Title
Double-strand-break-induced mutagenesis

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Publication Date
1994

Peer reviewed|Thesis/dissertation

Double-Strand-Break-Induced Mutagenesis:
Implications for Mechanisms of Genetic Recombination

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biophysics

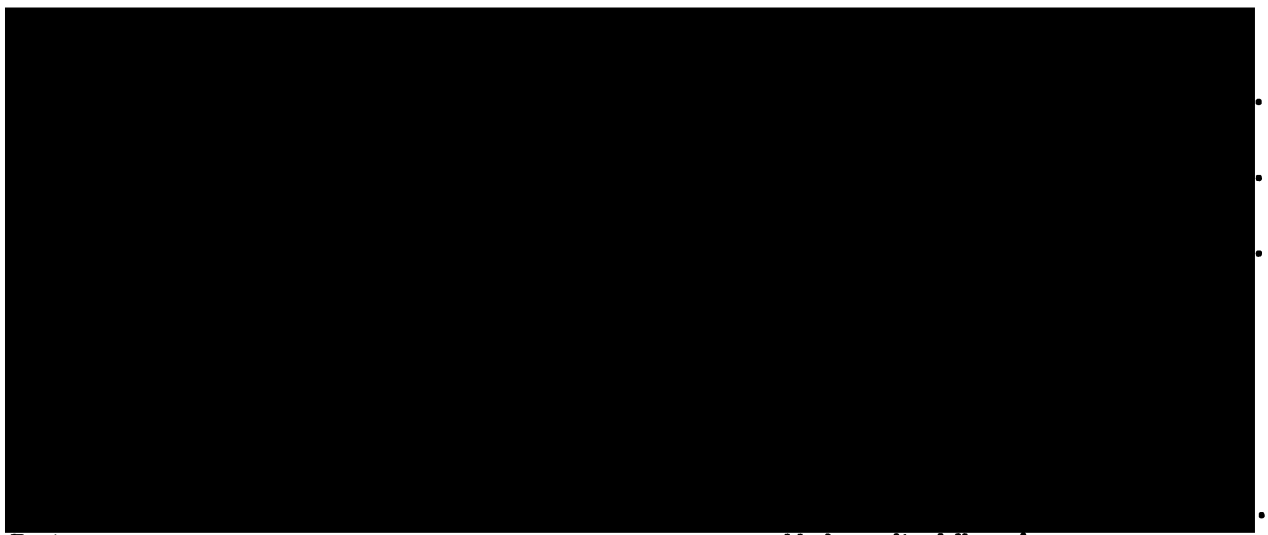
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



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Dedication

This work is dedicated to Jessica Gallegos, my wife and friend of many years.

Preface

My interest in the molecular biology of genetic material dates back to my first real introduction to DNA in a high school science class during the late 1970's. At that point I gained a true fascination for all aspects of genetics, from Mendelian genetics to DNA metabolism. It is, therefore, fitting that my dissertation should cover such a fundamental problem in the field of genetics as recombination of DNA in mammalian cells.

The text of chapter one is a reprint of the material as it appears in *Radiation Research: A Twentieth-Century Perspective. Volume II: Congress Proceedings* (W.C. Dewey, M. Edington, R.J.M. Fry, E.J. Hall, and G.F. Whitmore, Eds.) pp. 207-211. Academic Press (1991). The text of chapter two is a reprint of the material as it appears in *Environmental and Molecular Mutagenesis*, Volume 22, pp. 214-217 (1993). I wrote the text for both of these papers. The coauthor listed in these publications directed and supervised the research which forms the basis for the dissertation/thesis.

I would like to take the time now to thank the many individuals who made it possible for me to reach this point in academic science. First, I would like to thank Mike Mackey and Doug Spitz for inspiring me to stay in science. I would like to thank Karen Fu for giving me my first opportunity in a research laboratory; Bob Painter for providing a example of how a first class scientist should behave; John Murnane for teaching me how to become a molecular biologist; Shelly Wolff for teaching me the complexities of chromosome biology; Michael Banda for helping to cure all of my computer woes; Dennis Deen for helping to guide me through the graduate process; and Julie Ransom and the Graduate Group in Biophysics for putting the students first and fostering a truly

academic environment. I would like to thank all of the past and present members of Bill Morgan's laboratory who have helped me out at one time or another, especially Kate Land, Joan Rufer, Cee Cee Fairley, Barbie Yates, Joe Day, Caleb Wilson, and Jeff King. Last, I would like to thank my parents, Ted and Joan, for supporting me throughout all of my endeavors, and especially Bill Morgan for giving me the opportunity to undertake this venture and for his guidance and wisdom on how to complete it.

Abstract

Double-Strand-Break-Induced Mutagenesis: Implications

for Mechanisms of Genetic Recombination.

John W. Phillips

It is believed that the DNA double-strand break produced by treatment with many DNA damaging agents is the lesion responsible for those agents' ability to induce chromosome rearrangements. However the exact role of DNA double-strand breaks in such processes of genetic recombination is still not well defined. To study directly the role of DNA double-strand breaks in chromosomal recombination, I have examined the rearrangements induced in the endogenous adenine phosphoribosyl transferase (APRT) gene in cultured Chinese hamster ovary cells after exposure to restriction endonucleases. Restriction endonucleases recognize, bind to, and cleave specific DNA sequences to produce DNA double-strand breaks. This specificity of action makes them an ideal agent for use in examining genetic rearrangements induced by double-strand breaks. *Pvu*II, *Eco*RV, and *Stu*I, all of which produce blunt-end DNA double-strand breaks, were electroporated into CHO-AT3-2 cells hemizygous at the APRT locus. Colonies of viable cells containing mutations at APRT were expanded, and the recombination events that occurred during break repair were analyzed at the DNA sequence level. Restriction enzyme-induced rearrangements consisted of small deletions of 1 to 36 bp, insertions, and combinations of insertions and deletions at the cleavage sites. Most of the small deletions involved overlaps of one to four complementary bases at the recombination junctions. Southern blot analysis revealed more complex rearrangements, suggesting translocation, inversion, or

insertion of larger chromosomal fragments. These results indicate that blunt-end DNA double-strand breaks can induce illegitimate (nonhomologous) recombination in mammalian chromosomes and that they play an important role in mutagenesis, and genetic recombination.

Table of Contents

List of Tables

List of Figures

Introduction

In the mammalian genome, DNA double-strand breaks can occur during cellular processes (4, 80) or as the result of exposure to DNA-damaging agents (88). DNA double-strand breaks can cause chromosomal rearrangements (1), which can lead directly to cell killing (21), mutagenesis (44), and cell transformation (12), and can provide the initial step leading to genomic instability or carcinogenesis (54, 79). To understand how DNA double-strand breaks lead to genetic rearrangements, it is important to understand the mechanisms of DNA double-strand-break rejoining and how these processes are carried out at the DNA and chromosomal levels.

Studies of plasmid integration in a variety of mammalian cell types, along with SV40 recircularization studies in monkey cells, have shown that mammalian cells predominantly repair DNA double-strand breaks by end-joining mechanisms that do not require extensive homology between the molecules to be joined (72). Recombination between nonhomologous DNA substrates was first described in bacteria (24) and has been defined as illegitimate recombination (25). Most studies examining illegitimate recombination have used DNA substrates linearized with various restriction endonucleases to produce specific combinations of end structures. These substrates are cleaved *in vitro* and passed through various recombination systems, and the rejoined products are studied. Recircularization studies of a linearized SV40 genome passed through CV 1 monkey cells, and plasmid rejoining studies in *Xenopus laevis* egg and human cell extracts, have shown that these end-joining processes frequently use overlaps of one to six complemen-

tary bases (66, 71, 84). The products of illegitimate recombination between segments of mammalian chromosomes have been studied by examination of translocation breakpoints and deletion and insertion junctions at various genetic loci (43, 44). Although many of the breakpoint junctions show the small complementary base overlaps that are the signature of illegitimate recombination, the initial steps leading to the recombinational event and the mechanisms involved are not understood.

The purpose of this dissertation is to examine the events that occur at the molecular level when DNA double-strand breaks induce genetic rearrangements in a chromosomal gene, and to understand the relationship between the molecular processes of DNA end joining and recombination. I will start by describing the advantages of using restriction endonucleases as the double-strand breaking agent for studying genetic recombination (Chapter One), and then discuss what is known about the involvement of double-strand breaks in mutagenesis (Chapter Two). I will then examine experimentally the types of genetic rearrangements that specific, restriction endonuclease produced, DNA double-strand breaks are able to induce at a defined location in the mammalian genome, and discuss my results in terms of what is known about the mechanisms of double-strand break rejoining and illegitimate recombination (Chapter Three).

Chapter One: Restriction enzyme-induced DNA double-strand breaks as a model to study the mechanisms of chromosomal aberration formation.

Ionizing radiations are proficient at inducing chromosomal aberrations in mammalian cells at all stages of the cell cycle. Moreover, it has been shown that there is a direct correlation between chromosomal aberration formation and cell death in irradiated cells (21). However, the mechanism(s) of induced aberration formation are still unknown. Ionizing radiations cause a range of lesions in DNA, including DNA double- and single-strand breaks, DNA crosslinks, and DNA base damage (83). Of these lesions the DNA double-strand break appears to be the major lesion involved in chromosomal aberration formation (9, 57, 58).

To investigate the role of double-strand breaks in chromosomal aberration formation, we and others have been introducing restriction enzymes into cells (reviewed in 49). Restriction enzymes recognize specific DNA sequences, bind to the DNA, and cleave to produce a double-strand break in the absence of any other known DNA lesions (70). In this report we will review our recent research in which we used restriction enzymes to study chromosomal aberration formation, address some of the major issues involved in the use of restriction enzymes, and explore future directions for such studies.

