairs that flanked specific exons containing the unique restriction site. These regions of the *APRT* gene were amplified by polymerase chain reaction in a total volume of 100 μl, using a standard 40-cycle amplification protocol and reagents from the GeneAmp DNA amplification kit and 0.5 U of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT). Primers were 18-mers and used at 50 pmol per reaction in conjunction with 0.5 μg genomic DNA. Amplified regions were analyzed for mutations by restriction fragment length analysis; 20 μl aliquots of PCR product were digested with the same restriction enzyme used to induce the *APRT* mutation and resolved via 4% agarose gel electrophoresis. RE-induced mutations by definition lose the RE site and are refractory to subsequent enzyme cleavage (115).

Analysis of delayed reproductive cell death in restriction enzyme treated cells

To ascertain whether clones determined above to be RE-induced or non-RE-induced *APRT* mutants showed any differences in delayed reproductive cell death, clonogenic assays were performed to measure plating efficiency. CFAs were performed on 23 individual *Bsa*HI enzyme induced *APRT* mutants and 10 spontaneous *APRT* mutants by plating 100 cells/100 mm dish in triplicate.

Irradiation and chemical treatment of cells

X-irradiation: Log-phase cells were exposed to 10 Gy of X-rays at 250 cGy/min at ambient temperature using a Phillips RT250 X-ray machine (250-kV peak, 15 mA; half-value layer 1.0 mm Cu).

Hydrogen peroxide (H_2O_2, Sigma): GM10115$^{+/+}$ cells ($1x10^5$ cells/ml, 10 ml total) were incubated with the desired concentration of H_2O_2 for 30 minutes at 34°C. All tubes were maintained at equal cell densities during drug treatment to minimize intracellular variations in H_2O_2 concentration due to catabolic consumption by catalase. For simultaneous treatment of cells with H_2O_2 and the RE *Stu*I, cells were first electroporated with *Stu*I then immediately diluted into prewarmed medium containing the desired concentration of H_2O_2.

Bleomycin (Blenoxane Squibb): Twenty five cm^2 flasks containing $2-3x10^6$ GM10115$^{+/+}$ cells were incubated with the desired concentration of BLM (1.0-300 μg/ml) for four hours at 34°C.

Neocarzinostatin: NCS was a generous gift from Dr.'s Gene Joiner and L. Gayle Littlefield at the Oak Ridge National Laboratory. One million GM10115$^{+/+}$ ($1x10^5$

cells/ml) were incubated with the desired concentration of NCS (0.1-10 μg/ml) for one hour at 34°C.

Immediately following the appropriate treatment cells were rinsed to remove residual drug and resuspended in fresh complete medium before plating. Those cells not destined for experiments involving the isolation and identification of delayed genomic instability were replated for the analysis of first division metaphases.

Cell survival and colony isolation

Immediately following irradiation or chemical treatment, cells were diluted and plated in triplicate into 100 mm dishes containing 10 ml of medium to determine the surviving fraction. The number of cells plated was based on the expected level of kill for a particular agent, and on limiting the number of colonies per plate (~20) to minimize the chances of selecting more than one individual clone during colony isolation. After two to three weeks, distinct and well isolated colonies of ~100 cells had formed. Some of these colonies were picked at random from each of the plates using sterile dacron swabs dipped in trypsin and expanded to mass culture (~ 1-3x10^6 cells) in 25 cm^2 flasks. The remaining colonies were stained with 0.1% crystal violet in 25% ethanol and counted. Cell survival was determined as the number of colonies picked plus the number of colonies stained divided by the number of cells plated times the plating efficiency. Those colonies expanded to mass culture were prepared for analysis of potential chromosomal instability as described below.

Collection of metaphase cells

Metaphase cells derived from clonally expanded cell populations were collected by mitotic shake off following a two to three hour incubation in the presence of the mitotic spindle inhibitor colcemid (2x10^{-7} M final concentration). Cells were then swollen in

hypotonic 0.075 M KCl solution for 15 minutes at 37°C, dehydrated in 100% methanol, and fixed in a 3:1 mixture of methanol:acetic acid. Mitotic cells were dropped onto precleaned glass microscope slides and allowed to air dry at least two days at room temperature before storage at -20°C.

Fluorescence in situ hybridization (FISH)

Post-treatment analysis of chromosome aberrations at the first metaphase and of delayed chromosomal instability utilized FISH of a labeled probe to the human chromosome in the hybrid cells. The labeling of this bluescript vector-based library of human chromosome four-specific DNA sequences has been described (84).

Slides with metaphase spreads were washed in two changes of 2X SSC (0.3M NaCl and 0.03 M sodium citrate, pH 7) for 15 minutes, then dehydrated in 70, 90 and 100% ethanol for two minutes each at ambient temperature. After drying, chromosomes were denatured for 30 seconds at 80°C in 70% formamide and 2X SSC, then dehydrated again in the ethanol series described above. After drying, 35 µl of hybridization mix (70% formamide, 15% dextran sulfate, 2X SSC and 50 ng labeled probe) was applied to each slide, which was then covered with a glass coverslip and sealed with rubber cement. Slides were incubated a minimum of two days in a humidified incubator at 37°C.

All remaining steps were done at ambient temperature. Slides were washed in three changes of PBD, once briefly and twice for five minutes each. For detection of the biotinylated probe, slides were incubated with FITC-avidin (Oncor) for 15 minutes while covered with plastic coverslips (Oncor). Slides were washed again in three changes of PBD and the fluorescent signal amplified via analogous incubation using an anti-avidin antibody (Oncor) followed by a repeat incubation with the FITC-avidin. After a final rinse and two five-minute washes in PBD, propidium iodide in antifade (Oncor) was added to slides which were then covered with glass coverslips.

Cytogenetic analysis

Visualization of metaphase chromosomes following FISH was accomplished using a Zeiss Axioskop microscope equipped with a dual-band pass FITC/Texas Red filter set. With this system and the combination of fluorescent dyes used, the background of hamster chromosomes appear red (propidium iodide emission), while the biotinylated probe hybridized to the human chromosome four target appears yellow-green (fluorescein emission). Kodak 400 ASA slide film was used for all fluorescence photography, and no digital processing was used to modify any of the presented images.

Genomic instability for the purposes of this paper is defined as any clone derived from a single cell which shows at least three distinct metaphase subpopulations involving rearrangements of human chromosome four (84). Analysis of chromosomal instability entails scoring 200 individual metaphases from each clone. Only those rearrangements involving human chromosome four were scored and categorized as described by Tucker *et al.* (143).

RESULTS

Ionizing radiation treatment

To establish the frequency with which ionizing radiation induces genomic instability, as measured by delayed chromosomal destabilization, *APRT*$^{+/+}$ and *APRT*$^{+/o}$ cells were exposed to 10 Gy of X-rays. Table 3.1 shows the results of over 10 independent experiments where 152 individual clones were isolated from single progenitor cells surviving X-ray exposure. Examination of more than 30,000 metaphases indicates 10 Gy of X-rays induces genomic instability in 33% of the clones analyzed. 10 Gy of X-rays induced chromosomal instability in both *APRT*$^{+/+}$ and *APRT*$^{+/o}$ GM10115 cells at a similar frequency.

Generation and analysis of APRT$^{+/o}$ GM10115 sublines

Hemizygous *APRT*$^{+/o}$ sublines of GM10115 CHO cells were generated specifically for the experiments involving restriction enzymes. Clones selected under intermediate levels of 8-aza-adenine were expanded and analyzed by Southern blot analysis (Figure 3.1). CHO cells contain two copies of the *APRT* gene, one located on chromosome Z4 and the other on the Z7 chromosome (4, 5). The GM10115 strain is heterozygous for a *Bcl* I restriction fragment length polymorphism involving the presence or absence of a *Bcl* I site ≈ 6.8 kb downstream of the *APRT* gene (36). *Eco*RI/*Bcl* I double digests of genomic DNA from *APRT*$^{+/+}$ GM10115 cells (lane 1) show three bands that hybridize to a 3.9 kb *Bam*HI fragment *APRT* probe: 1) a dark (dual-copy) 7.1 kb *Eco*RI fragment present on both chromosomes; 2) an 8.0 kb *Eco*RI-*Bcl*I fragment present only on the Z4 chromosome; and 3) a 10.1 kb *Eco*RI-*Bcl*I fragment present only on the Z7 chromosome. Similar analysis performed on three independently derived GM10115 *APRT* mutants (lanes 2-4) show a less-intense (single copy) 7.1 kb *Eco*RI fragment and loss of the 8.0 kb *Eco*RI-*Bcl*I fragment indicating these clones, like other spontaneous deletion events characterized in CHO strains, have undergone complete loss of the CHO Z4 *APRT* allele (2, 3). Lane 5 shows wild-type CHO cells. The *APRT*$^{+/o}$ subline shown in lane 4 (subclone D2) was made specifically to monitor misrepair of RE induced DSBs and was used in all subsequent experiments utilizing REs.

Restriction enzyme treatment

Using RE concentrations determined previously to maximize the yield of *APRT* mutations (115), clonogenic survival for all enzymes used was reduced to 10-15%. Metaphase chromosomes were prepared from cells treated with RE at the first mitosis post-treatment to verify the clastogenic properties of REs in cells. Four hundred metaphases examined from two independent experiments in which cells were electroporated with equivalent volumes of RE storage buffer without enzyme did not show aberrations. Analysis of 200 metaphases from cells treated with 100U of *Bsa*HI or 25U of *Pvu*II, revealed that 62 and 71% respectively showed rearrangements involving human chromosome four.

APRT mutant colonies were picked and clonally expanded for both preparation of metaphase chromosomes and purification of genomic DNA. Using PCR amplification of the region of the *APRT* gene that surrounded the RE recognition sequence of interest and recleavage with the same RE that was used in the mutagenesis experiments, we were able to categorize the mutants according to whether they contained a change at the recognition sequence for that particular enzyme (Table 3.2). Figure 3.2 shows a typical analysis of 11 *APRT* mutants isolated after StuI treatment. Lanes 3-5 show mutations that did not involve the recognition sequence, and are referred to as non-RE-induced mutants. Lanes 6-12 show mutations that did involve the recognition sequence, and are now refractory to enzyme cleavage. These we classified as RE-induced mutants. They typically comigrate with the uncut control (lane 2) unless they have undergone recombination resulting in large insertions or deletion at the restriction site (lanes 6 and 11). Those mutants that were not amplified by PCR using primers flanking the recognition sequence, but could be amplified at other regions of the *APRT* gene were also considered RE-induced mutants (data not shown). Past work has established that these mutants contain complex rearrangements involving the *APRT* locus (115). Molecular analysis of 233 *APRT* mutants indicates that 25/40 (62%), 12/83 (14%), 25/36 (69%), and 61/74 (82%) of the clones exposed to

*Bsa*HI, *Pvu*II, *Eco*RV, or *Stu*I respectively, had changes at their respective recognition sites (Table 3.2). Collectively, 123 or 53% of the mutants were RE-induced.

For each of the *APRT* mutants isolated and characterized above, a subset of the clonal population was set aside for the preparation of metaphase chromosomes. None of the 233 mutant clones analyzed demonstrated chromosomal instability despite analysis of over 46,000 metaphases from the RE-induced and non-RE induced groups (Table 3.2).

Plating efficiency in restriction enzyme treated cells

Having determined the nature of the *APRT* mutation in a large number of RE-treated clones, we sought to determine whether clones known to be either RE-induced or non-RE-induced *APRT* mutants, showed any differences in delayed reproductive cell death. The results of the clonogenic assays used to measure plating efficiencies of each clone are shown in Table 3.3. A total of 23 RE-induced *APRT* mutants (treated with 100 units of *Bsa*HI) had a mean plating efficiency of 73 +/- 4.8 (+/- SEM), while a total of 10 non-RE-induced *APRT* mutants had a mean plating efficiency of 73 +/- 4.0 (+/- SEM). Statistical analysis (analysis of variance, and student t-test) performed between the data sets of RE-induced and non-RE-induced *APRT* mutants shown in Table 3.3 were not found to be significantly different.

Hydrogen peroxide treatment

To determine whether H_2O_2 was capable of eliciting delayed genomic instability, cells were exposed to a range of concentrations (1.0-300 μM) for 30 min at 34°C. The survival data for H_2O_2 exposure is shown in Figure 3.3. As before, metaphase chromosomes prepared at the first mitosis were examined for aberrations, and after scoring 200 metaphases derived from populations of cells exposed to H_2O_2 no chromosome aberrations were detected. Clones isolated from H_2O_2 concentrations which reduced

survival by 2-3 logs were expanded for analysis of genomic instability. Of the 18, 7, and 6 clones isolated after 100, 200, 300 μM H_2O_2 treatment respectively, none exhibited delayed chromosomal instability (Table 3.4).