Restriction enzyme-induced chromosomal aberrations. Restriction enzymes are extremely efficient inducers of chromosomal aberrations. A consistent feature of restriction enzyme-induced aberrations is that blunt-end double-strand breaks are more efficient at inducing chromosomal damage than are cohesive-end double-strand breaks (3,

9, 11). There is some controversy, however, over whether certain enzymes, such as *Bam*-HI, will induce cytogenetic damage; both positive (29, 82, 94) and negative (9, 10) results have been reported. In our hands *Cfo*I, *Hind*III, and *Kpn*I do not induce aberrations. Others, like *Eco*RI and *Hpa*II, are poor inducers of aberrations, probably because they are somewhat inhibited by the methylation pattern of Chinese hamster ovary (CHO) cells.

Variability in the efficiency of restriction enzyme action. The introduction of biologically active proteins, in this instance restriction enzymes, into cells can result in significant differences in chromosomal aberration yields in different experiments (reviewed in 49). Even when using the same permeabilization technique, e.g., electroporation, there can be differences between repeat experiments performed on the same day (51) or between experiments using the same batch of enzyme but performed on different days (50). This is of some concern in experiments attempting to modulate enzyme-induced breakage when different treatment groups must serve as controls, e.g., with inhibitors of DNA repair (15, 58, 60) or various salt solutions (62, 86). Unfortunately, there does not appear to be any obvious way around this problem at present.

When comparing the efficiencies of different enzymes with different recognition sequences that produce different types of double-strand break, one way of reducing the number of variables involved is to use enzymes with approximately the same number of potential cleavage sites in the target cells (89). Despite such precautions, each restriction enzyme is a unique protein with its own optimal reaction conditions, amino acid composition, protein structure, and methylation sensitivity. The reaction conditions in the nucleus of a mammalian cell might be optimal for one endonuclease in terms of salt concentration,

pH, etc., but inhibitory for another. Furthermore, under such conditions one enzyme may be stable for hours, while another may lose its activity in minutes because of oxidation, denaturation, or degradation.

Specificity of restriction enzyme action. DNA sequence recognition, binding, and cleavage are well worked out *in vivo* for bacteria and *in vitro* for eukaryotic DNA under ideal conditions of DNA purification, salt, pH, and temperature. But how does this relate to the specificity of restriction enzymes in the nuclei of cells in culture given the inherent complexity of the higher order structure of chromosomal DNA? This was addressed indirectly by Winegar et al. (91), who used the enzymes (isoschizomers) *Msp*I and *Hpa*II, both of which recognize the sequence CCGG. *Hpa*II will cut only if the internal cytosine is unmethylated, whereas *Msp*I will cut regardless of the methylation status. In CHO cells the CpG sequence is heavily methylated (34). As expected, *Msp*I was much more effective than *Hpa*II at inducing chromosomal aberrations. These data indicate that for these isoschizomers chromosomal aberration formation is consistent with their expected specificity in CHO cells.

Recently, the shuttle vector pHAZE, which is stably maintained as an episome in a human lymphoblastoid cell line (42), was used to demonstrate conclusively that restriction enzymes electroporated into these cells recognize, bind, and cut pHAZE with their reputed specificity in a nuclear environment (76, 92). Since shuttle vectors maintained in mammalian cells acquire the characteristics of eukaryotic chromatin (14, 27), it is reasonable to conclude that restriction enzymes will also cleave chromosomal DNA with the same specificity. It will be shown in chapter three that the restriction

enzymes *Pvu*II, *Eco*RV, and *Stu*I all cleave chromosomal DNA with their reputed specificities.

It is also possible that the packaging of DNA in chromatin blocks enzyme accessibility to many potential restriction enzyme recognition sites. If this were the case, then we should expect a saturation of damage once a threshold concentration of enzyme was reached. Using *Pvu*II at doses ranging from 10-1000 units per treatment, Yates et al. (93) observed saturation in cell killing, induction of aberrant cells, and chromosome exchange-type aberration yields. These endpoints all reached a maximum effect at 100 units of *Pvu*II per treatment and remained constant up to 1000 units. These dose-response data led to the conclusion that all potential *Pvu*II sites available in the cell had been cleaved and that saturation had indeed occurred.

Furthermore, using *Alu*I and *Sau*3AI, Morgan et al. (52) recently showed that restriction enzymes will effectively cleave chromosomal DNA during metaphase when the chromatin is condensed. It is of interest that the majority of aberrations observed were interstitial deletions. Restriction enzymes electroporated into mammalian cells generate DNA of low molecular weight (3). It is possible that the structure of metaphase chromosomes is responsible for bringing the free DNA ends of the endonuclease-induced low molecular weight DNA together in an intra-strand fashion, resulting in the formation of these minute acentric rings.

Restriction enzyme-induced DNA double-strand breaks. A major difficulty encountered while using restriction enzymes to study the consequences of double-strand breaks on various cellular endpoints is the quantification of the amount of DNA damage,

i.e., double-strand breaks induced by enzyme treatment. This difficulty is largely attributed to the period of time various enzymes are active after electroporation into the cell. We have used pulsed-field gel electrophoresis to investigate how long it takes for enzymes to cleave DNA after their introduction into the cell and how long enzymes are active in a nuclear environment (3). The three enzymes studied, *Pst*I, *Pvu*II, and *Xba*I, were all active within 10 min of electroporation. *Pst*I and *Pvu*II showed a distinct peak in break formation at 20 min, whereas *Xba*I showed a gradual increase in break frequency over time. Another increase in the number of breaks was observed with all three enzymes at 2 and 3 h after electroporation and was probably due to degradation of nonspecific DNA in a subpopulation of enzyme-damaged cells that lysed after enzyme exposure. In parallel studies using the same restriction enzyme concentration, we found that CHO cells exposed to the various restriction enzymes in G_1 showed only G_1-type chromosome aberrations (3). This led us to conclude that *Pst*I, *Pvu*II, and *Xba*I were active for only 2-3 h in cells. These conclusions contradict those of another recent study using neutral elution to assay the kinetics of *Pvu*II-induced double-strand breakage (20), in which *Pvu*II was said to remain active for > 24 h after electroporation into cells.

An interesting aspect of restriction enzyme-induced double-strand breaks is how long breaks remain open after cleavage and therefore how long they are available to interact with each other. These breaks have intact 5′ phosphate and 3′ hydroxyl termini and should be rapidly repaired by simple ligation. However, any analysis of the removal of breaks by cellular repair processes is further complicated by the kinetics of induction of restriction enzyme-induced double-strand breaks. This has been investigated in two

recent studies that examined the interaction between X-ray- and restriction enzyme-induced lesions in the formation of chromosomal aberrations (51, 82). Tanzarella et al. (82) demonstrated that enzyme-induced double-strand breaks are able to interact with X-ray-induced breaks immediately after introduction of the enzyme into the cell. In similar experiments we confirmed and extended these observations to show that restriction enzyme-induced double-strand breaks are available for interaction for only a short time. Breaks produced by the blunt-end cutter *Alu*I were available for interaction for less than 2 h, and breaks produced by the cohesive-end cutter *Sau*3AI for less than 30 min (51). These data can also be interpreted to show that neither *Alu*I nor *Sau*3AI is active for more than 2 h inside CHO cells.

Relationship between restriction enzyme-induced double-strand breaks and chromosomal aberrations. Attempts to relate restriction enzyme-induced double-strand breaks to chromosomal aberrations at metaphase have proved difficult because of the confusion over the duration of enzyme action, the stage of the cell cycle at which enzyme exposure occurs (DNA in S phase cells, being more decondensed, may be more accessible to enzyme cleavage), and the variability in the efficiency of introduction of enzymes into cells. Given these caveats, Ager et al. (3) found that break frequency and chromosomal aberration frequency were inversely related: Compared with the cohesive-end cutters *Pst*I and *Xba*I, the blunt-end cutter *Pvu*II gave rise to the most aberrations but the fewest breaks, suggesting that it is the type of break rather than the break frequency that is important for chromosomal aberration formation. These observations seem to agree well with early studies that attempted to relate double-strand breaks with chromosomal aberra-

tion formation. DNA strand breakage in CHO cells after exposure to *Pvu*II or *Bam*HI was measured by Bryant (9) using alkali unwinding. Both enzymes induced breaks at a similar frequency despite dramatic differences in chromosomal aberration yield. Natarajan et al. (59) used nucleoid sedimentation to determine the frequency of breaks induced by either 60 or 120 units of *Pvu*II. No dose-response relationship was observed.

Summary and conclusions. Clearly, there is ample evidence from our laboratory and others that restriction enzymes can be introduced efficiently into mammalian cells in culture. Once in a cell they will cleave DNA with their reputed specificity to produce double-strand breaks. In contrast to ionizing radiations, which induce observable damage almost immediately after irradiation, restriction enzymes act over a protracted period within the cell, in our hands 30 min to 3 h depending on the enzyme. Within the cell, then, there must be a balance between enzyme-induced cleavage and break rejoining by endogenous cellular repair processes. These processes can involve poly(ADP-ribosyl)ation (15) and, to a lesser extent, polymerase alpha (15, 58, 60). Nevertheless, an unknown fraction of restriction enzyme-induced double-strand breaks are either not repaired or are misjoined to lead to chromosomal aberrations. It is our feeling that an important direction for future research is to determine precisely when restriction enzymes induce double-strand breaks, how long the breaks persists within the cell, and what the relationship is between breakage, repair, and misjoining. The ultimate goal of these studies is to relate events at the DNA level to the formation of aberrations in metaphase chromosomes.

Chapter Two: The DNA double-strand break in mutagenesis.

Ionizing radiation was first shown to be a mutagen more than 65 years ago (53). However, the molecular events involved in processing radiation-induced damage into a mutational event are still not well understood. Ionizing radiations produce a variety of lesions in DNA, including double- and single-strand breaks, cross-links, and base damage (83). Molecular studies have revealed a wide range of mutations, including large rearrangements (i.e., those that are detectable by restriction fragment length polymorphism (RFLP) analysis on Southern blots), small deletions and/or insertions, base substitutions, and frameshift mutations, which generally are not detectable by Southern blot analysis (reviewed in 8, 77). Most ionizing radiation-induced mutations are large deletions (77).