Restriction enzyme and hydrogen peroxide treatment

The capacity to produce RE-induced DSBs in the nucleus and damage other cellular targets with H_2O_2 provided an attractive method of modeling the action of ionizing radiation. Consequently we investigated whether combined exposure to REs and H_2O_2 could lead to chromosomal instability in GM10115$^{+/o}$ cells. Of the 35 *APRT* mutant clones analyzed, 24 showed RE-induced mutations and the remaining 11 were non-RE-induced mutants. None were found to show delayed chromosomal instability (Table 3.4).

Bleomycin treatment

The ability of BLM to induce genomic instability was investigated by exposing cells to 1.0-250 μg/ml BLM for four hours at 34°C. The resulting cell kill is shown in Figure 3.4. To further establish the activity of the drug, metaphase chromosomes were prepared at the first mitosis following drug exposure. Two hundred metaphases were examined at the first mitosis where 25, 29, 43, and 51% contained multiple complex aberrations involving human chromosome four, following treatment with 100, 150, 200, and 250 μg/ml BLM, respectively.

At various drug concentrations survivors were isolated, expanded and analyzed for genomic instability. Table 3.5 lists data for 62 clones analyzed which survived exposure to 100-250 μg/ml BLM. At drug concentrations of 100, 150, 200, and 250 μg/ml, 4/19 (21%), 2/8 (25%), 9/16 (56%) and 11/19 (58%) of the clones, respectively, were found to be genomically unstable. Figure 3.5 depicts examples of two clones isolated after exposure

to 100 μg/ml BLM, one genomically stable clone (panel a), and one genomically unstable clone (panels b-f). Panel (a) shows the normal unrearranged human chromosome four (yellow) against a background of hamster chromosomes (red). Panels (b-f) represent the types of rearrangements involving the human chromosome which can be encountered in a single unstable clone; the multiple complex rearrangements involving various portions of human chromosome four demonstrate the dramatic and dynamic nature of the endpoint measured.

Neocarzinostatin treatment

To analyze the capacity of NCS to induce genomic instability, cells were exposed to 0.1-10 μg/ml NCS for one hour at 34°C. The cell survival curve is shown in Figure 3.6. As with BLM, metaphase chromosomes were isolated at the first mitosis to further confirm the intracellular activity of the drug. Sixteen and 40% of the 200 metaphase chromosomes examined after exposure to 1.0 and 3.0 μg/ml NCS, respectively, showed multiple complex aberrations.

Cytogenetic data gathered from clones surviving NCS treatment are shown in Table 3.6. Collectively, 40 clones isolated after 0.5 log kill were analyzed. Table 3.6 shows that a total of 0/14 (0%), 3/14 (21%), and 5/12 (42%) of the clones analyzed after exposure to 1, 3, or 10 μg/ml of NCS respectively, were found to be genomically unstable.

DISCUSSION

Cells surviving exposure to ionizing radiation demonstrate several endpoints collectively termed genomic instability. These include delayed reproductive cell death, which is revealed by variability in colony size and a persistent reduction in plating efficiency (heritable lethal mutations), giant cell formation, cell fusion, lowered cell

attachment ability, delayed mutation, clonal heterogeneity, transformation, and delayed chromosomal instability (reviewed in 93). Perhaps the most dramatic of these biologic endpoints is clonal chromosomal destabilization manifested several generations post-irradiation as visualized by FISH. Ten Gy of x-rays reduces clonogenic survival by 2-3 logs and elicits delayed chromosomal instability in 33% of all clones examined (Table 3.1). Table 3.1 shows that nearly half of the genomically unstable *APRT*$^{+/+}$ GM10115 clones could be considered very unstable; 15% had five or more populations of chromosome aberrations, while nine clones showing over eight classes of aberrations were extremely unstable, having markedly complex rearrangements in nearly all metaphases examined. Of the 11 *APRT*$^{+/o}$ clones examined, six were unstable, thereby substantiating that genetic modification of the *APRT* locus does not preclude the manifestation of delayed chromosomal instability.

Cellular exposure to ionizing radiation results in a variety of directly and indirectly induced DNA lesions, including DNA base alterations, DNA-DNA and DNA protein cross-links, and single- and double-strand breaks (146). The random nature and variety of lesions inherent to radiation complicates elucidation of the primary target and initiating lesion responsible for inducing genomic instability. The effectiveness with which ionizing radiation induces delayed genomic instability, and the preponderance of evidence implicating DSBs as the critical lesions responsible for radiation-induced chromosome damage (13), mutagenesis (148), gene amplification (48), and cell kill (107), suggests that the DNA DSB may be the critical lesion responsible for genomic instability. To understand the nature of the molecular change which sends cells down the pathway of genomic instability, agents known to produce DNA DSBs with different end structures were investigated.

REs produce DSBs of known structure having 5' phosphate and 3' hydroxyl termini at defined sequences in the genome in the absence of any other DNA lesion.

APRT$^{+/o}$ hemizygous sublines of GM10115 cells were constructed specifically for this study to provide the appropriate genetic background to identify cellular cleavage by the RE as defined by RE-induced mutation in the *APRT* gene (Figure 3.2) (115). Although we verified the presence of only one genomic DSB, it's likely that many more DSBs were produced throughout the genome by RE treatment, and the frequency of complex aberrations observed in first division metaphases supports this notion. Genomic instability was not observed after numerous treatments using four different RE each with qualitatively different termini. Table 3.2 shows that regardless of whether mutations were RE-induced or not, none of the 233 clones analyzed was found to exhibit delayed chromosomal instability. Interestingly, related work has found REs unable to induce novel aberrations at early divisions post-treatment (158). Furthermore, data suggests that mutation frequency as measured at the *APRT* locus does not correlate with delayed genomic instability.

To investigate the capacity of REs to modulate another endpoint of genomic instability, i.e. delayed reproductive cell death, RE-induced and non-RE-induced *APRT* mutant clones were analyzed for persistent reductions in plating efficiency. Results shown in Table 3.3 reveal no significant difference in the plating efficiencies measured between RE-induced and non-RE-induced mutants. These results are in contrast to those reported by Chang and Little (23), who found REs capable of eliciting a persistent reduction in plating efficiency. The discrepencies noted above are likely the result of differences between our two experimental systems.

Exposure to ionizing radiation results in damage to nuclear as well as non-nuclear targets in part through the action of hydroxyl radicals. To mimic the indirect action of radiation on target molecules, without the formation of DNA DSBs, we investigated the effects of H_2O_2 which produces global cellular damage indiscriminately by virtue of the same reactive species (149, 150, 152). Cells exposed to H_2O_2 did not show chromosome aberrations at the first mitosis post-treatment, despite the production of prodigious

quantities of DNA single-strand breaks (SSB) and base damages (152). Treatment of cells with micromolar levels of H_2O_2 at 34°C results in substantial cell kill (Figure 3.3) and, despite isolating clones surviving greater than 2-logs kill, none of the 31 clones analyzed demonstrated chromosomal instability (Table 3.4). This indicates that the action of reactive H_2O_2 derived hydroxyl radicals throughout both nuclear and non-nuclear intracellular compartments was insufficient to elicit genomic instability, or at a minimum, H_2O_2 induces this phenotype far less frequently than other treatments producing equivalent levels of cell kill.

We expanded this study by including a combination of RE and H_2O_2 treatments to determine whether they may accomplish together what neither could do alone. Our rationale was that addition of H_2O_2 might damage a critical non-nuclear target, which in conjunction with RE-induced DSBs might elicit genomic instability. Alternatively, addition of H_2O_2 could generate hydroxyl radicals which would produce additional damage at the site of restriction cleavage, thereby generating DSBs more similar to those produced by ionizing radiation. These premises were not borne out experimentally, as the dual action of each agent was again unable to induce any observable genomic instability (Table 3.4). This dual-treatment required the use of H_2O_2 levels lower than treatment with H_2O_2 alone, otherwise cytotoxicity was too pronounced which reduced the yield of *APRT* mutants to impractically low levels. Nonetheless, the fact that we did observe RE-induced mutations argues against the possibility that the RE was inactivated by the oxidizing activity of H_2O_2.

The inability to induce genomic instability with REs and/or H_2O_2, compared to the high frequency with which ionizing radiation was able to induce this phenomenon, suggested a fundamental difference in the types and/or locations of the lesions produced. To model more faithfully the types and qualities of DSBs produced by radiation we turned to the radiomimetic antibiotics BLM and NCS. These clastogenic compounds react in concerted fashion to mediate site-specific free-radical attack on deoxyribose moieties in

both strands of DNA, resulting in DNA DSBs of defined structure, in addition to SSBs and closely opposed abasic sites (118). BLM produces strand breaks with 5'-phosphate and 3'-phosphoglycolate termini, and abasic sites formed in roughly equal proportions (42, 68, 118, 140). NCS induced DNA damage produces DNA DSBs with 3'-phosphate and 5'-aldehyde or 5'-carboxylic acid termini as well as abasic sites (28, 44, 64, 118). A small fraction of the NCS chromophore is also found crosslinked to the DNA (119).

Analysis of 62 clones, surviving BLM concentrations which resulted in at least 1.5 logs kill (Figure 3.4), shows that BLM was able to induce delayed genomic instability. The observed frequency of instability was proportional to drug levels; raising the concentration of BLM from 100 to 250 μg/ml more than doubled the percentage of unstable clones from 21 to 58% (Table 3.5). Many of these clones (7 total) were markedly unstable, with five or more aberrant populations predominating the net population. The complexity of these rearrangements is illustrated in Figure 3.5, which depicts a fraction of the aberrations detected in an unstable clone classified to contain over 16 distinct rearranged populations after a 100 μg/ml treatment of BLM.

Similarly, analysis of 40 clones isolated after NCS treatment indicates that NCS also elicits delayed genomic instability (Table 6). However, only those clones isolated after 1.5 logs kill (>3.0 μg/ml NCS, Figure 3.6) exhibited genomic instability. As with BLM, the observed frequency of instability was proportional to drug levels (Table 3.6). Of the eight unstable clones identified, six had five or more aberrant populations representing the majority of all metaphases scored. Of these, three were remarkably unstable, where all metaphases examined were aberrant with well over 20 distinct subpopulations of rearrangements identified within each clonal population.

Our findings that delayed genomic instability can be induced by exposure to BLM and NCS demonstrate for the first time the persistent destabilization of chromosomes following chemical treatment. DNA damage in the form of complex DSBs may constitute

at least one of the signals which initiate the onset of genomic instability. As with ionizing radiation, DSB damage resulting in both 3' (BLM) and 5' (NCS) chemical modification of DNA termini require some degree of enzymatic processing before gap filling and strand resealing. Such lesions may challenge cellular repair, as opposed to the readily reparable termini formed by restriction enzymes and single strand nicks induced by H_2O_2.

Our results suggest that any agent capable of producing complex types of DNA DSBs will induce genomic instability. The frequency with which genomic instability is observed, particularly after ionizing radiation or treatment with radiomimetic antibiotics, suggests multiply redundant pathways for induced instability. Our data using RE suggest that DNA double strand breakage *per se* does not necessarily lead to chromosomal instability. Furthermore, the potentially lethal DNA lesions induced by H_2O_2 do not appear to lead to instability. Instead, our data indicate that the complexity or quality of DNA DSBs may be important in initiating chromosomal instability. Other downstream cellular processes that occur in response to DNA damage may modulate further the extent, frequency, and heterogeneity of genomic instability, and can include induction of genes promoting instability, deletion of genes controlling stability, activation of endogenous viruses, or epigenetic factors such as signal transduction cascades.

Table 3.1. Delayed chromosomal instability induced by 10 Gy of ionizing radiation

GM10115$^{+/+}$ Cells

# of Clones	# Metaphase Subpopulations[a]	% Aberrant Metaphases[b]
69	1-2	0 - 3
6	1-2	7 - 8
22	1-2	97 - 100
8	3-4	0 - 6
3	3-4	12 - 38
12	3-4	85 - 100
2	5-7	3 - 9
5	5-7	19 - 71
5	5-7	100
9	8-17	84 - 100

GM10115$^{+/o}$ Cells

# of Clones	# Metaphase Subpopulations[a]	% Aberrant Metaphases[b]
5	1 - 2	0 - 3.5
2	3 - 5	3 - 4
1	3	80
1	5	3.5
2	7	99 - 100

[a]Number of different abnormal metaphase subpopulations showing distinct types of chromosome aberrations.
[b]Percentage of metaphases scored showing chromosomal aberrations.