The DNA double-strand break appears to be the major lesion involved in chromosomal aberration formation (9, 57, 58). It is also very likely that the DNA double-strand break is the dominant lesion involved in radiation-induced mutagenesis. The double-strand break has been implicated as the primary lesion responsible for the induction of large deletions (46) and, to a lesser extent, large insertions, small deletions, and frameshift mutations (28, 45, 46). It is still unclear, however, whether or not the double-strand break is directly induced by the ionizing radiation or whether it is the result of a step in the cellular repair pathways for other types of DNA lesions. There is still too little information available to propose a definitive mechanism for the production of the observed alterations.

Here we will review the types of mutations induced by low-linear energy transfer

(LET) ionizing radiations, with an emphasis on those DNA sequence alterations and genomic rearrangements that are thought to be associated with double-strand breaks. We will then discuss the techniques being used to understand the involvement of DNA double-strand breaks in mutagenesis when no other DNA lesions are induced.

Background (spontaneous) mutations. In mammalian cells, ionizing radiation-induced mutagenesis has been studied at four primary endogenous loci: the hypoxanthine phosphoribosyl transferase (*HPRT*) gene, the adenine phosphoribosyl transferase (*APRT*) gene, the *HLA-A* gene in human T lymphocytes, and the thymidine kinase (*TK*) gene. To understand the mechanisms of ionizing radiation-induced mutagenesis, it is important first to understand the types of mutations that occur spontaneously in a particular cell system and genetic locus of interest. In mammalian cells most spontaneous mutations (70-80%) are small DNA changes that do not alter RFLPs by Southern blot analysis, and the remainder are primarily deletions (reviewed in 8, 77).

Ionizing radiation-induced mutations. In contrast to what is observed in unirradiated cells, mutations induced by exposure to sparsely ionizing radiations are predominantly deletions (77). However, the nature of the mutation spectrum depends on the loci studied. In a recent study taking advantage of newly available fine-structure RFLP maps of the Chinese hamster ovary (CHO) *HPRT* gene, Fuscoe et. al. (26) found that 73% of X-ray-induced mutations were of the deletion type, with 10% of those showing partial deletion of the gene and 63% showing total deletion of the gene. In contrast, X-ray-induced mutations at the CHO *APRT* gene were predominantly small changes: 79% resulted in no RFLP change on Southern blots (77). Almost all of the deletions that

were detected by Southern blot analysis were at the 5′ end of the gene; no mutations were observed that involved deletion of breakpoints downstream of the *APRT* gene (44, 77). This difference in mutation spectrum is believed to be a function of the hemizygous cell lines used for the *APRT* studies, including the CHO-AT3-2 cell line used in chapter three of this study. It appears that the direction of the deletions seen, all extending in the upstream direction from *APRT*, are the result of some important sequence information lying downstream of *APRT* (reviewed in 44). Although possibly affecting the total distribution of mutations seen at this locus, this characteristic of the hemizygous CHO cell lines makes them ideal for examining mutations at the nucleotide sequence level. When 27 γ radiation-induced *APRT* mutants from one study that showed no alterations by Southern blot analysis were analyzed at the DNA sequence level, 56% of the mutations were base substitutions (45). Of the spontaneous mutations from unirradiated cells in a study examining 89 mutant *APRT* genes (68), 63% were base substitutions. Thirty percent of the γ radiation- induced *APRT* mutations were small deletions ranging in size from 1 to 30 base pairs (bp) (45), and 19% of the spontaneous *APRT* mutations were small deletions ranging in size from 1 to 20 bp (68). One major difference in the spectrum of γ radiation-induced mutations at the *APRT* locus was in a class called complex rearrangements (7, 44, 46). This class of mutations constituted 10% of the γ radiation-induced mutations but none of the spontaneous mutations. These complex rearrangements showed RFLPs on Southern blots that were much more complicated than simple deletions, and it was speculated that they involved inversions, translocations, or very large insertions (44).

A role for the DNA double-strand break in mutagenesis. The DNA double-strand break is the most likely lesion to be involved in producing the ionizing radiation- induced partial- and total-gene deletions seen at all of the loci studied to date. The double-strand break is also implicated in the γ radiation-induced insertions found at *APRT* (46), in the X-ray- induced insertion found at *HPRT* (26), and in the γ radiation-induced complex rearrangements found at *APRT* (44). Because the double-strand break is thought to be the major lesion involved in chromosome aberration formation, it is a logical candidate for inducing rearrangements such as gene deletions and large insertions. The most comprehensive data on the small-scale alterations induced in an endogenous mammalian gene by sparsely ionizing radiations come from the *APRT* locus. The small deletions and insertions found at this locus may be the result of mis-rejoined double-strand breaks, with small amounts of genetic material being deleted or inserted during the repair or rejoining process. Of the ionizing radiation-induced alterations, the least likely type to involve DNA double-strand breaks would be base substitutions. These mutations are thought to be induced at apurinic and apyrimidinic sites (45).

Types of DNA strand breaks and their potential mutagenic effects. When comparing various types of mutations and trying to elucidate molecular mechanisms for their formation, one question that arises is whether the initial DNA double-strand breaks are always going to be similar in structure and whether any differences that may exist will present lesser or greater challenges to the cell trying to repair them. A double-strand break is thought to be the result of two single-strand breaks in close proximity on opposite strands (reviewed in 88). Studies conducted with double-stranded DNA fragments *in vitro*

showed that γ radiation produced two main types of single-strand breaks in the DNA (31, 32). For these two types of breaks, the 3′ end groups were found to be either a 3′ phosphate group (31) or a 3′ phosphoglycolate group (32). Both types of breaks involved the loss of one entire nucleotide, and both were believed to be initiated by an attack of free radicals. Both types of breaks ended in 5′ monophosphate groups, with neither ending in 3′ hydroxyl groups. Henner et al. (33) postulated that faithful repair of such breaks would require, at a minimum, cellular systems to remove the 3′ phosphate and 3′ phosphoglycolate end groups, exposing the 3′ hydroxyl groups, to resynthesize the missing nucleotide, and to ligate the broken strands. *Escherichia coli* exonuclease III (exo III) is capable of removing these 3′ groups (33). Under specific conditions in which exo III will behave in a nonprocessive manner, Henner et. al. (33) showed that the first step by exo III is the removal of the 3′ phosphate or phosphoglycolate moiety, leaving a 3′ hydroxyl group without removing additional nucleotides. However, under other conditions or at higher enzyme concentrations, exo III acts in a processive manner by removing additional nucleotides. Whether this enzymatic activity is regulated *in vivo* to keep the exonucleases from removing extra nucleotides is not known. These types of mechanisms may explain the small deletions seen at *APRT* and other loci. If exonuclease activity is necessary before ligation of radiation-induced strand breaks, the small deletions seen may be a function of the amount of time the exonuclease stays on the strand to remove nucleotides after removing the 3′ end groups.

There is evidence for other types of end structure at the site of ionizing radiation-induced single-strand breaks (reviewed in 63), but they appear to be much less

prevalent than those discussed above (31). Studies of DNA break rejoining after treatment with large doses of ionizing radiation have shown that almost all measurable DNA strand breaks are rejoined (23, 30). Regardless of the structure of the broken DNA ends, cells have the enzymatic systems available to modify them for ligation. If a break cannot be rejoined, it is most likely lethal to the cell and irrelevant to the study of mutagenesis. So if cells are able to rejoin the broken DNA ends, the problems leading to mutation from DNA double-strand breaks would tend to involve small losses of DNA at the sites of breaks, or the rejoining of the broken DNA ends to incorrect ends, leading to such endpoints as deletions, translocations, inversions, and insertions.

Restriction endonuclease-induced DNA double-strand breaks. Restriction endonucleases have been used to investigate the biological consequences of DNA strand breakage, in particular, the role of DNA double-strand breaks in chromosome aberration formation (reviewed in 49, and in chapter one). When introduced into mammalian cells by electroporation, restriction endonucleases are efficient inducers of chromosome aberrations, cell killing, and cell transformation (6, 90). Not only are double-strand breaks the only lesions that restriction endonucleases produce in DNA, but they produce precise DNA ends of known sequence and structure, i.e., blunt, 5′, or 3′ overhanging ends with ligatable 5′ phosphate and 3′ hydroxyl groups. Because of the properties of restriction endonucleases and their ability to be introduced into and function in mammalian cells (48, 76), they are a useful tool for studying DNA sequence alterations induced by specific double-strand breaks in the absence of any other lesion.

Restriction endonucleases have been found to induce mutations at certain

mammalian loci (reviewed in 63). The first such study found that treatment with *AluI* led to an increase in mutation frequency at the *HPRT* locus in Chinese hamster V79 cells, but not at the Na/K-ATPase locus, as measured by ouabain resistance (61). Another study has shown that treatment with restriction endonucleases increased the mutation frequency at the *TK* locus in CHO KI cells (78). Neither of these studies examined the induced mutations at the molecular level.