Table 3.2. Induction of *APRT* mutations and delayed chromosomal rearrangements by REs

Restriction Enzyme	Enzyme Conc.	# Clones Analyzed	# Metaphase Subpopulations[a]	% Aberrant Metaphases[b]	Enzyme Induced Mutations
Bsa HI	25 U	24	1-2	0 - 2.5	Yes
		1	1	100	Yes
		14	1-2	0 - 2.5	No
		1	1	100	No
Pvu II	25 U	11	1-2	0 - 10	Yes
		1	1	100	Yes
		68	1-2	0 - 5	No
		2	2	8 - 10	No
		1	1	100	No
Eco RV	300 U	15	1-2	0 - 3	Yes
		9	1	0	No
	450 U	10	1-2	0 - 2.5	Yes
		7	1	0 - 0.5	No
Stu I	10 U	31	1-2	0 - 2	Yes
		2	1	23 - 57	Yes
		5	1-2	100	Yes
		8	1-2	0 - 2.5	No
	15 U	23	1	0 - 0.5	Yes
		5	1	0	No

[a]Number of different abnormal metaphase subpopulations showing distinct types of chromosome aberrations.

[b]Percentage of metaphases scored showing chromosomal aberrations.

Table 3.3. Plating efficiencies of RE-induced and non-RE-induced *APRT* mutants

RE-Induced[a] APRT Mutants	Plating Efficiency[c] (%)	RE-Induced[a] APRT Mutants	Plating Efficiency[c] (%)	Non-RE-Induced[b] APRT mutants	Plating Efficiency[c] (%)
2A1	61 +/- 2	5B2	72 +/- 4	1A	64 +/- 6
3A1	85 +/- 2	6B2	57 +/- 8	1B	87 +/- 2
5A1	73 +/- 8	2C1	38 +/- 6	1C	69 +/- 8
2A2	70 +/- 6	3C1	72 +/- 7	1D	67 +/- 5
3A2	84 +/- 12	4C1	92 +/- 20	1E	79 +/- 8
4A2	62 +/- 9	5C1	98 +/- 3	1F	67 +/- 11
5A2	32 +/- 4	2C2	74 +/- 12	1G	82 +/- 13
6A2	62 +/- 3	3C2	86 +/- 5	1H	68 +/- 8
2B1	102 +/- 25	4C2	68 +/- 1	1I	71 +/- 8
5B1	103 +/- 3	5C2	71 +/- 9	1J	76 +/- 2
6B1	95 +/- 8	6C2	73 +/- 16		
3B2	87 +/- 1				

[a]APRT mutants derived from cells treated with 100 units of BsaHI.

[b]APRT mutants derived from cells treated with BsaHI storage buffer alone.

[c]Percent average plating efficiency with standard deviation.

Table 3.4. Delayed chromosomal instability analyzed following exposure to H_2O_2 or the combination of H_2O_2 and the RE *StuI* (15 units)

H_2O_2 Conc. (μM)	# Clones Analyzed	# Metaphase Subpopulations[a]	% Aberrant Metaphases[b]	Enzyme Induced Mutations
100	18	1-2	0 - 4.5	N.A.[c]
200	7	1-2	0 - 5	N.A.
300	6	1-2	0 - 12.5	N.A.
30	14	1-2	0 - 2.5	Yes
	2	1-2	0 - 2	No
60	10	1-2	0 - 4.5	Yes
	9	1-2	0 - 1.5	No

[a]Number of different abnormal metaphase subpopulations showing distinct types of chromosome aberrations.

[b]Percentage of metaphases scored showing chromosomal aberrations.

[c]N.A. = Not applicable.

Table 3.5. Delayed chromosomal instability induced by BLM

Bleomycin Conc. (mg/ml)	# Clones Analyzed	# Metaphase Subpopulations[a]	% Aberrant Metaphases[b]
100	15	1 - 2	0 - 2
	1	4	3
	1	8	35
	1	13	100
	1	>16	87
150	6	1 - 2	0 - 2
	2	3	2.5
200	7	1 - 2	0 - 5
	6	3 - 4	0 - 6
	1	5	3.5
	1	5	100
	1	7	18
250	7	1	0 - 1
	1	1	100
	9	3 - 4	0 - 6
	1	6	15.4
	1	8	56

[a]Number of different abnormal metaphase subpopulations showing distinct types of chromosome aberrations.
[b]Percentage of metaphases scored showing chromosomal aberrations.

Table 3.6. Delayed chromosomal instability induced by NCS

Neocarzinostatin Conc. (μg/ml)	# Clones Analyzed	# Metaphase Subpopulations[a]	% Aberrant Metaphases[b]
1.0	14	1 - 2	0 - 3
3.0	9	1 - 2	0 - 2
	2	1 - 2	100
	1	5	100
	1	9	99
	1	>25	100
10.0	7	1	0 - 2
	2	3 - 4	2 - 9
	1	7	18
	1	22	100
	1	>35	100

[a]Number of different abnormal metaphase subpopulations showing distinct types of chromosome aberrations.

[b]Percentage of metaphases scored showing chromosomal aberrations.

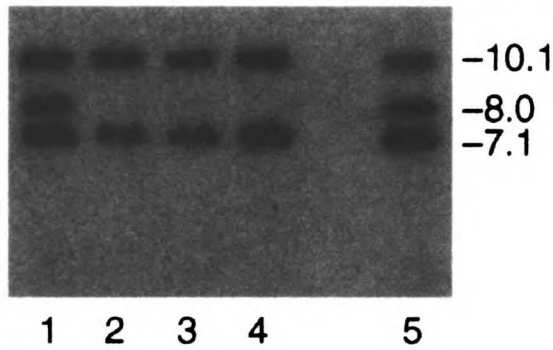

Figure 3.1. Southern blot hybridization analysis of the three *APRT*$^{+/o}$ hemizygous sublines that were derived from the *APRT*$^{+/+}$ GM10115 cell line. *Eco*RI/*Bcl*I double digests of genomic DNAs, hybridized with a 3.9 kb *Bam*HI fragment Chinese hamster *APRT* probe. Lane 1, parental GM10115 cell line; lanes 2-4, three independently derived *APRT*$^{+/o}$ hemizygous sublines; lane 5, wild-type CHO cells.

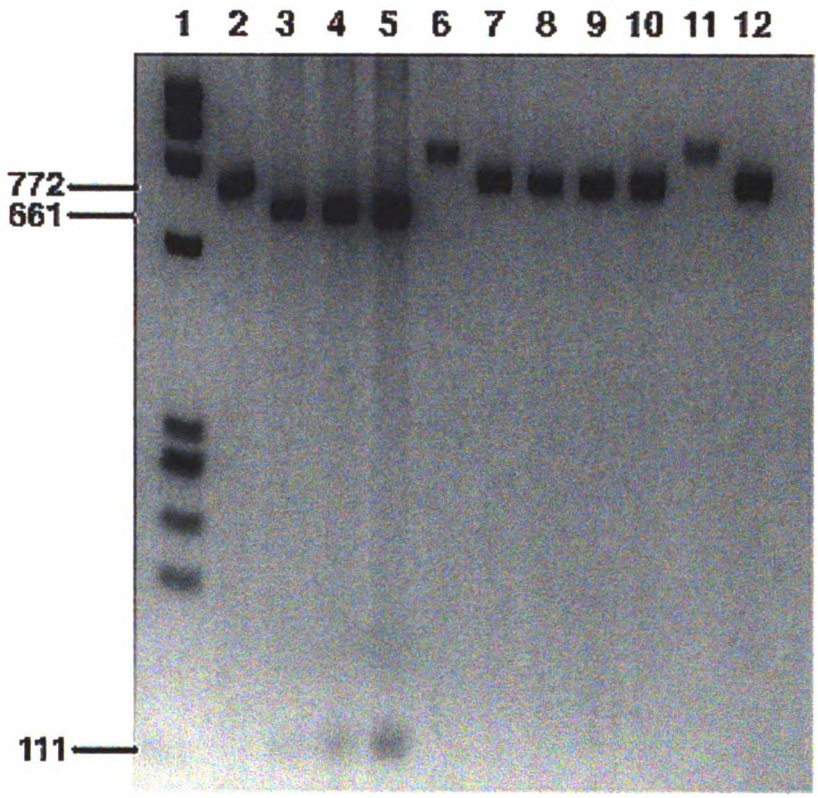

Figure 3.2. *Stu*I fragments resolved on a 4% agarose gel. Lane 1 marker φX174 *Hae*III digested DNA; lane 2 undigested PCR amplified *Stu*I fragment; lanes 3-5 show mutations not involving the RE recognition site and are referred to as non-RE-induced mutants; lanes 6-12 show mutations that did involve the RE recognition sequence and are referred to as RE-induced mutants. RE-induced mutants shown typically comigrate with the uncut control (lane 2) unless illegitimate recombination of DNA ends results in large insertions (lanes 6 and 11) or large deletions (none shown).

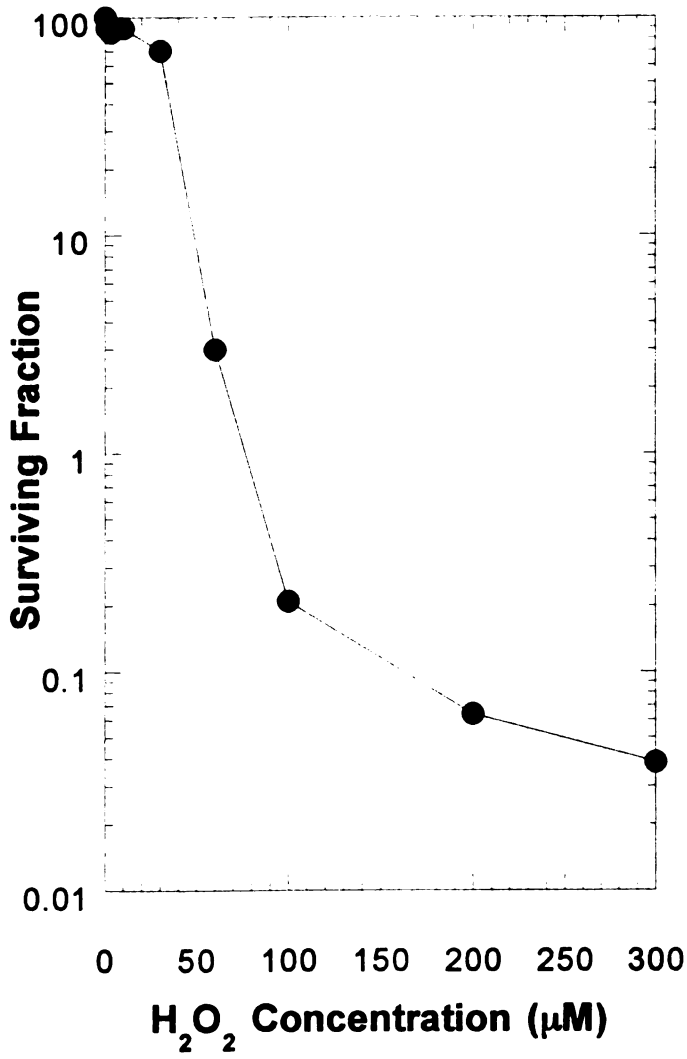

Figure 3.3. Cell survival following a 20 minute exposure to micromolar levels of hydrogen peroxide at 34^{o}C.

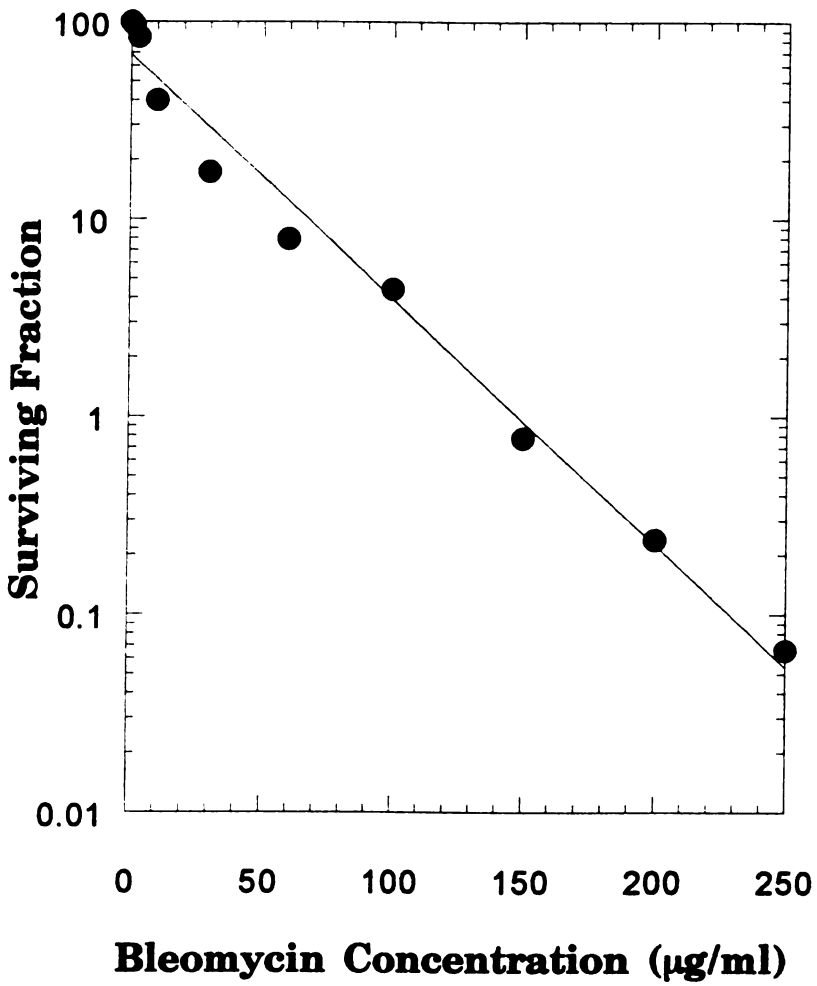

Figure 3.4. Cell survival following a four hour exposure to bleomycin at 34°C. All data is fitted with a least mean square straight line of the form $y=ce^{-mx}$. From the slope m of this line, the D_{37} (the drug concentration needed to reduce the fraction of surviving cells from 1 to $1/e$) under the described conditions was calculated to be 35 μg/ml.