A recent study has attempted to address the molecular events involved in mutation production by examining the mutations induced by restriction endonuclease-produced double-strand breaks in the shuttle vector pHAZE, an Epstein-Barr virus-based shuttle vector containing the *lacZ* gene, stably maintained in a human lymphoblastoid cell line (92). In this study, 71% of the mutations were due to large rearrangements and 29% were due to small alterations. Of the large rearrangements, most (79%) were large deletions between two different enzyme sites and involved no modification of the end of the break before rejoining; 4% were large insertions (> 1 kb); and the remaining 17% were inversions or both inversions and deletions between different enzyme sites. Seventy-one percent of the small alterations were deletions ranging from 1 to 36 bp, and 29% were nucleotide insertions of 1 or 2 bp. None of the restriction endonuclease-induced double-strand breaks produced base substitutions that would be observed as mutations in this system. Although it is unlikely that double-strand breaks induce significant numbers of base substitutions, the results of this study do not rule out this possibility. The study does, however, shed light on the involvement of well-defined double-strand breaks in mutagenesis. By knowing the precise end structure of the double-strand break that induced

the mutation and then sequencing the end result of the cellular processing of that break, we can begin to speculate on the molecular mechanisms of double-strand break-induced mutagenesis. To date these investigations have primarily involved shuttle vectors episomally maintained in mammalian cells. The precise description of mutational events in mammalian cells requires similar studies in an endogenous chromosomal gene. Such studies are described in the next chapter. If we can understand the types of mutations induced by a simple ligatable double-strand break, e.g., a restriction endonuclease-induced double-strand break, then we can begin to understand the complexities introduced by ionizing radiations and other potential environmental mutagens.

Chapter Three: Illegitimate recombination induced by DNA double-strand breaks in a mammalian chromosome.

To study the products of illegitimate recombination induced by DNA double-strand breaks in a mammalian chromosome, I chose to examine the mutations that restriction enzymes induce in the endogenous adenine phosphoribosyl transferase (APRT) gene when introduced into cultured Chinese hamster ovary cells. In this chapter, I report on the types of alterations that were induced in the APRT gene by blunt-end DNA double-strand breaks produced by *Pvu*II, *Eco*RV, and *Stu*I at different locations within the gene. Using three restriction enzymes allowed me to examine the various types of recombination events that a blunt-end DNA double-strand break can induce in the APRT gene, and to determine whether the location of the double-strand break within the gene, or the base sequences flanking the double-strand break, have any effect on the mutation process.

MATERIALS AND METHODS

Cell culture. A Chinese hamster ovary (CHO) cell line hemizygous at the APRT locus, CHO-AT3-2, was used (2). In this cell line, deletions spanning downstream of the functional allele of the APRT gene are thought to be lethal (44). This characteristic has the effect of selecting for rearrangements that have at least one breakpoint within the gene and allows us to easily clone the recombinational junction by using the available sequence information for the APRT gene. Cells were maintained as monolayer cultures in alpha-modified minimum essential medium supplemented with 10% dialyzed fetal bovine serum, 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 µg/ml). Cells were

maintained in exponential growth at 37°C in an atmosphere of 5% CO_2 in air.

Restriction enzyme treatment. The three restriction endonucleases used in this study were obtained from Boehringer Mannheim (Indianapolis, IN). *Pvu*II and *Stu*I were obtained at a concentration of 10 U/μl. *Eco*RV was obtained at a concentration of 40 U/μl. The restriction enzymes were introduced into CHO-AT3-2 cells by electroporation as described previously (90), with some modification. Briefly, exponentially growing cells were trypsinized and washed once at ambient temperature with phosphate-buffered sucrose (7 mM KH_2PO_4, pH 7.4, 1 mM $MgCl_2$, 272 mM sucrose), then resuspended at a final concentration of ~5 x 10^6 cells per ml in phosphate-buffered sucrose. Aliquots (0.8 ml) of this cell suspension were placed in individual electroporation cuvettes with a 0.4-cm electrode gap. Restriction enzymes in their storage buffer or equivalent amounts of storage buffer alone were added to the cuvettes and mixed. These suspensions were then electroporated at ambient temperature in a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA) with a field strength of 750 V/cm and 125 μFD capacitance. Several groups of cells were electroporated for each restriction enzyme, and the data presented for each enzyme were obtained from at least two experiments.

Survival assays and mutant selection. After electroporation, cells were either plated for survival or divided into groups for mutant selection. For clonogenic survival, cells were seeded at a density of 200 cells per 60-mm dish in 5 ml of medium. Plates were incubated for 10-14 days before fixation and staining of colonies in 0.1% crystal violet (Allied Chemical, Morristown, NJ) in 25% ethanol, and colonies were counted for plating efficiency and survival. Cells used for mutant selection were maintained in exponential

growth for a 5- to 7-day expression period before being plated into selective medium. APRT$^-$ cells were selected by plating ~2 x 10^5 cells per 100-mm dish in medium containing 80 μg/ml 8-aza-adenine (Sigma Chemical Company, St. Louis, MO). After 10-14 days APRT$^-$ colonies were counted for mutation yields, and two or three colonies from each group were isolated for analysis.

Analysis of mutations. Genomic DNA was isolated by a salting out method (47). Regions of the APRT gene that included the restriction enzyme recognition sequences were amplified by the polymerase chain reaction (PCR) in a total volume of 100 μl, with the use of the GeneAmp DNA Amplification Reagent Kit and AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT). Primers used for amplification were 18-mers, with one of the primers biotinylated at the 5′ end. Primers were added to a final concentration of 0.5 μM, and ~ 1 μg of genomic DNA was used for each reaction. PCR conditions consisted of 40 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, in a DNA thermal cycler (Perkin-Elmer). The amplified regions were analyzed for mutations by restriction fragment length polymorphism (RFLP) analysis of 10-μl aliquots on 1.5% agarose gels.

DNA sequencing. Sequencing was performed according to the direct solid-phase sequencing technique of Hultman et al. (35). Briefly, single-stranded DNA for sequencing was generated by incubating 80 μl of PCR product with 300 μg of Dynabeads (Dynal, Inc., Great Neck, NY) that had been washed twice in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 M NaCl for 15 min at ambient temperature. Supernatant was removed by means of the Dynal MPC-E Magnetic Particle Concentrator, and the beads were washed once in TE buffer and incubated for 15 min at ambient temperature in 0.10 M NaOH to generate the

single-stranded DNA. The bound strand was washed once in 0.10 M NaOH and twice more in TE buffer before being resuspended in 15 µl of H_2O for sequencing. Sequencing was performed by the dideoxy sequencing method with Sequenase version 2.0 (U.S. Biochemical, Cleveland, OH) according to the manufacturer's instructions for single-strand sequencing, with the use of [α-^{35}S]dATP from Amersham (Arlington Heights, IL).

Southern blot analysis. High molecular weight genomic DNA for Southern blot analysis was prepared as described by Murnane et al. (55). Restriction enzyme digestion of genomic DNA was carried out according to the manufacturer's instructions (Boehringer Mannheim). Gel electrophoresis, Southern blot transfer, and nucleic acid hybridization were performed as described previously (48). [α-^{32}P]dCTP-labeled probes were prepared by random-primed DNA labeling (22) with the 3.9-kb *Bam*HI fragment of the cloned APRT gene.

RESULTS

Restriction enzyme-induced cell killing and mutagenesis. Three restriction endonucleases (*Pvu*II, *Eco*RV, and *Stu*I) were introduced separately by electroporation into CHO-AT3-2 cells hemizygous at the APRT locus. All have six-base recognition sequences and cleave in the center of the recognition sequences to produce blunt-end DNA double-strand breaks. Each restriction enzyme has one recognition sequence within the coding sequences of the APRT gene (Fig. 1). No restriction enzyme recognition sequences outside the APRT gene were analyzed for this study.

At the concentrations used, the restriction enzymes reduced clonogenic survival to between 12 and 20% of control values (Table 1). This degree of cell killing resulted in

Fig. 1. Restriction map of the 3.9-kb *Bam*HI clone of the CHO APRT gene. The cleavage sites indicated above the line are those of the enzymes used to induce mutations. Their recognition sequences are: *Pvu*II, CAGCTG; *Eco*RV, GATATC; *Stu*I, AGGCCT. The cleavage sites indicated below the line are those of the enzymes used for Southern blot analysis. The boxes represent the five exons that constitute the coding sequences for the APRT gene (5).

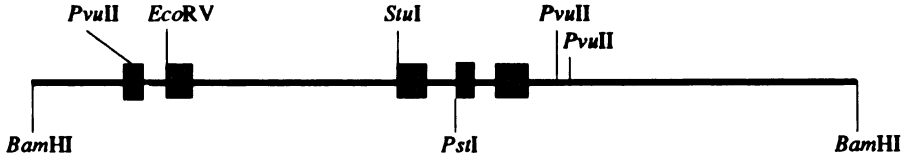

PvuII
EcoRV
StuI
PvuII
PvuII
BamHI
PstI
BamHI

TABLE 1. Survival and mutation yield in CHO-AT3-2 cells after exposure to restriction enzymes

Restriction enzyme	% Survival[a]	No. of mutant colonies per 10^6 clonogenic cells	
		Control	Treated
*Pvu*II, 25 U	12	3.6	46
*Eco*RV, 300 U	17	5.3	34
*Stu*I, 10 U	20	3.8	29

[a] Survival was determined by dividing the plating efficiency of treated cells by the plating efficiency of control cells and multiplying by 100%.

[b] Control cells were electroporated with the storage buffer for each restriction enzyme.

maximum mutation yield at the APRT locus (data not shown). *Pvu*II and *Stu*I were highly efficient at inducing cell killing and mutagenesis at relatively low concentrations, 25 and 10 units, respectively. *Eco*RV required 300 units to produce similar levels of cell killing and mutation yield (Table 1).

Screening of restriction enzyme-induced mutations. Mutant colonies were picked and expanded for frozen storage and DNA purification and analysis. We used PCR to amplify a region of the APRT gene that surrounded the restriction enzyme recognition sequence of interest and subjected the amplified material to RFLP analysis. By using the same restriction enzyme for the RFLP analysis that was used in the mutagenesis experiments, we were able to categorize the mutants according to whether or not they contained a change at the recognition sequence for that particular enzyme (Table 2). About 70% of the mutants induced by all three enzymes had changes at their respective recognition sequence. The remaining mutants had mutations that either did not involve the recognition sequence or did not allow amplification across the restriction enzyme cleavage site (Table 2). Considering the mutation yields that were obtained (Table 1), it seems likely that the mutations that did not involve the restriction enzyme recognition sequence were spontaneous in origin. PCR analysis of 25 mutants from control populations (untreated cells or cells electroporated only with storage buffer) showed that none of the spontaneous mutations involved the recognition sequence for the restriction enzyme used in the corresponding experiments (data not shown). For the mutations that inhibited amplification across the cleavage site, additional PCR analysis was performed with a combination of primer sets. In all cases we determined that the restriction enzyme

TABLE 2. RFLP analysis of restriction enzyme-induced mutations in the APRT gene

Restriction enzyme	Total no. of mutants analyzed	No change[a]	No PCR product[b]	Alteration recognition sequence[c]
*Pvu*II	42	8	4	30
*Eco*RV	43	5	5	33
*Stu*I	42	9	4	29

[a] The PCR product retained the recognition sequence.

[b]No PCR product was obtained when primers that would amplify the recognition sequence were used, but other regions of the APRT gene were successfully amplified.

[c]The PCR product had an altered recognition sequence that was refractory to enzyme cleavage.

cleavage site could still not be amplified, although the downstream regions of the gene were intact and subject to amplification (data not shown).

DNA sequence alterations induced at restriction enzyme recognition sequences. Only those mutations that affected restriction enzyme recognition sequences were characterized by DNA sequencing. PCR products were sequenced directly. The three restriction enzymes induced a wide range of DNA sequence alterations, from small deletions to various-sized insertions and combinations of insertions and deletions (Table 3). Because I cloned multiple colonies from each treatment group, several of the mutants that were characterized may have arisen from the same mutation. For this reason the values given in Table 3 have been separated into the total number of mutations analyzed and the number of mutations that arose from independent events. The independent mutations are defined as those that arose either in separate treatment groups or in the same treatment group but with distinct DNA sequence alterations. Unless otherwise noted, all values reported in the text refer to the independent mutations.

The most striking difference in the types of alterations induced by blunt-end DNA double-strand breaks in various locations in the APRT gene was between those found at the *Pvu*II recognition sequence and those found at the *Eco*RV and *Stu*I recognition sequences (Table 3). Most of the *Pvu*II-induced alterations were single-nucleotide insertions (Table 3). All of the single-nucleotide insertions at the *Pvu*II recognition sequence were found at the cleavage site, with no deletion of wild-type sequence (Fig. 2A). Most of these were C/G insertions, with the remaining types being either T/A or A/T insertions (Fig. 2A). In contrast, only a total of four single-nucleotide insertions were found at the *Eco*RV and *Stu*I

TABLE 3. DNA sequence alterations induced at restriction enzyme recognition sequences

Restriction enzyme	No. of mutations analyzed[a]		Single nucleotide insertions		Deletions[b]		Small insertions[c]		Large insertions[d]	
	Total	Ind.	Total	Ind.	Total	Ind.	Total	Ind.	Total	Ind.
*Pvu*II	30	22	21	14	8	7	0	0	1	1
*Eco*RV	33	24	3	3	24	15	3	3	3	3
*Stu*I	29	26	1	1	23	21	3	2	2	2

[a]The numbers in the "total" columns represent all of the mutations analyzed at the DNA sequence level and may include some duplicates. The numbers in the "independent" columns represent only those mutations that arose from independent events.

[b]The deletions ranged in size from 1 to 36 bp.

[c]Single-nucleotide insertions are not included in this category. The small insertions ranged in size from 4 to 13 bp and were accompanied by deletions of 1 to 26 bp.

[d]The large insertions ranged in size from 34 to 273 bp, and half of them were accompanied by deletions of 6 to 23 bp.

Fig. 2. Single-nucleotide insertions found at the (A) *Pvu*II and (B) *Eco*RV cleavage sites that were not accompanied by deletions. Bases in bold italics represent the insertions. The numbers represent the frequency of each type of insertion arising from independent events. The numbers in parentheses represent the total number of insertions observed when different from the number of independent events.

A	*Pvu*II	-CAG CTG-	
	-CAG*C*CTG-		8 (13)
	-CAG*T*CTG-		4 (6)
	-CAG*A*CTG-		2
B	*Eco*RV	-GAT ATC-	
	-GAT*G*ATC-		1
	-GAT*A*ATC-		1

recognition sequences. Two of these were located at the cleavage site with no deletion of wild-type sequence (Fig. 2B).

Small deletions were also induced by *Pvu*II and were the largest class of alterations induced by *Eco*RV and *Stu*I. These deletions ranged in size from 1 to 36 bp (Table 3). The *Pvu*II-induced deletions occurred on either side of the cleavage site and ranged in size from 2 to 18 bp, with complementary overlapping bases found at two of the seven junctions (Fig. 3A). The *Eco*RV-induced deletions occurred on either side of the cleavage site and ranged in size from 1 to 36 bp, with overlaps of 1-3 bases at all but one of the fifteen junctions that were not associated with small insertions (Fig. 3B). The most common alteration induced by *Eco*RV was a 2-bp AT deletion in the middle of its recognition sequence. This site was flanked by a 2-bp AT direct repeat (Fig. 3B).

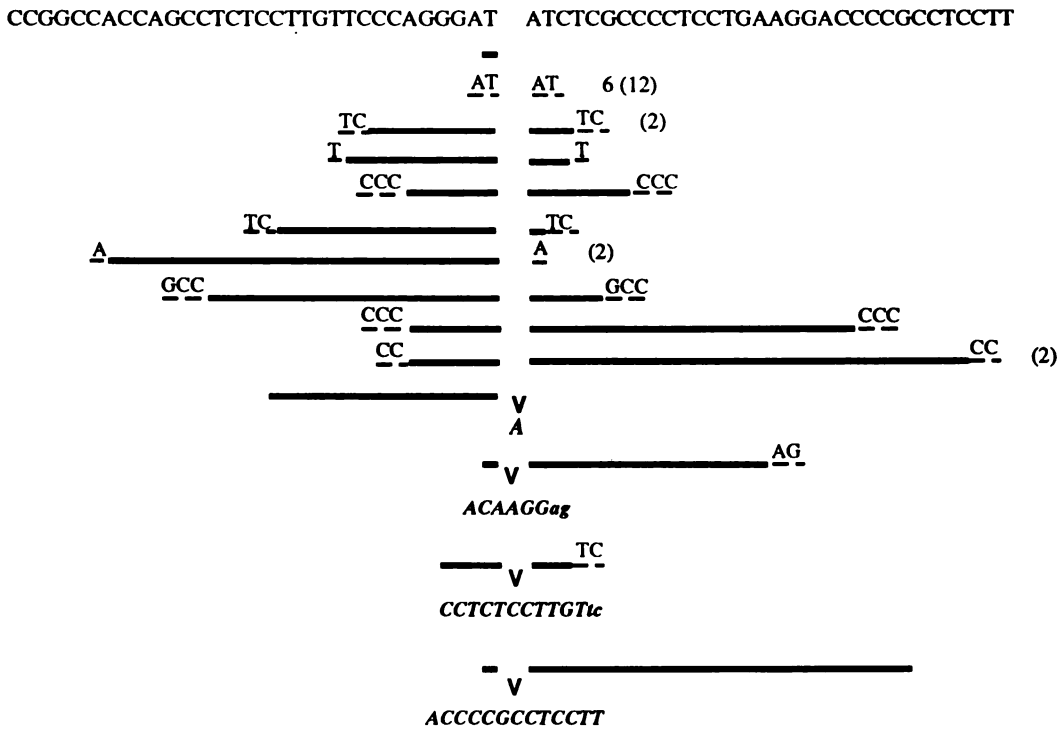
The deletions induced by *Stu*I also occurred on either side of its cleavage site and ranged in size from 2 to 24 bp, with 13 of the 21 deletions having overlapping bases at the break junctions that were not associated with small insertions (Fig. 3C). Several *Stu*I-induced deletions occurred multiple times, with the most common ones having 4 overlapping bases at the junctions (Fig. 3C).

Both *Eco*RV and *Stu*I induced single-nucleotide and other small insertions that were accompanied by deletions (Table 3). These insertions ranged in size from 1 to 13 bp, with deletions of 1 to 26 bp occurring on one or both sides of the cleavage site (Fig. 3B,C). Two of the *Eco*RV-induced small insertions were direct repeats of regions near the *Eco*RV cleavage site (Fig. 4A). In addition, one of the *Eco*RV- and one of the *Stu*I-induced small insertions were inverted repeats of regions near their respective cleavage sites (Fig. 5A).

Fig. 3. Deletions and small insertions induced by (A) *Pvu*II, (B) *Eco*RV, and (C) *Stu*I. The sequences at the top represent the wild-type sequence of that region of the APRT gene. The gap in the sequence represents the enzyme cleavage site. The thick solid lines represent the deleted base pairs. The numbers following a deletion show how many times each rearrangement occurred in independent mutants. Those not followed by a number were observed only once. The numbers in parentheses represent the total observed when this value was different from independent. The thin hatched lines represent overlaps found at the deletion junctions where it is unclear on which side of the break the bases were deleted. The letters above the hatched lines show the overlapping complementary bases. For example, the AT overlaps that were found six times at the *Eco*RV site (B) represent a 2-bp deletion that changed the wild-type sequence from -GATATC- to -GATC- in the mutant genes. The bold italic letters under certain deletions represent nucleotides inserted into the rejoined junction. Bases shown in lower case letters are speculated to have been part of the insertion and used in overlap formation.