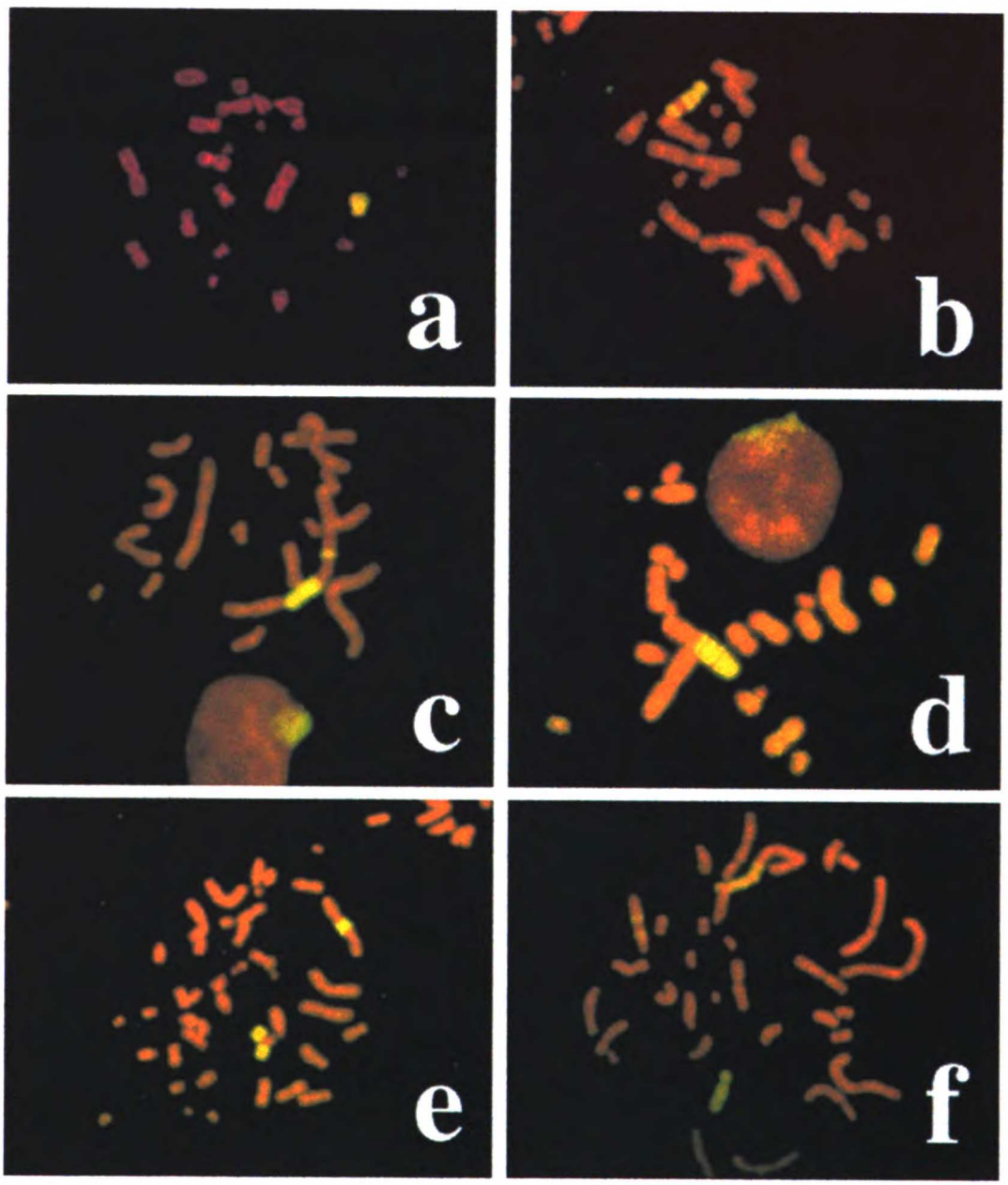

Figure 3.5. Examples of chromosome rearrangements found in one normal and one unstable clone, isolated from individual progenitor cells surviving a four hour exposure at 34^{o}C to 100 μg/ml of bleomycin. a) normal metaphase showing nonrearranged human chromosome 4. b-f) series of photos showing the types of complex rearrangements involving human chromosome 4 that can be encountered in just one unstable clone.

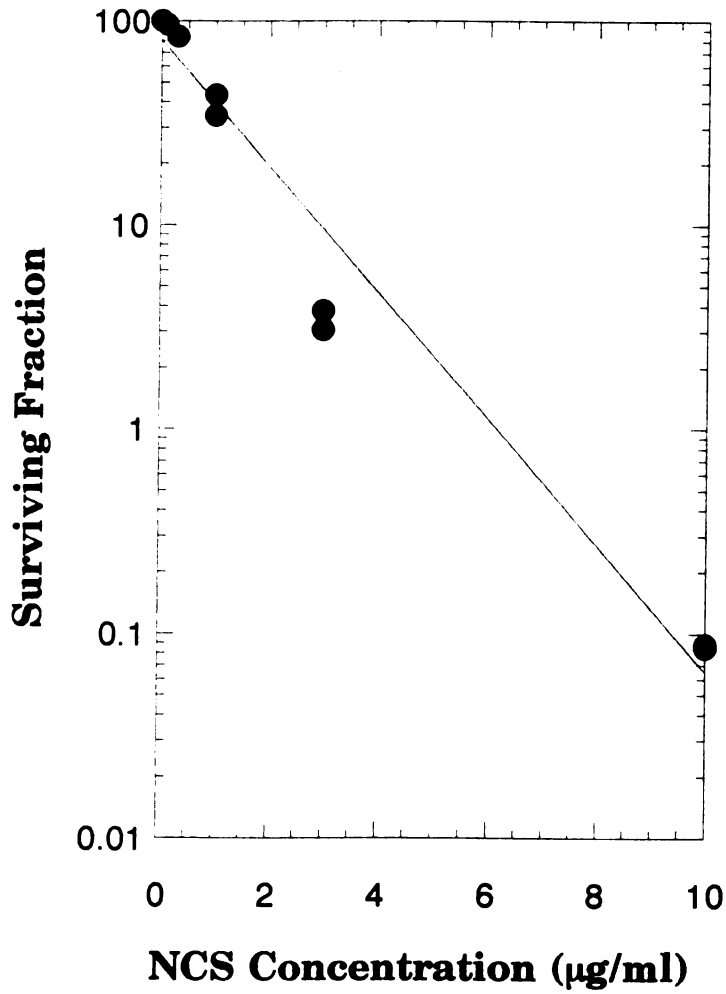

Figure 3.6. Cell survival following a 1 h exposure to neocarzinostatin at 34°C. All data is fitted with a least mean square straight line of the form $y=ce^{-mx}$. From the slope m of this line, the D_{37} (the drug concentration needed to reduce the fraction of surviving cells from 1 to $1/e$) under the described conditions was calculated to be 1.4 μg/ml.

Chapter 4 Chromosomal Instability and Its Relationship to Other End Points of Genomic Instability

SUMMARY

Chromosomal destabilization is one endpoint of the more general phenomenon of genomic instability. We previously established that chromosomal instability can manifest in clones derived from single progenitor cells several generations after x-irradiation. To understand the potential relationship between chromosomal destabilization and the other endpoints of genomic instability, we generated a series of chromosomally stable and unstable clones by exposure to X-rays. All clones were derived from the human-hamster hybrid line GM10115, which contains a single copy of human chromosome four in a background of 20 to 24 hamster chromosomes. These clones were then subjected to a series of assays to determine if chromosomal instability is associated with a general "mutator phenotype" and if it modulates other endpoints of genomic instability. Thus, we analyzed clones for sister chromatid exchange, delayed reproductive cell death, delayed mutation, mismatch repair, and delayed gene amplification. Statistical analyses performed on each group of chromosomally stable and unstable clones indicated that, although individual clones within each group were significantly different from unirradiated clones for many of the endpoints, there was no significant correlation between chromosomal instability and sister chromatid exchange, delayed mutation, and mismatch repair. Delayed gene amplification was found to be marginally correlated to chromosomal instability ($p< 0.1$), and delayed reproductive cell death (the persistent reduction in plating efficiency after irradiation) was found to be significantly correlated ($p<0.05$). These correlations may be explained by chromosomal destabilization, which can mediate gene amplification and can result in cellular lethality. These data implicate multiple molecular and genetic pathways leading to different manifestations of genomic instability in GM10115 cells surviving exposure to DNA damaging agents.

INTRODUCTION

Chromosomal instability, an endpoint of genomic instability, is one of the many delayed effects associated with acute exposure to ionizing radiation. The multiple phenotypes of genomic instability can include a variety of karyotypic abnormalities, such as chromosomal destabilization, sister chromatid exchange (SCE) and aneuploidy, gene mutation and amplification, clonal heterogeneity, delayed reproductive cell death, alterations in DNA repair capacity, neoplastic transformation, and metastatic progression (93). These events may contribute to an increased rate of genetic change, characteristic of genomic instability, and are hypothesized to be crucial in multistep carcinogenesis (76, 104).

The persistent destabilization of chromosomes has been described in both clonal and nonclonal populations for a variety of mammalian cell lines surviving exposure to both sparsely and densely ionizing radiations (55, 60, 62, 84, 85, 124). Of particular interest is the necessarily dynamic nature of this phenotype, which can be observed in clonal descendants of single progenitor cells several generations after irradiation (84). Some fraction of the gross chromosomal change observed in unstable clones might provide the driving force behind a "mutator phenotype" that could further compromise normal cellular physiology. Support for this notion is found in the large number of neoplasms that exhibit many of the same endpoints, collectively called genomic instability (76, 93, 104,77, 91, 95). Other forms of genetic instability have been hypothesized to account for a heritable mutator phenotype that can lead to several types of delayed mutation (24, 34, 39, 58, 70, 74, 109, 111), altered tissue responses (51), persistent reduced cloning efficiency (21-23), and neoplastic transformation and carcinogenesis (27, 87, 88).

Despite extensive research into each of these cellular processes, very little is known about the potential relationships among the molecular and cellular endpoints associated with both the mutator phenotype and genomic instability. In this study, we investigated the potential relationships between chromosomal instability and other endpoints of genomic

instability, specifically, SCE, delayed reproductive cell death, delayed mutation at the *HPRT* locus, DNA mismatch repair (MMR), and delayed gene amplification at the *CAD* locus.

MATERIALS AND METHODS

Cell culture

All chromosomally stable and unstable subclones described in this report were derived from the parental human/hamster hybrid cell line GM10115, which contains one copy of human chromosome four in a background of 20 to 24 hamster chromosomes. Cells were maintained as monolayers in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 0.2 mM L-proline, and grown at 34°C in humidified incubators containing 5% $C0_2$ in air. Under these conditions, cell doubling times of 21 to 23 hours were obtained.

The Chinese hamster ovary cell lines CHO-MT and CHO-B, generous gifts from Michael Armstrong (Merck Research Laboratories, Philadelphia, PA) and Margerita Bignami (Istituto Superiore di Sanita, Rome, Italy), were used as the positive and negative controls, respectively, in the MMR assay. Cells were grown in αMEM, 10% FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM L-glutamine.

Isolation and characterization of subclones

We generated the following three distinct classes of clones: (*a*) x-irradiated and chromosomally stable, (*b*) x-irradiated but chromosomally unstable, and (*c*) unirradiated. Chromosomally stable and unstable subclones were isolated from exponentially growing populations of GM10115 cells after irradiation with 10 Gy of X-rays, delivered at 250 cGy/min at ambient temperature by using a Philips RT250 X-ray machine (250 kVp, 15

mA, half-value layer 1.0 mm Cu). Immediately after irradiation, sufficient cells were plated (~50,000/dish) such that 5-10 colonies (~100 cells/colony) originating from single cells survived in each 100-mm tissue culture dish. Subclones of unirradiated control cells were obtained by plating 20 log-phase cells in each of twenty 100-mm dishes. After two to three weeks of growth, colonies were picked at random by using sterile dacron swabs dipped in trypsin and were expanded to mass culture (1-3 x 10^6) in 25 cm^2 flasks. Clonally expanded cultures were then harvested for the analysis of potential chromosomal instability (see below).