A

B



C

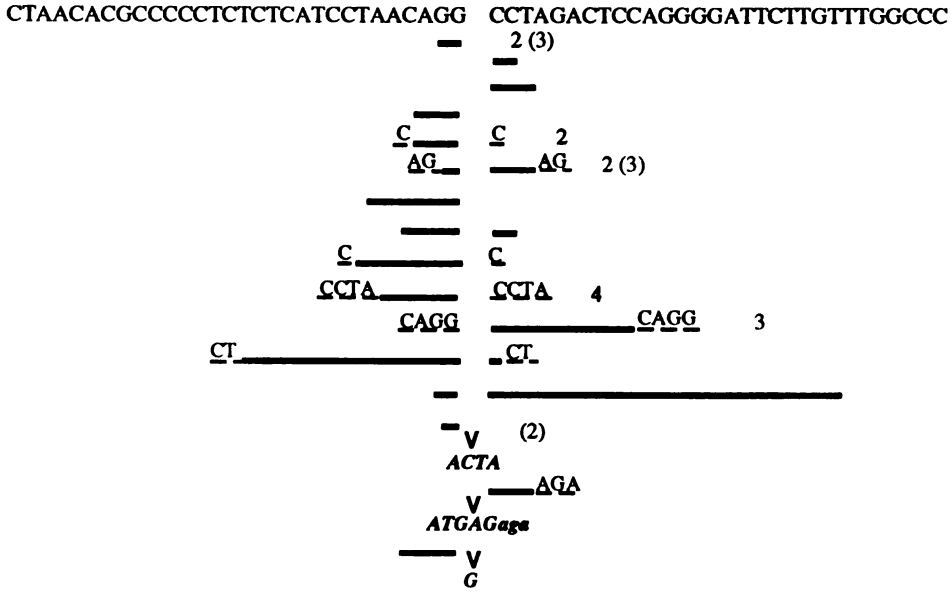


Fig. 4. (A) Nucleotide sequences of *Eco*RV-induced insertions that are direct repeats of adjacent regions. The wild-type APRT sequence is shown above each altered sequence. Arrows represent the restriction enzyme cleavage sites. The underlined bases on the wild-type sequences show the region that has been duplicated. The repeated nucleotides in the altered sequence are shown in bold italics. (B) Proposed slippage and misalignment model for the production of the mutation observed in the mutant RVA-9. (B,i) DNA double-strand break produced by *Eco*RV. (B, ii) Deletion of 4 bp from the upstream DNA end. (B, iii) Slippage and misalignment of the four-base CCAG repeat. (B, iv) DNA synthesis (arrow) from the misaligned base pairs. (B, v) Realignment of the CCAG repeats back to their original positions, followed by alignment of complementary bases for break rejoining.

A

-CCAGCCTCTCCTTGTTCCCAGGGAT↓ATCTCGCCCCTCCTGAAGGACCCCGCCTCCTTCCG-

RVA-9 -CCAGCCTCTCCTTGTTCCCAG*CCTCTCCTTGTTC*GCCCCTCCTGAAGGACCCCGCCTCCTTCCG-

-CCAGCCTCTCCTTGTTCCCAGGGAT↓ATCTCGCCCCTCCTGAAGGACCCCGCCTCCTTCCG-

R-14 -CCAGCCTCTCCTTGTTCCCAGGGAACCCCG*CCTCCTT*CCTCCTTCCG-

B

```
i)   -CCAGCCTCTCCTTGTTCCCAGGGAT     ATCTCGCCCCTCCTGAAGGACCCCG-
     -GGTCGGAGAGGAACAAGGGTCCCTA     TAGAGCGGGGAGGACTTCCTGGGGC-

ii)  -CCAGCCTCTCCTTGTTCCCAG
     -GGTCGGAGAGGAACAAGGGTC

iii) -CCAGCCTCTCCTTGTTCCCAG
     ------------------GGTCGGAGAGGAACAAGGGTC

iv)  -CCAGCCTCTCCTTGTTCCCAGCCTCTCCTTGTTC→
     ------------------GGTCGGAGAGGAACAAGGGTC

v)   -CCAGCCTCTCCTTGTTCCCAGCCTCTCCTTGTTCGCCCCTCCTGAAGGACCCCG-
     -GGTCGGAGAGGAACAAGGGTC             AGCGGGGAGGACTTCCTGGGGC-
```

Fig. 5. (A) Nucleotide sequences of *Eco*RV- and *Stu*I-induced insertions that are inverted repeats of adjacent regions. The wild-type APRT sequence is shown above each altered sequence. Arrows represent the restriction enzyme cleavage sites. The underlined bases on the wild-type sequences show the region that has been duplicated. The inverted repeats in the altered sequence are shown in bold italics. (B) Model for the formation of the inverted repeat insertion found in the mutant Stu-49. Bold italics represent nucleotides added by DNA synthesis. (B, i) DNA double-strand break produced by *Stu*I. (B, ii) Production of a single-stranded region. (B, iii) Foldback DNA formation followed by DNA synthesis (arrow). (B, iv) Relaxation of the foldback structure and alignment of complementary bases for break rejoining.

A

-CCAGCCTCTCCTTGTTCCCAGGGAT↓ATCTCGCCCCTCCTGAAGGACCCCGCCTCCTTCCG-

R-15 -CCAGCCTCTCCTTGTTCCCAGGG***AACAAGGAG***GACCCCGCCTCCTTCCG-

-GCCCCCTCTCTCATCCTAACAGG↓CCTAGACTCCAGGGGATTCTTGT-

Stu-49 -GCCCCCTCTCTCATCCTAACAGG***ATGAGAGA***CTCCAGGGGATTCTTGT-

B

i) -GCCCCCTCTCTCATCCTAACAGG CCTAGACTCCAGGGGATTCTTGT-
-CGGGGGAGAGAGTAGGATTGTCC GGATCTGAGGTCCCCTAAGAACA-

ii) -GCCCCCTCTCTCATCCTAACAGG

iii) -GCCCCCTCTCTCATCCT ^{A}
← ***AGAGAGTA***GGA$_{C}$ ^{A}

iv) -GCCCCCTCTCTCATCCTAACAGG***ATGAGAGA***CTCCAGGGGATTCTTGT-
TCTGAGGTCCCCTAAGAACA-

All three restriction enzymes also induced larger insertions at their recognition sequences (Table 3). *Pvu*II induced a 273-bp insertion at its cleavage site, with no deletion of the flanking host DNA sequences and no regeneration of *Pvu*II recognition sequences at either junction (Fig. 6A; Pvu-56). *Eco*RV induced three large insertions at its cleavage site. Two of these insertions, 123 and 88 bp in length, had no accompanying deletions and no regeneration of recognition sequences at the insertion junctions (Fig. 6A; RVA-28 and R-10). The third *Eco*RV large insertion was 180 bp in length and was accompanied by a 23-bp deletion, with 7 bp deleted upstream of the site and 16 bp deleted downstream (Fig. 6B; R-12). This insertion contained a GT direct repeat that was repeated fifteen times and ended at the 3′ insertion junction. *Stu*I induced two large insertions of 93 and 34 bp at its cleavage site, and both insertions were accompanied by deletions (10 and 6 bp, respectively) from the upstream side of the cleavage site (Fig. 6B; Stu-10, Stu-20). There were no deletions from the 3′ side of the *Stu*I cleavage site in either mutant, and *Stu*I recognition sequences were not regenerated at the 3′ insertion junctions.

Southern blot analysis of nonamplifying alterations. The 13 mutants that contained alterations that prevented PCR amplification across cleavage sites were examined by Southern blot analysis to detect rearrangements involving the APRT gene. All of the mutants showed altered RFLP patterns. The most interesting of these rearrangements were in the five mutants that showed wild-type RFLP patterns when digested with the restriction enzyme that was used to induce mutagenesis (Fig. 7A) but abnormal RFLP patterns when digested with the restriction enzymes *Pst*I or *Bam*HI (Fig. 7B). *Pvu*II, *Eco*RV, and *Stu*I each induced rearrangements of this type. The wild-type RFLP pattern

Fig. 6. Junctions formed by the large insertions at the restriction enzyme cleavage sites. Clone names and cleavage sites are shown at the left. At the right are the insertion junctions, where the normal letters represent APRT sequences and the bold italic letters represent inserted nucleotides. The total length of each insertion is shown above the junctions. (A) Large insertions that were not accompanied by deletions of APRT sequences. (B) Large insertions that were accompanied by deletions. The numbers following the Δ symbol below the junctions indicate how many base pairs were deleted from either side of the cleavage site.