Collection of metaphase cells

After two to three hours of exposure to the mitotic spindle inhibitor Colcemid (2 x 10^{-7} M final concentration), mitotic cells were collected from each of the clonally expanded cultures by mitotic shake-off and swollen in hypotonic 0.075 M KCl solution for 15 min at 37^{o}C, dehydrated in 100% methanol, and fixed in methanol:acetic acid (3:1 vol/vol). Mitotic cells were dropped onto precleaned glass microscope slides and allowed to air dry for 1 day at ambient temperature before storage at -20^{o}C.

Fluorescence in situ hybridization

For analysis of delayed chromosomal instability we used fluorescence *in situ* hybridizataion of a labeled probe to the human chromosome in the human-hamster hybrid cells. Labeling of this Bluescript vector-based library of human chromosome four-specific DNA sequences (pBS4) and subsequent hybridization protocols were performed as described previously (7). Briefly, slides were hybridized at 37°C for two days with 35 µl of hybridization mixture containing 50 ng of biotinylated probe. Indirect detection of the hybridized probe to the human chromosome four target involved sequential 15-min incubations with fluorescein-conjugated avidin (Oncor) and an anti-avidin antibody,

interspersed with five minute washes in phosphate-buffered detergent. Chromosomes were counterstained with propidium iodide in Antifade (Oncor), fitted with glass coverslips, and stored at 4°C until analysis by fluorescence microscopy.

Cytogenetic analysis

After fluorescence *in situ* hybridization, metaphase chromosomes were analyzed by means of a Zeiss Axioskop microscope equipped with a dual-band pass fluorescein isothiocyanate/Texas Red filter set. In this system, the hamster chromosomes appear red (propidium iodide emission), and the human chromosome four that hybridizes with the biotinylated probe appears yellow-green (fluorescein emission).

Chromosomally unstable clones were defined as those having at least three distinct aberrant metaphase subpopulations involving rearrangements of human chromosome four (84). Any clone showing fewer than three such rearrangements was considered chromosomally stable. All clones were derived from single progenitor cells, and 200 individual metaphase spreads were analyzed for each clone. Chromosome rearrangements were categorized as described by Tucker *et al.* (143), and only those rearrangements involving human chromosome four were scored.

Experimental protocol for comparison of clones

In all experiments unirradiated control clones were compared with subclones surviving 10 Gy of X-rays. The x-irradiated clones were classified as chromosomally stable (a total of 14 clones showing <3 aberrant metaphase subpopulations) or unstable (a total of 12 clones with ≥5 aberrant metaphase subpopulations). Individual clones were expanded into six 150cm^2 tissue culture flasks then analyzed in a series of parallel experiments designed to facilitate a side-by-side comparison of results (Figure 4.1).

Plating efficiency

For determination of the plating efficiencies for each of the three groups of clones, cells were diluted and 100 cells were plated in triplicate in 100-mm dishes containing 10 ml of αMEM supplemented with 10% dialyzed FBS. Cells formed colonies over one to two weeks, after which time the plates were fixed and stained (0.1% crystal violet in 25% ethanol), and colonies with >50 cells were scored.

Delayed gene amplification assay

Delayed gene amplification was assayed at the *CAD* gene locus. Each clone was thawed and grown in the presence of αMEM supplemented with 10% dialyzed FBS for 3-4 days. *CAD* amplification was assayed by plating 3×10^5 cells into each of five 100-mm dishes containing 10 ml of medium and 1×10^{-4} M N-(phosphonacetyl)-L-aspartate (PALA), a transition state inhibitor of ATCase (obtained from the drug synthesis branch of the National Cancer Institute). To optimize the selection of clones having genuine *CAD* amplicons, the concentration of PALA was based on a level determined previously to be nine times the LD_{50} (129). Three to four weeks of growth were generally required before PALA-resistant cells grew into colonies of sufficient size for counting, isolation, and expansion. Fresh medium containing PALA was replenished two times each week throughout the selection period.

Sister chromatid exchange analysis

Each group of clones was cultured in Dulbecco's modified Eagle's medium in the presence of 2×10^{-5} M BrdU (Sigma) for 44 hours, which was optimal for two complete replication cycles and the subsequent detection of SCE (94, 96). Metaphase cells were prepared as described above, and BrdU-substituted chromosomes were stained by a slightly modified fluorescence-plus-Giemsa procedure (112). Briefly, slides were washed

twice in Sorensen's buffer (15 mM potassium phosphate, pH 7.6) for 10 minutes before incubating them in Sorensen's buffer containing 20 μg/ml of Hoechst dye 33258 (Sigma) for 20 minutes. Slides were rinsed, covered with glass coverslips, exposed to UVA light (27 Wm^{-2} incident output) at 55°C for four minutes, and stained for 10 minutes with a 5% solution of Giemsa in water.

Delayed mutation assay

Delayed mutation was assayed at the *HPRT* locus for each of the three groups of clones. Clones were thawed and grown in αMEM supplemented with 10% dialyzed FBS for 3 to 4 days. ***HPRT***$^-$ mutants were selected by plating 3 x 10^5 cells into each of five 100-mm dishes containing 10 ml of medium and 10 μg/ml of 6-thioguanine. This concentration of 6-thioguanine had been determined to minimize the selection of false positives. Two to three weeks of growth were generally required before 6-thioguanine-resistant cells grew into colonies of sufficient size for counting, isolation, and expansion.

Gel shift assay for mismatch binding

For examination of the potential relationship between MMR and chromosomal stability, clones were grown for the preparation of cell extracts with which mismatch binding assays and immunologic blot analyses of protein levels could be performed. Whole-cell extracts of all clones were prepared as described by Jiricny *et al.* (59). Extracts made in this manner routinely gave high yields of biologically active protein as determined by topoisomerase I unwinding following the procedure provided by the manufacturer (Sigma), and polymerase mediated extension assays (78). Protein levels were determined by the method of Bradford (16).

For determination of whether cell extracts contained an activity capable of binding to DNA mismatches, a ^{32}P-end-labeled oligonucleotide 5'-^{32}P-

GGGAAGCTGCCAGGCCCCAGTGTCAGCCTCCTATGCTC-3'

was annealed to a complementary strand that was either an exact match (homoduplex) or contained a single noncomplementary thymine opposite the highlighted and underlined guanine (G) in the oligonucleotide shown above (heteroduplex) (34). For annealing of both homo- and heteroduplexes, a 1:2 mixture of labeled and unlabeled oligos was heated to 90°C for two minutes in 30 μl of STE (100 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA). The solution was then slowly cooled to 60°C for 60 minutes, then to 55°C for 45 minutes, and finally to ambient temperature.

Mismatch binding reactions and nondenaturing polyacrylamide gel electrophoresis were performed as described by Stephenson and Karran (138). Briefly, for binding reactions, homo- and heteroduplex DNA constructs were incubated with cell extracts prepared from each of the three groups of clones. Reactions were run at ambient temperature in a total volume of 20 μl in 25 mM HEPES-KOH (pH 8.0), 0.5 mM EDTA, and 0.5 mM dithiothreitol. Each reaction contained one μg of poly(dI•dC)-poly(dI•dC) and 40 fmol of unlabeled homoduplex as noncompetitive inhibitors. Fifteen micrograms of cell extract was added to the reaction mixture, which was incubated for five minutes at ambient temperature; 20 fmol of end-labeled heteroduplex was then added for an additional 20 minutes. After incubation, 10-μl aliquots were removed from the final reaction mixture, and DNA-protein complexes were resolved on a 6% polyacrylamide gel. After electrophoresis, gels were dried and exposed on a Phosphorimager (Molecular Dynamics) for data digitization and image analyses. For competition experiments, the indicated

amount of nonradioactive homo- or heteroduplex was substituted for the 1 μg of poly(dI•dC)-poly(dI•dC) and 40 fmol of unlabeled homoduplex.

Immunologic analysis of MSH2 and MLH1 proteins

By using standard techniques (89), five μg of each cellular extract was separated on sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Danvers, PA). Blots were blocked and incubated with antibodies to hMSH2 and hMLH1 in TBST buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 0.5% Tween-20) containing 10% nonfat dry milk. Polyclonal hMSH2 and hMLH1 antibodies were generated by Oncogene Sciences (Cambridge, MA) using overproduced and purified proteins and were characterized for specificity in total cell extracts. Antibodies were used at dilutions from 1:1000 to 1:2500. Proteins were detected by means of enhanced chemiluminescence as recommended by the manufacturer (Amersham, Arlington Heights, IL). Differences in MSH2 and MLH1 protein levels in genomically stable and unstable subclones were quantified by comparison to levels of an α-tubulin loading standard, as described previously (89).

Statistical analysis

Analysis of variance (ANOVA) was used first to ascertain whether the difference between the means in the subsets of clones was significant. When ANOVA indicated significance, data were scrutinized further by performing unpaired two-tailed t-tests, applying the Bonferroni correction when necessary (43).

RESULTS

Chromosomal instability

Cytogenetic classification of the clones selected for further study is shown in Table 4.1. While the stable clones, by definition, showed no more than two sub-populations of metaphase cells containing rearrangements involving human chromosome four, the unstable clones showed up to 21 distinct subpopulations containing rearrangements of the human chromosome. Included in the group of stable clones, were two clones showing a rearrangement of human chromosome four. The rearrangements were identical in all metaphases from the clone and were presumably induced directly by radiation, indicating that while the progenitor cell had been damaged it did not become genomically unstable. The absence of rearrangements involving human chromosome four in > 50,000 metaphases from unirradiated GM10115 cells (data not shown) indicates the marked stability of this chromosome in the hamster background and shows that spontaneous rearrangement of the chromosome was not a consideration.

Delayed reproductive cell death

To determine whether the clones exhibited differences in long-term survival, reflecting delayed reproductive cell death, we diluted and plated cells in triplicate and determined plating efficiencies by clonogenic assay (Table 4.2). Not only did clones surviving x-irradiation exhibit reduced plating efficiencies compared to unirradiated controls, but irradiated chromosomally unstable clones showed reduced plating efficiencies compared to irradiated stable clones ($p<0.05$), reflecting a significantly higher incidence of delayed reproductive cell death in the unstable clones.

Gene amplification

The stable and unstable clones were then analyzed for their ability to amplify the *CAD* gene in response to PALA selection. Calculated amplification frequencies for all

clones were corrected for differences in plating efficiency. *CAD* gene amplification frequencies for irradiated clones were significantly higher than those for the unirradiated parental clones (Table 4.3). A marginally significant p value was obtained ($0.05<p<0.1$) in a comparison of stable and unstable clones indicating a positive correlation between the delayed effects of gene amplification and chromosomal instability.

Sister chromatid exchange

To test whether any of the clones exhibited instability at a second cytogenetic endpoint, we measured SCE frequency in metaphase chromosomes (Figure 4.2). For each clone, the number of chromosomes and the number of SCE in 30 individual metaphases were scored (Table 4.4). Statistical analysis indicated no significant difference between stable or unstable clones.

Delayed mutation

Stable and unstable clones were then analyzed for mutation frequency at the *HPRT* locus (Table 4.5). Mutation frequencies were calculated according to the number of plated cells and were corrected for differences in plating efficiency as indicated in Table 4.2. For those clones in which no mutations were observed, (clones 7, 10, 24, and 129) upper estimates of the mutation frequency are provided. As with *CAD* gene amplification frequency, significant increases in mutation frequency were observed for irradiated compared to unirradiated clones. Unlike *CAD* however, no significant increases in mutation frequency were observed for unstable compared to stable clones.

Mismatch repair

Stable and unstable clones were then examined for G-T mismatch binding activity. Cell extracts were incubated with a radioactively end-labeled DNA heteroduplex containing a G-T mismatch at a central base pair, and reactions were resolved by polyacrylamide gel

electrophoresis (Figure 4.3). A higher molecular weight band indicated the presence of a more slowly migrating heteroduplex bound by protein. As expected, cell extracts isolated from CHO-MT cells were proficient in mismatch binding, whereas cell extracts prepared from CHO-B cells were not capable of retarding the mobility of the heteroduplex through the gel (Figure 4.3) (10). All stable and unstable clones demonstrated proficiency for binding the heteroduplex.

Cell extracts from CHO-MT cells were then used to confirm that the heteroduplex binding activity was specific for the presence of the G-T mismatch (Figure 4.4). At equal concentrations, the heteroduplex was more effective at competing G-T mismatch binding than was the homoduplex. Similar results were obtained when competition experiments were run in the presence of noncompetitive inhibitors or nonspecific DNA templates (data not shown).