A

|← 273 bp →|

PVU-56 -CAG CTG- → -CAG***AAAAA----------TCTCT***CTG-

|← 123 bp →|

RVA-28 -GAT ATC- → -GAT***CGGTG---------ATGTG***ATC-

|← 88 bp →|

R-10 -GAT ATC- → -GAT***AAAAG---------CAATT***ATC-

B

|← 180 bp →|

R-12 -GAT ATC- → -TCC***TTTTC----------(GT)$_{15}$***AGG-

Δ 7bp Δ 16bp

|← 93 bp →|

STU-10 -AGG CCT- → -TCA***ACACC---------AATAC***CCT-

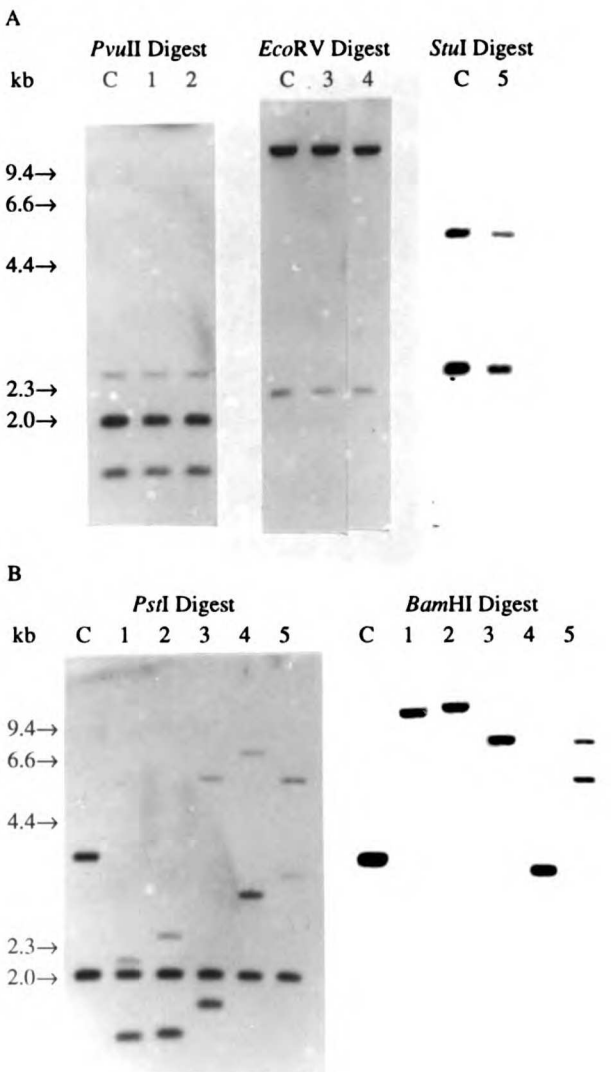
Δ 10bp

|← 34 bp →|

STU-20 -AGG CCT- → -CCT***CCGGA--------GTGGG***CCT-

Δ 6bp

Fig. 7. Southern blot analysis of mutants that contained rearrangements that did not allow PCR amplification across the restriction enzyme cleavage site. (A) Genomic DNA from wild-type CHO-AT3-2 cells (lanes C) and mutant cells (numbered lanes) was digested with the restriction enzymes that were used to induce the rearrangements. *Pvu*II-induced mutants Pvu-7 and Pvu-42 are shown as lanes 1 and 2, respectively. *Eco*RV-induced mutants RVA-15 and R-11 are shown as lanes 3 and 4, respectively. *Stu*I-induced mutant Stu-43 is shown as lane 5. Molecular weight markers (in kb) are shown at the left. (B) The 4.1-kb band from the *Pst*I digestion of wild-type CHO-AT3-2 DNA is upstream from the *Pst*I site in APRT and contains the *Pvu*II, *Eco*RV, and *Stu*I recognition sequences that are located in the first three exons of the APRT gene. The 3.9-kb band from the *Bam*HI digestion of wild-type DNA contains the entire APRT gene.

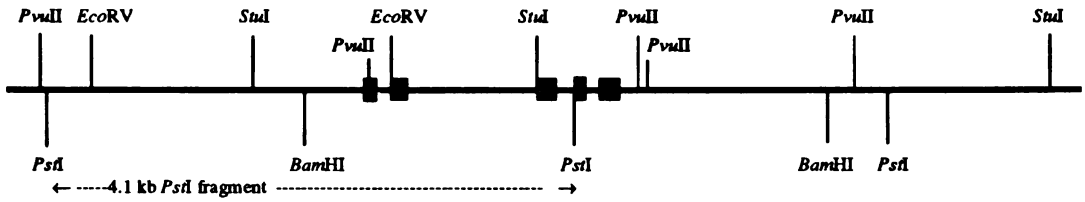

A
PvuII Digest
EcoRV Digest
StuI Digest
kb
C 1 2
C 3 4
C 5
9.4→
6.6→
4.4→
2.3→
2.0→
B
PstI Digest
BamHI Digest
kb
C 1 2 3 4 5
C 1 2 3 4 5
9.4→
6.6→
4.4→
2.3→
2.0→

produced after digestion with *Pst*I had two bands of 2.2 and 4.1 kb. The 4.1-kb band contained the first three exons of the APRT gene (Fig. 8). All five of the mutants had rearrangements that led to the disappearance of the 4.1-kb band and its replacement with two bands (Fig. 7B). The 2.2-kb band containing the 3′ end of the gene remained unchanged. Digestion with *Bam*HI produced a wild-type RFLP pattern that consisted of one 3.9-kb band (Fig. 7B) that contained the entire APRT gene (Fig. 8). The five mutants had rearrangements that led to the disappearance of the 3.9-kb *Bam*HI band and its replacement with two bands that in all cases added up to substantially more than 3.9 kb (Fig. 7B).

DISCUSSION

When introduced into CHO-AT3-2 cells by electroporation, the restriction endonucleases *Pvu*II, *Eco*RV, and *Stu*I were all able to produce DNA double-strand breaks at their recognition sequences within the endogenous APRT gene. These double-strand breaks induced illegitimate recombination that resulted in mutations at the APRT locus consisting mainly of small deletions and various-sized insertions. The complementary base overlaps found at most of the deletion junctions indicate that these blunt-end DNA double-strand breaks induced rejoining events in a mammalian chromosome that were the same as those seen in SV40 and plasmid rejoining studies in a variety of in vivo and in vitro systems (36, 66, 71, 84). Recently, it has been shown that DNA double-strand breaks are able to induce similar illegitimate recombination events in the chromosomes of haploid *rad52 Saccharomyces cerevisiae* strains that cannot repair the DNA double-strand breaks by homologous recombination (38). Therefore, it appears that chromosome breaks can be rejoined by similar mechanisms in yeast and mammalian cells and that the characteristics of

Fig. 8. Restriction map of region surrounding the CHO APRT gene. The cleavage sites indicated above the line are those of the enzymes used to induce mutations. The cleavage sites indicated below the line are those of additional enzymes used for Southern blot analysis. The boxes represent the five exons that constitute the coding sequences for the APRT gene (5). The exons and the cleavage sites are drawn to scale. The two *Bam*HI sites are located 3.9 kb apart. The 4.1 kb *Pst*I fragment contains the first three exons of the APRT gene.


PvuII
EcoRV
StuI
EcoRV
StuI
PvuII
PvuII
PvuII
StuI
PvuII
PstI
BamHI
PstI
BamHI
PstI
← -----4.1 kb PstI fragment ---------- →

illegitimate recombination that have been described may reflect basic processes in DNA metabolism.

The most common mutation induced by *Eco*RV was a 2-bp deletion in the middle of the recognition sequence. The interesting characteristic of this mutation was the 2-bp AT direct repeat flanking either side of the cleavage site and providing the 2-base overlap used in the rejoining process. There was a tendency for the break-rejoining process to use at least one overlap that was either at or very near one of the ends of the broken DNA. Seventy percent of the deletion junctions induced at all three restriction enzyme recognition sequences contained complete wild-type sequences on one side of the cleavage site. Roth and Wilson (71) found that 97% of the rejoining events of recircularized SV40 genomes were within 15 nucleotides of the ends of the linear substrate molecules and that more than 60% of the junctions retained either the terminal nucleotides from a blunt end or the 5′ or 3′ extensions from an overhanging end. At the restriction enzyme-induced recombination junctions in the APRT gene, 88% of the rejoining events were within 15 nucleotides of the ends. By studying the junctions from nonhomologous substrates rejoined in *X. laevis* egg extracts, Pfeiffer and coworkers (67) found that base mismatches external to the complementary bases used in overlap formation were more inhibitory to the rejoining process than were internal base mismatches, which were virtually neglected by the rejoining system. This inhibitory effect of external base mismatches may drive the reactions to use at least one overlap that is situated at or near the end of the broken DNA.

Although small deletions predominated in the mutation spectrums induced by

*Eco*RV and *Stu*I, single-nucleotide insertions were the predominant mutation induced by *Pvu*II. Of the insertions found at many of the illegitimate recombination junctions that have been studied in a variety of mammalian systems, there is a preference for single-nucleotide insertions (73). Although terminal transferase is a candidate for facilitating single-nucleotide insertions in lymphoid cells, it is not believed to be responsible for those seen at recombination junctions in nonlymphoid cells (73). Because CHO cells are not of lymphoid lineage, it is likely that the single-nucleotide insertions seen at all three restriction enzyme cleavage sites were the result of some other process. DNA polymerases are able to add single nucleotides to the ends of blunt-end duplexes in vitro (16) and hence may play a role in the single-nucleotide insertions found at some of the restriction enzyme-induced recombination junctions.

It is unclear why single-nucleotide insertions were found at so many of the *Pvu*II-induced breakpoint junctions. A study of illegitimate recombination junctions induced by *Pvu*II in the *lac*Z gene carried on a stably maintained episomal shuttle vector in human cells showed that *Pvu*II is capable of generating different spectrums of recombination junctions at its three cleavage sites within *lac*Z (92). Single-nucleotide insertions accounted for 7 of the 19 mutations found at one of the *Pvu*II cleavage sites in *lac*Z but only 2 of the 20 mutations found at the other two *Pvu*II cleavage sites (92). It is possible that the positioning of the bases to be used in overlap formation may affect steps in the rejoining process. Differences in nucleotide sequence or chromatin structure surrounding the site of a double-strand break could also have an effect.

DNA polymerases clearly play a role in some of the restriction enzyme-induced

small-insertion mutations in the APRT gene. *Eco*RV mutants RVA-9 and R-14 have small insertions that are direct repeats of regions near the *Eco*RV cleavage site (Fig. 4A). The insertion in mutant RVA-9 appears to be a duplication that occurred by slippage and misalignment of a 4-bp CCAG direct repeat followed by DNA synthesis. After duplication the chromosome break appears to have been rejoined by alignment of two complementary nucleotides synthesized during duplication (Fig. 4B).

Fill-in DNA synthesis is required for many nonhomologous end-rejoining processes (85). Furthermore, it has been shown that both the Klenow fragment of DNA polymerase I from *Escherichia coli* and native Taq DNA polymerase are capable of synthesizing across discontinuous templates in vitro (17, 37). It has been speculated that such activity makes DNA polymerases a candidate for the protein that aligns broken DNA ends during illegitimate recombination (17, 37). If DNA polymerases are an integral part of the end-rejoining machinery, they may be responsible not only for these clearly template-derived insertions but also for other small insertions seen at illegitimate recombination junctions. A 4-nucleotide ACTA insertion induced at the *Stu*I site was not duplicated from any template near the cleavage site. This insertion may have arisen by synthesis from some further-removed template or by other mechanisms such as capture and ligation of small oligonucleotides into the break before rejoining (74).