To ascertain whether the mismatch binding activity might be due to the presence of the MSH2 protein, we analyzed cell extracts from unstable clones and unirradiated clones by immunologic blot analysis with an antibody probe specific to the MSH2 protein. Scanning densitometry indicated no major differences in the levels of MSH2 between unstable and unirradiated clones (Figure 4.5). Similar results were found for all stable clones. In related experiments, cell extracts from the clones were probed for the presence of hMLH1, again no significant differences in hMLH1 levels between stable and unstable clones were detected (data not shown).

DISCUSSION

We undertook this investigation to address the hypothesis that chromosomally unstable clones surviving x-irradiation exhibit other endpoints associated with genomic instability. We rationalized that chromosomal instability could provide the driving force to directly or indirectly compromise various mechanisms that maintain the integrity of the genome, from the nucleotide to the chromosomal level. The quantity, quality, and

complexity of the observed chromosome rearrangements underscore the dynamic nature of this phenotype, and suggest that radiation initiates a series of events that can mediate the persistent destabilization of chromosomes. Although, the precise molecular mechanisms leading to gross chromosomal destabilization are not known, they do not appear to lead to the formation of SCE. Since no significant difference in SCE frequency between stable and unstable clones was found, the mechanisms of SCE formation are different from those that promote gross interchromosomal recombination.

Determining plating efficiency by clonogenic assay is the means for detecting heritable lethal mutations (8, 11, 12, 33, 97, 135) and delayed reproductive cell death (21) and provides a measure of the replicative integrity of a cell. In this study, all clones surviving 10 Gy of X-rays exhibited significantly reduced plating efficiencies compared to unirradiated controls. These findings corroborate those of earlier studies (21, 22, 84, 135). Chromosomally unstable cells also exhibited a reduced plating efficiency compared to stable clones. The positive correlation observed between delayed reproductive cell death and chromosomal instability suggests that the dynamic formation and missegregation of abnormally rearranged chromosomes in the unstable clones contributes to reproductive failure.

A number of studies have emphasized the importance of chromosome destabilization and breakage and refusion events in mediating the processes responsible for gene amplification (47, 117, 122, 157). Thus, we reasoned that gene amplification, as measured by PALA resistance, might be increased in those clones that exhibited marked chromosomal instability. Irradiated clones did show a significantly higher amplification frequency compared to unirradiated controls, and chromosomally unstable clones also showed a substantially higher amplification frequency than did stable clones. Its uncertain why the level of gene amplification observed was not higher in those clones (115, CS9, LS1) showing the most chromosome fusions. We should point out that we are only looking at one chromosome comprising 5% of the genome, and that fusion events

involving other chromosomes, including the hamster chromosome with the *CAD* gene, were not scored. Nevertheless, these observations suggest that chromosome instability may be necessary but not sufficient for gene amplification.

Chang and Little (24) have shown that clones derived from CHO cells surviving exposure to x-irradiation demonstrate a persistent increase in mutation frequency at the *HPRT* locus. They hypothesized that some form of genetic instability results in a heritable mutator phenotype. To investigate whether a change in mutation frequency coincides with a change in chromosomal stability, we assayed chromosomally stable and unstable clones for delayed mutation at the *HPRT* locus. Mutation frequencies were increased significantly in some clones surviving radiation exposure, but there was no overall correlation between chromosome stability and delayed mutation.

Because an inverse relationship between plating efficiency and *HPRT* mutation frequency was reported by Chang and Little (24), we plotted plating efficiency against *HPRT* mutation frequency and *CAD* amplification frequency (Figure 4.6). We found no discernible relationship between plating efficiency and mutation frequency (Figure 4.6A) or between plating efficiency and gene amplification (Figure 4.6B).

Since it is well established that faithful MMR activity is critical in maintaining genomic integrity (65, 92, 125), we examined stable and unstable clones for MMR binding capacity and MSH2 and MLH1 protein levels. We investigated the functionality and presence of representative gene products of the MMR pathway in cell extracts derived from our clones by testing their ability to bind to a DNA heteroduplex containing a mismatched base pair. All cell extracts were capable of binding and retarding the mobility of the DNA heteroduplex through the gel (Figure 4.3). This binding was specific for the mismatched heteroduplex, because homoduplex and poly(dI•dC) templates were unable to compete for binding activity as effectively as the heteroduplex. Subsequent analysis of cell extracts on immunologic blots showed no significant differences in levels of hMSH2 or hMLH1 protein.

Our results indicate that all GM10115 clones contain functional MSH2 protein, which can presumably bind specifically to DNA templates containing mismatched base pairs. These data indicate that MMR and chromosomal stability are not interdependent. Nonetheless, the lack of a correlation between MMR, delayed mutation, and chromosomal stability reported here is consistent when seen in the context that functional MMR protects cells against the enhancement of mutation but not necessarily against gross chromosomal change.

Loeb (76) has argued that a mutator phenotype is required for multistep carcinogenesis. This is based on the observation that the spontaneous mutation rate is insufficient to account for the high frequency of mutations in cancer cells and that the vast majority of cancer cells show multiple chromosomal abnormalities. The emerging concept is that the genomes of cancer cells are unstable and that this instability results in a cascade of mutations, some of which enable cancer cells to bypass host regulatory processes (77). Our data suggest that chromosomally unstable GM10115 clones do not possess a mutator phenotype. Nonetheless, some clones did exhibit increases in several of the endpoints associated with genomic instability, such as gene mutation (i.e., clones 118, 132, 146, and LS12) and gene amplification (i.e., clones 2, 7, 24, 132, 138 and LS1). Collectively, however, chromosomally unstable and stable clones were not significantly different. This is not altogether unexpected, because the multiple endpoints associated with genomic instability may not be driven by the same set of upstream events or the same set of downstream processes.

We have presented a comprehensive set of parallel experiments using human/hamster hybrid GM10115 cells in which the same subsets of irradiated and chromosomally stable, irradiated and chromosomally unstable, and unirradiated subclones were examined and compared. The data unambiguously demonstrate a close relationship between chromosomal instability and delayed reproductive cell death and, to a lesser extent,

Table 4.1. Cytogenetic classification of chromosomally stable and unstable clones

Stable clones	Types of chromosome aberrations[a]					No. Metaphase subpopulations[f]	% Aberrant metaphases[g]
	RT[b]	NRT[c]	Dicentrics	AF[d]	Misc.[e]		
102	0	0	0	0	0	0	0
103	99	1	0	0	0	2	100
110	99	0	0	1	0	2	100
114	100	0	0	0	0	1	100
118	0	0	0	0	0	0	0
126	0	7.5	0	0	0	1	7.5
129	98	2	0	0	0	2	100
130	0	0	0	0	0	0	
132	100	0	0	0	0	1	100
133	100	0	0	0	0	1	100
141	1	0	0	0	0	1	1
145	0	0	0	0	0	0	0
146	0	0	0	0	0	0	0
152	0	4	1	0	0	2	5

[a] Aberration types given as a percentage of the total metaphases scored.

[b] RT, reciprocal translocations.

[c] NRT, nonreciprocal translocations.

[d] AF, acentric fragments.

[e] Miscellaneous aberrations, including rings, triradials, quadraradials, etc.

[f] Number of different abnormal metaphase subpopulations showing distinct types of chromosome aberrations.

[g] Percentage of metaphases scored showing chromosomal aberrations.

Table 4.1 (continued)

Unstable clones	Types of chromosome aberrations[a]					No. Metaphase subpopulations[f]	% Aberrant metaphases[g]
	RT[b]	NRT[c]	Dicentrics	AF[d]	Misc.[e]		
2	6	81	4	7	0	14	98
7	39	60	1	0	0	10	100
10	95	0.5	4	0.5	0	5	100
11	0.5	57.5	2	40	0	11	100
23	93	4.5	2.5	0	0	8	100
24	95.5	3.5	0.5	0	0.5	8	100
115	12	45	40	2	1	21	100
138	22	14	0	0	2	6	38
147	1	98	0	1	0	7	100
CS9	5	19	74	0	0	11	98
LS1	2.5	0	79	2.5	0	9	84
LS12	4.5	89	6.5	0	0	9	100

[a] Aberration types given as a percentage of the total metaphases scored.
[b] RT, reciprocal translocations.
[c] NRT, nonreciprocal translocations.
[d] AF, acentric fragments.
[e] Miscellaneous aberrations, including rings, triradials, quadraradials, etc.
[f] Number of different abnormal metaphase subpopulations showing distinct types of chromosome aberrations.
[g] Percentage of metaphases scored showing chromosomal aberrations.

Table 4.2. Plating efficiency of chromosomally stable and unstable clones

Stable clones	% Plating efficiency	Unstable clones	% Plating efficiency[a]
GM10115[b]	83 +/- 2.5	2	31 +/- 3.2
GM10115[b]	92 +/- 3.1	7	46 +/- 3.9
GM10115[b]	85 +/- 1.2	10	50 +/- 2.6
102	72 +/- 6.2	11	67 +/- 4.7
103	65 +/- 3.0	23	45 +/- 1.2
110	64 +/- 5.5	24	28 +/- 1.4
114	74 +/- 8.2	115	36 +/- 3.4
118	69 +/- 2.0	138	28 +/- 2.9
126	52 +/- 1.7	147	51 +/- 3.5
129	60 +/- 5.7	CS9	46 +/- 3.5
130	57 +/- 0.88	LS1	72 +/- 3.2
132	69 +/- 7.5	LS12	62 +/- 8.1
133	84 +/- 5.3		
141	51 +/- 2.6		
145	47 +/- 0.67		
146	59 +/- 2.6		
152	51+/- 2.9		

[a]Values are expressed as mean +/- standard error.

[b]One of three independently derived subclones of the unirradiated parental cell line.

Table 4.3. Delayed *CAD* amplification frequency for chromosomally stable and unstable clones

Stable clones	CAD amplification frequency[a]	Unstable clones	CAD amplification frequency[a]
GM10115[b]	17 +/- 3.5	2	220 +/- 26
GM10115[b]	18 +/- 3.1	7	390 +/- 30
102	78 +/- 11	10	23 +/- 6.4
103	11 +/- 3.8	11	34 +/- 4.2
110	5.2 +/- 1.6	23	4 +/- 3.3
114	3.6 +/- 2.2	24	530 +/- 52
118	15 +/- 4.4	115	110 +/- 22
126	5.1 +/- 3.7	138	820 +/- 42
129	19 +/- 2.2	147	41 +/- 11
130	23 +/- 4.9	CS9	160 +/- 16
132	360 +/- 12	LS1	290 +/- 6.9
133	26 +/- 4.1	LS12	180 +/- 19
141	90 +/- 8.2		
145	100 +/- 15		
146	21 +/- 2.5		
152	130 +/- 13		

[a] Amplification frequencies corrected for plating efficiency and expressed per 10^6 cells; mean +/- standard error.

[b] One of three independently derived subclones of the unirradiated parental cell line.

Table 4.4. SCE in chromosomally stable and unstable clones

Stable clones	No. Chromosomes/ metaphase	No. SCE/ metaphase[a]	No. SCE/ chromosome
GM10115[b]	23 +/- 0.16	6.1 +/- 0.55	0.26
GM10115[b]	22 +/- 0.33	5.1 +/- 0.42	0.23
GM10115[b]	22 +/- 0.28	5.3 +/- 0.55	0.24
102	23 +/- 0.17	4.7 +/- 0.34	0.20
103	22 +/- 0.24	4.9 +/- 0.40	0.22
110	22 +/- 0.25	6.9 +/- 0.43	0.31
114	22 +/- 0.36	5.0 +/- 0.39	0.23
118	21 +/- 0.38	6.0 +/- 0.51	0.28
126	23 +/- 0.21	8.9 +/- 0.56	0.39
129	23 +/- 0.29	6.4 +/- 0.42	0.28
130	22 +/- 0.24	5.3 +/- 0.44	0.24
132	23 +/- 0.21	7.0 +/- 0.54	0.30
133	22 +/- 0.27	6.3 +/- 0.48	0.29
141	23 +/- 0.23	4.8 +/- 0.41	0.21
145	23 +/- 0.13	4.6 +/- 0.37	0.21
146	22 +/- 0.31	5.7 +/- 0.39	0.25
152	22 +/- 0.19	5.4 +/- 0.38	0.24

[a]Values are expressed as mean +/- standard error.

[b]One of three independently derived subclones from the unirradiated parental cell line.

Table 4.4 (continued)

Unstable clones	No. Chromosomes/ metaphase	No. SCE/ metaphase[a]	No. SCE/ chromosome
2	21 +/- 0.33	8.5 +/- 0.52	0.40
7	22 +/- 0.24	5.5 +/- 0.47	0.25
10	21 +/- 0.24	9.2 +/- 0.66	0.43
11	21 +/- 0.23	7.0 +/- 0.44	0.33
23	23 +/- 0.22	5.4 +/- 0.38	0.24
24	22 +/- 0.20	6.2 +/- 0.57	0.27
115	22 +/- 0.19	6.9 +/- 0.65	0.32
138	21 +/- 0.31	7.2 +/- 0.53	0.35
147	22 +/- 0.23	3.7 +/- 0.36	0.17
CS9	21 +/- 0.28	7.5 +/- 0.51	0.35
LS1	23 +/- 0.14	5.3 +/- 0.41	0.23
LS12	20 +/- 0.24	7.0 +/- 0.47	0.35

[a]Values are expressed as mean +/- standard error.

[b]One of three independently derived subclones from the unirradiated parental cell line.

Table 4.5. Delayed *HPRT* mutation frequency for chromosomally stable and unstable clones

Stable clones	HPRT mutation frequency[a]	Unstable clones	HPRT mutation frequency[a]
GM10115[b]	1.6 +/- 0.98	2	8.6 +/- 5.1
GM10115[b]	1.4 +/- 1.4	7	<1.4
GM10115[b]	0.78 +/- 0.78	10	<1.3
102	10 +/- 2.3	11	2.0 +/- 1.2
103	6.9 +/- 2.0	23	3.0 +/- 3.0
110	8.3 +/- 3.5	24	<2.4
114	2.7 +/- 1.8	115	3.3 +/- 1.7
118	16 +/- 3.1	138	4.8 +/- 2.9
126	6.4 +/- 3.5	147	1.3 +/- 1.3
129	<1.11	CS9	5.2 +/- 2.3
130	1.2 +/- 1.2	LS1	1.8 +/- 1.1
132	32 +/- 7.4	LS12	12 +/- 2.9
133	0.79 +/- 0.79		
141	1.3 +/- 1.3		
145	2.8 +/- 1.7		
146	33 +/- 7.3		
152	2.6 +/- 1.6		

[a] Mutation frequencies corrected for plating efficiency and expressed per 10^6 cells; mean +/- standard error.

[b] One of three independently derived subclones of the unirradiated parental cell line.

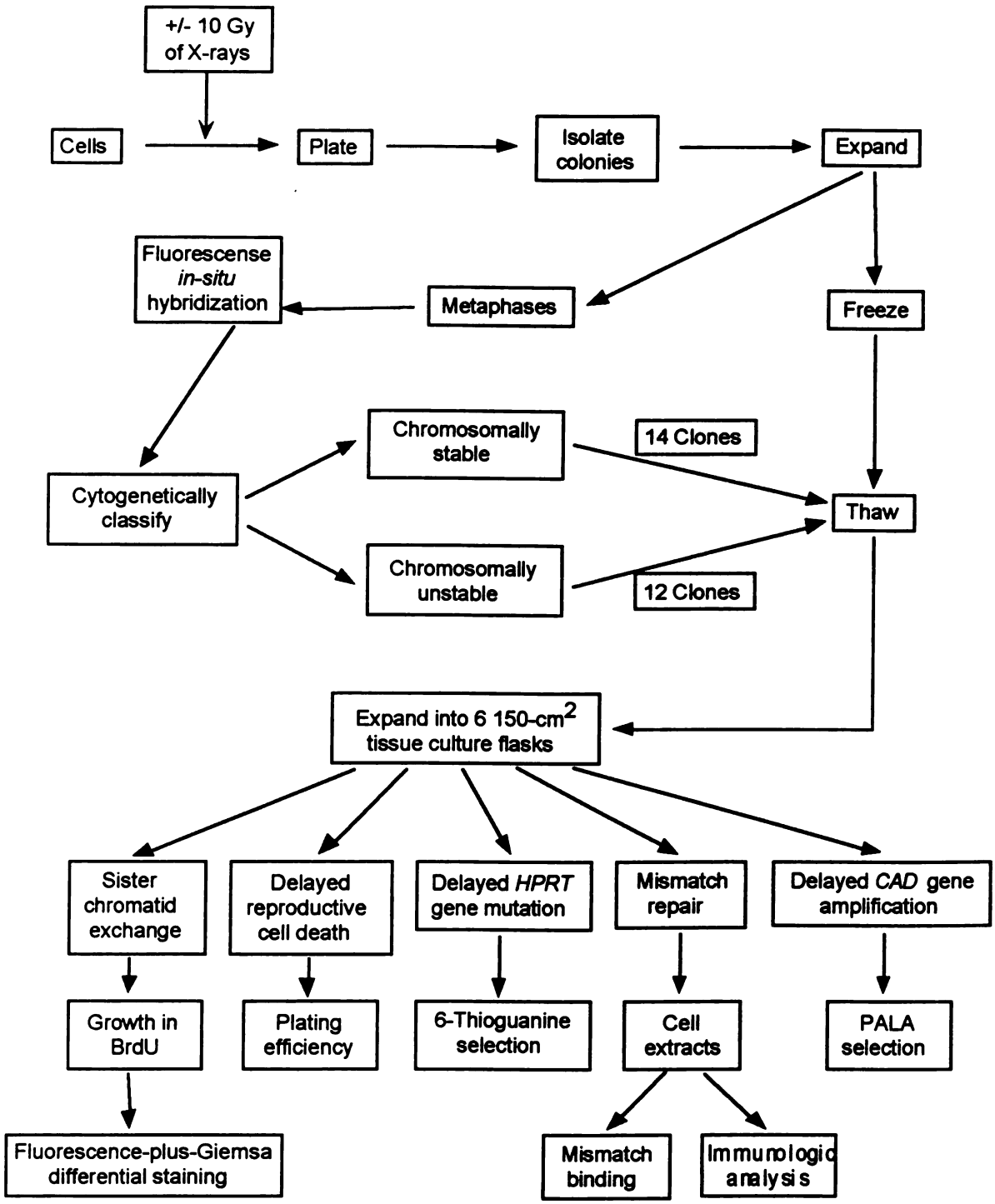

Figure 4.1. Schematic of the experimental protocol, outlining the generation and isolation of chromosomally stable and unstable subclones and the assays to which each subclone was subjected.

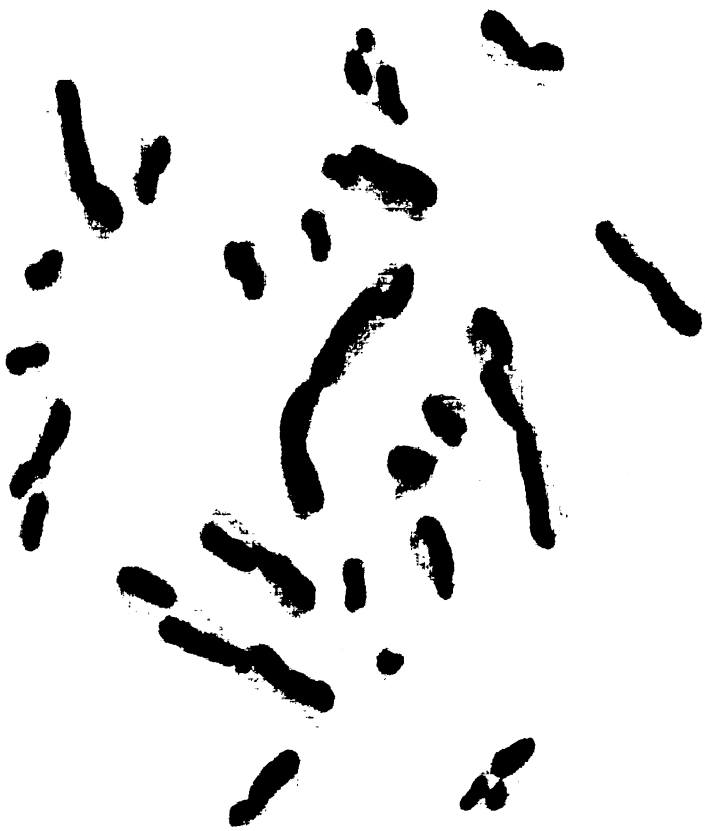

Figure 4.2. Metaphase of an unstable clone (LS12) showing SCE.

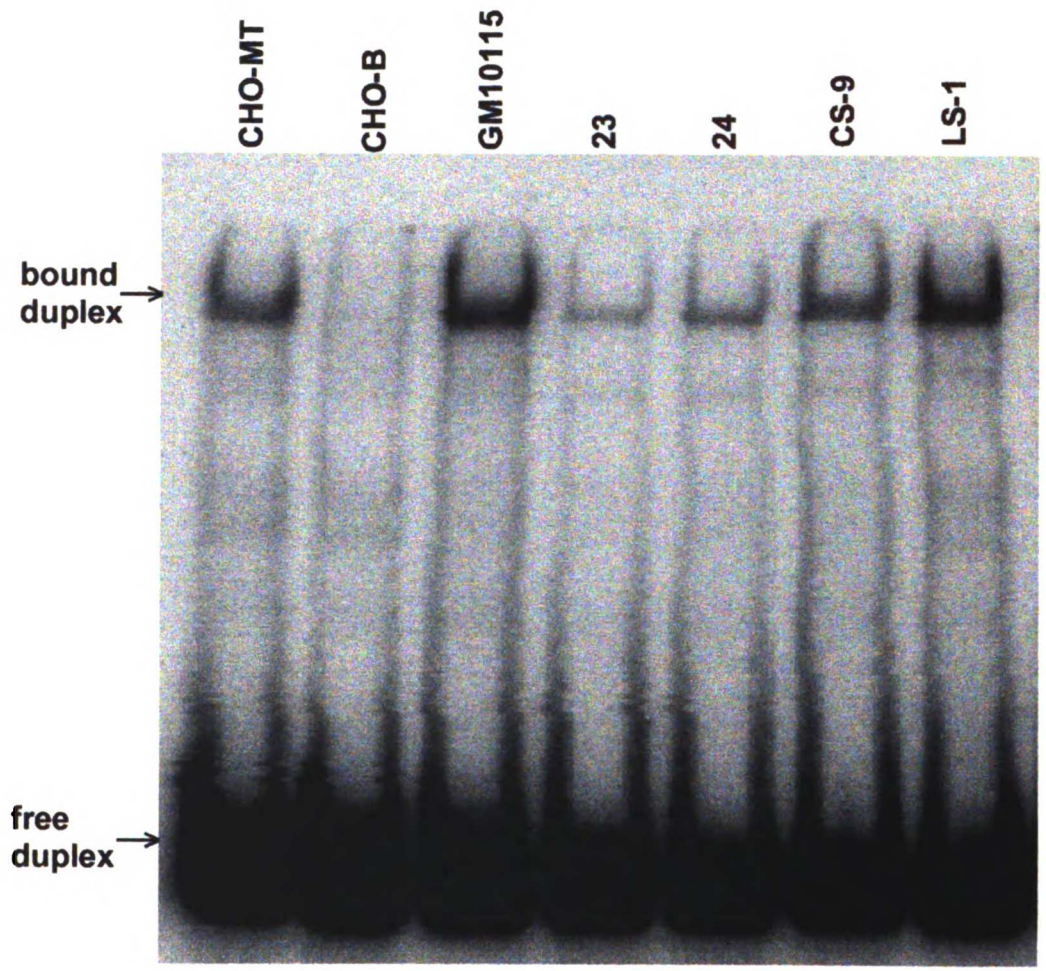

Figure 4.3. Mismatch binding in cell extracts prepared from CHO-MT cells (positive control), CHO-B cells (negative control), unirradiated GM10115 cells, and 4 chromosomally unstable clones (23, 24, CS9, LS1). Arrows indicate the positions of free and bound heteroduplex.

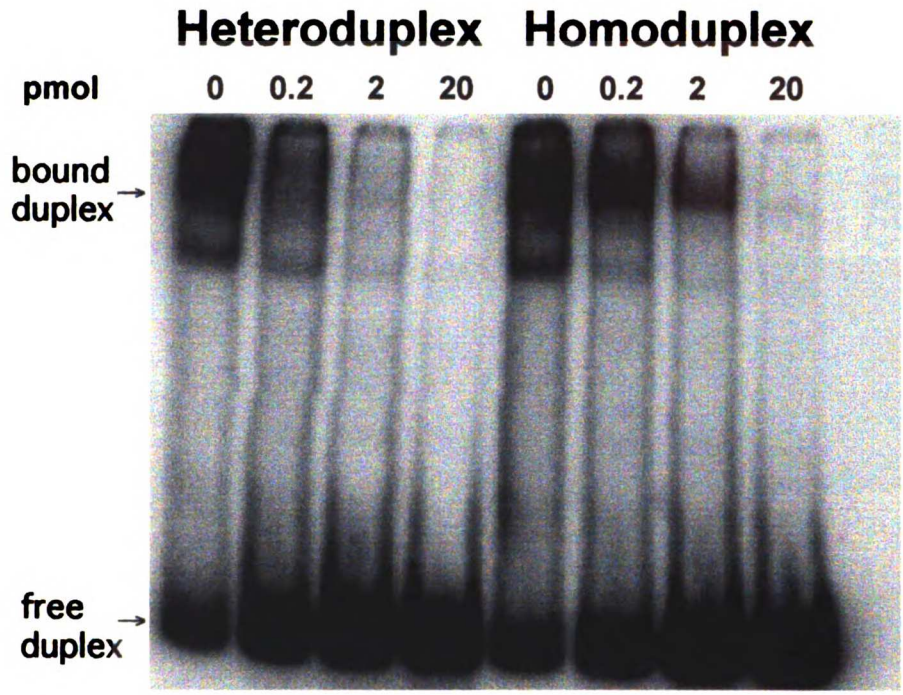

Figure 4.4. Specificity of G-T mismatch binding in CHO-MT extracts. Extracts were incubated with radiolabeled heteroduplex and the indicated amount of either heteroduplex or homoduplex competitor.