Both *Eco*RV and *Stu*I induced small-insertion mutations that were inverted repeats of regions near the cleavage sites. Both these mutations may have occurred in a template-derived fashion. As shown for the mutant Stu-49, restriction enzyme cleavage could be followed by liberation of one of the DNA strands, by either melting or degradation of the

other strand, allowing the free DNA strand to then fold back on itself to prime DNA synthesis and duplicate the region (Fig. 5B, i-iii). Duplication could then be followed by relaxation of the foldback DNA and alignment of complementary bases for break rejoining (Fig. 5B, iv). Similar models involving foldback DNA intermediates have been proposed for complex frameshift and deletion mutations produced in vitro by *E. coli* DNA polymerase I and its large-fragment derivative and by yeast DNA polymerase I (39, 64). Another possibility is that these inverted repeat mutations were the result of the cleaved DNA ends being covalently sealed into hairpins before rejoining. Such a model would be analogous to those proposed for inverted repeat formation during the excision of plant transposable elements (18) and coding joint formation during V(D)J recombination (40, 75). In these models the inverted repeats form as the result of unequal nicking of the sealed hairpin. Recently, it was shown that several mammalian cell mutants defective in DNA double-strand-break repair also show defects in V(D)J recombination (65, 81). It has been proposed that hairpin formation might occur at the sites of random chromosome breakage and that the scid gene product may be responsible for proper rejoining of the hairpin-sealed chromosome breaks as well as those breaks generated during V(D)J recombination (41).

The larger insertions found at the restriction enzyme cleavage sites ranged in size from 34 to 273 bp (Fig. 9). None of these inserted sequences were derived from the APRT gene or its immediately surrounding regions. Nor did any of the sequences show significant homology to those contained in the Genebank database (version # 81). Presumably, one mechanism of insertion formation would be to integrate fragments generated by restriction enzyme cleavage elsewhere in the cellular genome into one of the double-strand

Fig. 9. DNA sequences of large insertions induced at restriction endonuclease cleavage sites in the APRT gene. All sequences are given in the 5′ to 3′ direction and correlate with the coding strand of APRT. PVU-56 is a 273 bp insertion induced at the *Pvu*II site. RVA-28, R-10, and R-12 are insertions of 123 bp, 88 bp, and 180 bp, respectively, induced at the *Eco*RV site. STU-10 and STU-20 are insertions of 93 bp and 34 bp induced at the *Stu*I site.

PVU-56

AAAAATAACT TCATGGATAG GCATCATCAC AACATTAAGG TTTACAACCA
TTGTTCTAGG AAAATTCCCA TCAATTTATA CTTATTTTCC ATTGTTAAAC
CAAAACCCCT TCAGTTTGAT CCCGCTTTTA GTTATATATA CTGATAAATT
TTCCAATGTA TTCCACCTAG TTGTTTTCGA TTTATCGGTT GTTATGCCCT
ATTTAAAAGT ATGTAAGTCA TAGACACTTG AGTTTGAGGC TTAACCTTCC
CAGTCCACAC ATTTAACATC TCT

RVA-28

CGGTGGTGAT TATGATTAAA TTACATTATG ACGTATATGA AAATGTCCTA
GTAAACCCAT TTTCTAAACA TAATTACTAC ATGTTAATAA AAAGCATTTT
GAAAAAGCAA AGAAGAAAAT GTG

R-10

AAAAGGAGAG CAGGCTAACT TATCCTGAGA AAAGTCATTG AATTGATAAT
GAGAAAAAAC GGAAGCCTGG TGGCATTTCT GATCAATT

R-12

TTTTCCCCCC AGTCTATCTT TTCAGCATAA GGTACAGTAA GGAAACTAAT
TTCTTGTAAT GGTACCAAGG AAAGAAACAT AATAACAAGC ATGGAATGGT
GCCTCTAGGC TGTAGATAAG GCAGTATGAG TAAGGTGATG GAAGATTTCT
GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT

STU-10

ACACCTTGAA AGAAAGATCC AAGAAAAGAC AAAGACAGTC CCCAAATTCT
TCTCTTATTC TGTGTTACAT CAATTGGCTT CTTGATACAA TAC

STU-20

CCGGAGTGGA CTGGCGTTTT GCCTGGTGGG TGGG

breaks in the APRT gene. If the larger insertion sequences did originate as restriction enzyme cleavage products, one would expect them to be inserted and joined by either blunt-end ligation or processes involving noncomplementary end rejoining. Because no restriction enzyme recognition sequences were regenerated at the insertion junctions, it is unclear whether these insertions originated as enzyme-cleaved fragments of chromosomal DNA. Half of the large insertions seen at the restriction enzyme cleavage sites were accompanied by small deletions at the site of insertion. Small deletions (9 and 12 bp) of chromosomal sequences have been seen in human cells at two plasmid integration sites (56).

Five mutants contained rearrangements that prevented PCR amplification across the restriction enzyme cleavage site and exhibited both wild-type and abnormal RFLP patterns when analyzed on Southern blots. Because the entire APRT gene is still present in these mutants, and because it has been rearranged in such a manner as to retain a wild-type RFLP pattern only when digested with the restriction enzyme used to induce the rearrangements, I believe that the rearrangements are a consequence of restriction enzyme-produced DNA double-strand breaks. Furthermore, the rearrangements appear to have been joined by blunt-end ligation, regenerating the respective restriction enzyme recognition sequences at the junctions. The altered RFLP patterns seen by digestion with *Pst*I or *Bam*HI are consistent with the idea that the mutations responsible caused an interruption in the APRT gene at the cleavage sites of the enzyme used to induce the rearrangements. Such an interruption of the gene could be explained if the rearrangements were chromosome translocations, inversions, or large insertions. The inversions or inser-

tions would need to be large enough to provide at least one *Pst*I site and one *Bam*HI site between the interrupted regions of the APRT gene. Gamma irradiation induces a class of mutations at the CHO APRT locus, called complex rearrangements, that are also believed to be either chromosome translocations, inversions, or large insertions (44). This class of rearrangement has not been seen in the spontaneous mutation populations studied at the APRT locus (44) and probably results from interactions between multiple chromosome breaks.

Because this system identifies only those rejoining events that result in loss-of-function mutations, it is unclear how many of the restriction enzyme-produced double-strand breaks were rejoined in ways that did not destroy APRT protein function, such as blunt-end ligation of the broken DNA to restore the wild-type restriction enzyme recognition sequence. A study of the rejoining of the SV40 viral genome that had been linearized by the restriction endonuclease *Fnu*DII, which produces blunt ends, found that 77% of the vectors were rejoined by blunt-end ligation to restore the *Fnu*DII recognition sequence, whereas 23% of the infective vectors had lost the *Fnu*DII recognition sequence (87). *E. coli* can rejoin linearized plasmid substrates by mechanisms of nonhomologous recombination (36). Plasmids linearized by restriction enzymes that produce blunt-end DNA double-strand breaks and used to transform *E. coli* cells are recircularized by blunt-end ligation to restore the restriction enzyme recognition sequences < 20% of the time (19). The complex rearrangements analyzed on Southern blots provide evidence that mammalian cells can rejoin double-strand breaks in chromosomal DNA by blunt-end ligation, but whether these reactions are carried out as frequently as those seen with the SV40

genome in monkey cells is unknown. The proportion of breaks at the APRT locus rejoined by blunt-end ligation along with the other types of rejoining events may be influenced by the genetic damage, in the form of DNA double-strand breaks, that the restriction enzyme-treated cells will have sustained at other locations in their genomes. However, these effects should at least be tempered by the fact that none of the DNA damage can be so severe that it would preclude cell survival.

Conclusions

This study of DNA double-strand-break-induced genetic recombination illustrates the complexity of break rejoining in mammalian chromosomes. A blunt-end DNA double-strand break with intact 3′ hydroxyl and 5′ phosphate ends produced in chromosomal DNA can lead to insertion and deletion of nucleotides and more complex genetic rearrangements. Short stretches of complementary bases seem to play a pivotal role in the rejoining process. Whether the complementary bases are aligned during the rejoining process by a specialized alignment protein (85) or by DNA polymerases (17, 37) remains unclear. Many of the mutations induced by the restriction enzyme generated double-strand breaks appear to be polymerase mediated errors, some of which are consistent with known DNA polymerase misalignment mutations. When a DNA polymerase is bound to the end of a DNA duplex it has a primer and must next find a template to synthesize from. A search for template, whether active, or passive, could lead to the observed misalignment errors, and provides the basis by which DNA polymerases might function to align complementary bases during double-strand break rejoining.

Because the mechanisms of rejoining chromosomally located DNA double-strand breaks are similar to those observed in model systems, the rules for break joining established in such basic systems should be applicable to complex mammalian problems like chromosome aberration formation. SV40 rejoining studies have shown that mammalian cells can join any set of noncomplementary DNA ends and do it with the same efficiency as complementary end rejoining (87). Studies of chromosome aberration forma-

tion after combined exposure of CHO cells to restriction enzymes and X rays have shown that quite different kinds of double-strand breaks are able to interact (51, 82), lending credence to the idea that mammalian cells can join very different types of DNA double-strand breaks. If mammalian cells can join any two broken ends, then what factors influence whether two breaks are repaired faithfully, or whether they will interact to lead to exchange type chromosome aberrations? It may be that time and space are the only important considerations and that cells will indiscriminately join any two DNA ends that are in close proximity.

That blunt-end DNA double-strand breaks can induce such a wide spectrum of alterations at the APRT locus demonstrates the important role that such lesions play in mutagenesis. Failure to repair breaks is incompatible with cell survival, and none of the recombination events observed here compromised cellular viability. However, the long-term biological consequences of illegitimate recombination for genomic stability, particularly in terms of gene amplification, transformation, and chromosomal destabilization, remains unknown.

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