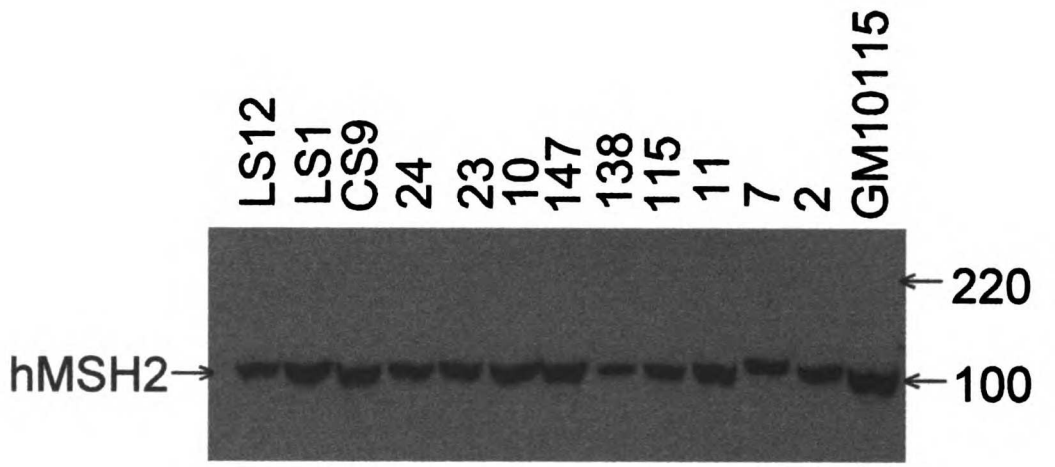

Figure 4.5. Immunologic blot of unstable clones and unirradiated GM10115 cell extracts using an antibody probe to MSH2. Arrows on the right indicate molecular mass (in kDa).

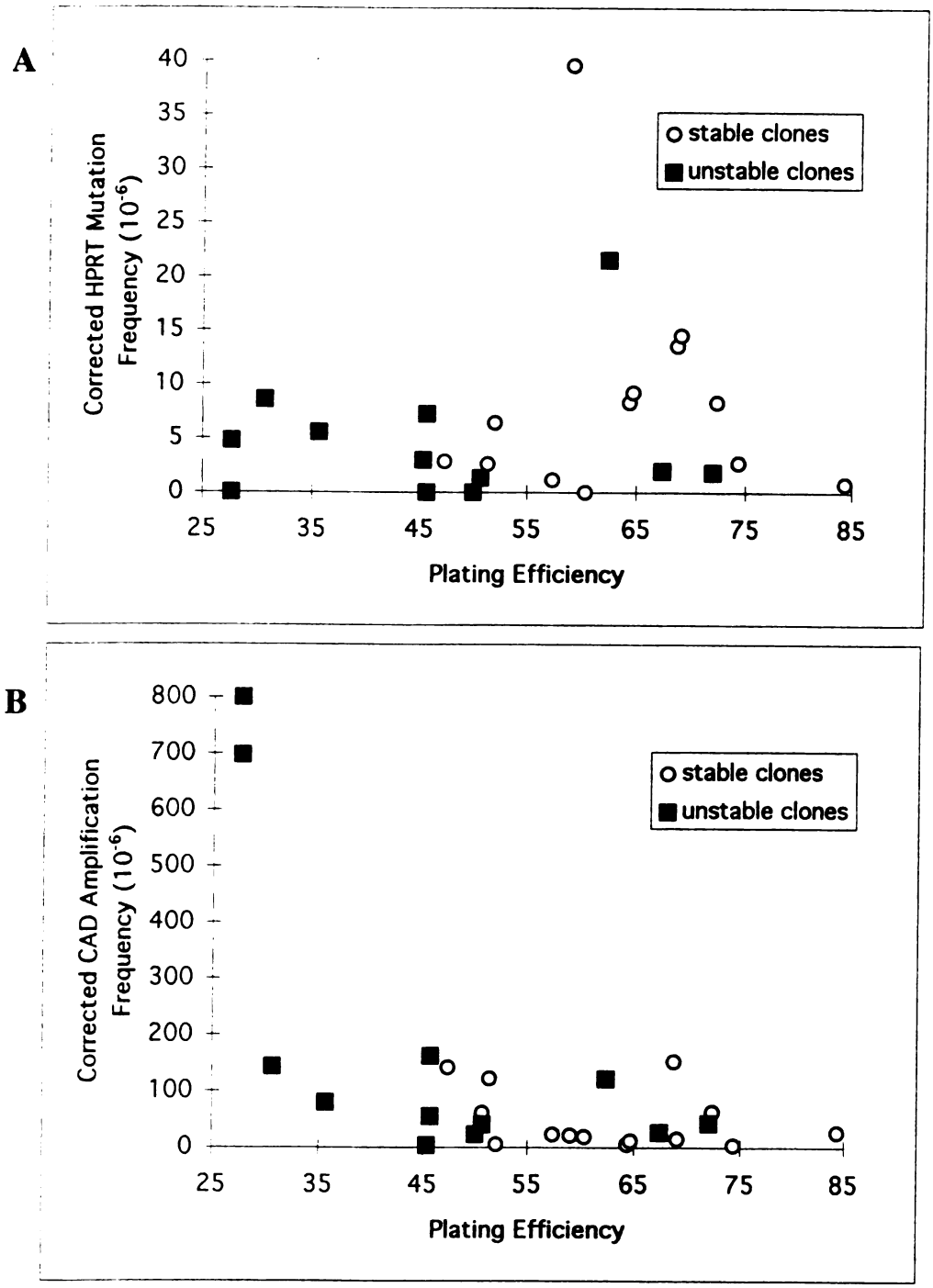

Figure 4.6. Scattergram of plating efficiency plotted against *HPRT* mutation frequency (A) or *CAD* amplification frequency (B) for all chromosomally stable (open circles) and chromosomally unstable (closed squares) clones.

Chapter 5 Conclusion

GENERAL

Chromosomal instability, operationally defined as an increase in the rate of chromosomal change, can be manifested in many mammalian cell types following exposure to ionizing radiation. As chromosomal rearrangements are seen in the majority of tumors, and the carcinogenic phenotype is thought to require an increased rate of genetic change, chromosomal instability has the potential to be a driving force for multistep carcinogenesis. Hence, it is important to understand both the mechanisms underlying the phenomenon of chromosomal instability and the cellular and organismic consequences of chromosomal instability. The major goals of this thesis were 1) to define the target for chromosomal instability 2) to determine the role of DSBs in inducing chromosomal instability and 3) to determine the relationship of chromosomal instability to other end points of genomic instability.

The major conclusions to result from this work are 1) the target for radiation-induced chromosomal instability in GM10115 cells is primarily, if not exclusively, nuclear 2) not all agents that cause DSBs induce chromosomal instability 3) except for delayed reproductive cell death, the manifestation of chromosomal instability does not correlate with other end points of genomic instability.

LOCATION OF THE CELLULAR TARGET

The high frequency of radiation-induced chromosomal instability indicates a large target size for initiating the phenomenon. One hypothesis was that the nucleus was the target for instability, as there is much evidence showing the biological effects of ionizing radiation are mediated by DNA damage. Irradiation of the DNA and associated nuclear strcutures by ^{125}IdU was sufficient to induce chromosomal instability. Similarly, we found that agents capable of causing complex DNA damage, such as BLM, NCS and X-

rays, were able to induce chromosomal instability, while DNA damaging agents which did not produce complex DNA damage, such as restriction enzymes and hydrogen peroxide, were not. We also investigated whether extranuclear damage would induce chromosomal instability, for there is indirect evidence that the target for chromosomal instability is larger than the nucleus (62, 83). We found neither extranuclear radiation from ^{125}I-suc-con A nor free radicals generated from hydrogen peroxide could induce chromosomal instability. Hence, the target for the induction of chromosomal instability in GM10115 cells is primarily, if not exclusively, nuclear.

INITIATING LESIONS AND MECHANISMS FOR PERPETUATION

The nature of the DNA damage which initiates chromosomal instability remains to be determined. Many immediate effects of ionizing radiation, such as cell killing, mutations and chromosome aberrations, are mediated by DNA double-strand breaks. We hypothesized that any agent capable of inducing DSBs could initiate chromosomal instability. While NCS, BLM and X-rays do initiate chromosomal instability, restriction enzymes, which cause simple DSBs, do not. Our results with ^{125}IdU are even more perplexing. ^{125}IdU, which causes complex DSBs (25), did initiate chromosomal instability, but the frequency was lower than anticipated and did not show a dose response. Further experiments suggested that ^{125}I decays in the nucleus of frozen cells do not contribute to chromosomal instability, even though they did cause cell killing and chromosomal rearrangements. It seems that DSBs are necessary but not sufficient for the eventual manifestation of chromosomal instability.

This work also yields insights into mechanisms which can perpetuate chromosomal instability. Two mechanisms proposed to perpetuate chromosomal instability are increased recombination, as evidenced by higher than expected frequencies of three color junctions, and bridge-breakage-refusion cycles. As both of these mechanisms require chromosome rearrangements between the human and a hamster chromosome, it is logical to assume that

increasing the frequency of chromosome rearrangements would increase the frequency of chromosomal instability. Increasing the number of decays from ^{125}IdU, however, increases the frequency of directly-induced chromosomal rearrangements without increasing the frequency of chromosomal instability, and restriction enzymes are capable of producing chromosomal rearrangements without inducing chromosomal instability. Therefore, directly-induced chromosomal rearrangements, in and of themselves, do not seem to be sufficient for perpetuating chromosomal instability.

BIOLOGICAL CONSEQUENCES OF CHROMOSOMAL INSTABILITY

It is unknown how individual mechanisms of genomic instability (chromosomal instability, gene amplification, etc.) contribute to the overall genomic instability seen in cancer. To investigate the hypothesis that the manifestation of chromosomal instability correlates with other end points of genomic instability, we characterized a battery of chromosomally stable and unstable clones in terms of delayed reproductive cell death, delayed mutations, gene amplification, sister chromatid exchange, and a functional mismatch repair system. The only phenotype which correlated strongly with chromosomal instability was delayed reproductive cell death. This is readily explained by the fact that many of the chromosomal rearrangements seen at delayed times are incompatible with cell survival in normal cells. Chromosomal instability correlated only weakly with gene amplification, and there was no correlation with sister chromatid exchanges, mutation frequencies or changes in mismatch repair capabilities. Hence, the manifestation of chromosomal instability after ionizing radiation is governed by a different set of upstream events as those causing other end points of genomic instability in irradiated cells. Clearly, chromosomal instability is not the only mechanism driving increased rates of genetic change in cells surviving ionizing radiation.

FUTURE DIRECTIONS

Our results with ^{125}IdU and restriction enzymes argues that DNA DSBs per se do not induce chromosomal instability. Perhaps only DSBs in certain regions of the genome are effective for inducing chromosomal instability. One way to cause radiation damage to different regions of the genome would be to pulse-label S phase cells with ^{125}IdU in either early or late S phase in order to induce double-strand breaks in either early or late-firing replicons. A similar experiment would be to pulse-label with ^{125}IdU and then chase for various lengths of time. Longer chase periods would allow the newly replicated DNA with incorporated ^{125}IdU to assemble into chromatin.

We argue here that there must be other mechanisms for perpetuating chromosomal instability in addition to bridge-breakage-refusion and hyperrecombination. Positive identification of other mechanisms involved in perpetuating chromosomal instability will require finding differences between chromosomal stable and unstable clones, or else modulating the manifestation of instability by activating/inhibiting particular cellular pathways. These may include activation of signal transduction pathways, activation of endogenous viruses, alterations in patterns of methylation, and epigenetic changes in chromatin structure.

A long term goal would be a faster way of identifying chromosomally unstable clones, as our current methods involve screening clones around twenty generations after radiation exposure. One potential strategy exploits the correlation between chromosomal instability and an increase in the frequency of apoptotic cells (C.L. Limoli and co-workers, manuscript in preparation). Thus, a plasmid containing the green fluorescent protein gene controlled by apoptotic regulatory elements could be introduced into GM10115 cells, and higher frequencies of instability would be manifested in green than in non-green colonies.

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