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Factors Affecting Mutational Specificity Induced by Ionizing Radiation and Oxidizing radicals

PROGRESS REPORT

1. Reminder of objectives

The ABSTRACT of our 1988 Proposal continues to give the objectives of our work.

We propose to analyze the factors affecting the specificity of mutational change as induced by ionizing radiation and oxidizing radicals. We want to understand not only the rules that affect base substitution but also the mechanism(s) by which additions and deletions are produced, since deletions are a common consequence of radiation. We wish to carry out this analysis in an in vitro mutation system that permits us to analyze the role of base sequence, of polymerase and of mutagenic agent. Our system is designed to screen out most direct breaks as a cause of mutation and should indicate the changes resulting from base damage to the DNA. We want to answer the following questions:

1. What types of base substitution mutations are induced by ionizing radiation and oxidizing radicals?
2. Are deletions and/or additions produced?
3. Is there a difference in the type of mutation produced dependent on the polymerase used? Do mammalian polymerases plus their accessory factors result in different patterns of mutation.
4. What is the mechanism by which base damage is converted to mutation.

Our proposal was based on utilization of an in vitro system in which mutations generated by the in vitro copying of a reporter gene sequence could be readily scored. This system is described in our previous proposals and in the reprint accompanying this report.

2. Specific Progress in the past year.

We published the results of our first study of ^{60}Co - γ -ray-induced mutation using the in vitro system.

Turkington, E. and Strauss, B. 1991. In vitro mutagenesis as a result of ^{60}Co - γ -ray-induced base damage. Mutation Res. 251: 187-199.

SUMMARY: We utilized a model system to study the mechanism(s) of mutation resulting from gamma ray-induced DNA base damage. ^{60}Co -irradiated, uracil-containing M13mp2 DNA was hybridized to normal (non-uracil) linearized double stranded virus DNA minus the lac reporter region. Only DNA without

strand breaks in the reporter region will circularize. This DNA was used as a substrate for a modified T7 DNA polymerase with no residual 3'-->5' exonuclease activity (Sequenase 2). The reaction product was transfected into a *rec⁻* bacterial host to minimize the occurrence of bypass events *in vivo*, and mutant progeny were selected. DNA irradiated with 400 or 800 Gy from a ⁶⁰Co γ source gave about a 5-fold increase in the percentage of mutants recovered after synthesis with Sequenase as compared to the recovery of mutants using control DNA. About twenty percent of the mutants recovered from both irradiated and control templates contained multiple mutations in the target area sequenced. The irradiated samples had an excess of mutations which resulted from changes at pyrimidines. C-->T transitions were most common. Mutations at T were mostly (-1) and (-2) frameshifts, particularly at sequences of repeated T's.

The distribution of mutations appeared non-random with some regions appearing particularly mutable. In particular, minus one (-1) frameshift mutations seemed particularly prevalent at runs of pyrimidines, particularly runs of T's. This particular distribution of mutations is not that which has been observed in studies from other laboratories of plasmids irradiated with either high or low LET radiation and transfected into cells.

Several different explanations of the difference suggest themselves.

- a) There is a difference in the response of single strand (our work) and double strand (other laboratories) DNA to radiation.
- b) The use of Sequenase (T7 DNA polymerase devoid of 3'-->5' exonuclease activity) as polymerase leads to a bias in the types of mutation observed,
- c) the use of a different target DNA sequence leads to a difference in the mutations obtained.

During the past year we have made the following progress in addressing these issues.

1. Determination of the nature of the damage leading to inactivation of the transfection activity of irradiated DNA.

- a. We have developed a method for estimating the proportion of linear single stranded and circular single stranded DNA in a sample. The separation of relaxed (nicked) and supercoiled double stranded DNA is well known, but a simple gel method for separating single stranded circular and linear DNA seems not to have been available. We observed that separation of these species is easily obtained by electrophoresis in a borate buffer.

- b. Given this methodology, we have determined that loss of M13 transfection activity is not correlated with the opening of circles of single stranded DNA. We therefore conclude that base damage rather than chain breakage is a major source of lethality (loss of transfection activity) in irradiated single strand DNA. Strand breakage of irradiated DNA is produced by alkali treatment. We are determining whether the alkali-labile sites, determined by the

conversion of circular to linear single stranded DNA in the presence of alkali is correlated with inactivation.

2. Preparation of purified substrate for mutation and enzyme studies.

In our previous studies on chemically induced mutation and in our published study with γ -ray induced mutation we produced gapped hybrid by hybridizing a limiting amount of treated single strand DNA with an equimolar amount of a double stranded molecule from which the reporter gene region had been removed. The hybridization mixture was used for experiments without further preparation. Although breaks are not the (sole) cause of lethality, they are produced and we have noticed that the yield of hybrid diminishes with increased dose of radiation. In order to make possible a better estimate of the kinetics of response to radiation dose, we have developed a method (based on that used by Modrich in preparation of substrate for mismatch repair analysis) for the preparation of purified gapped, irradiated substrate. The method maximizes the utilization of the linear DNA fragment which is the most expensive part of the substrate to prepare.

- a. Single stranded circular DNA is irradiated.
- b. The DNA in about tenfold molar excess is hybridized with double stranded fragment.
- c. After hybridization the mixture is passed through hydroxylapatite to remove excess single stranded DNA.
- d. The DNA is treated with an ATP dependent exonuclease from *M luteus* which hydrolyzes linear molecules. Gapped circular DNA survives the treatment.
- e. the remaining DNA is deproteinized with phenol and precipitated with ethanol.

Our first experiments indicate that after irradiation of single stranded DNA with 400 Gray, the yield of hybrid is reduced to about 50% relative to the unirradiated sample.

Since it is only gapped hybrid which can serve as a productive template for DNA synthesis and mutation production, we suppose that use of this purified template will provide a substrate to test for dose effect relationships.

3. Use of altered substrates for mutation studies.

Our studies show a region of the reporter gene with the sequence

5' CTG GCC GTC GTT TTA CAA CGT 3'

to be a hotspot for the deletion of one or two T's in the run of 4 T's. In our experiments we measure forward "mutation" from blue to white plaques. Following the lead of Kunkel, we have synthesized a substrate with an extra T in this region. This substrate yields colorless plaques. Revertants can be detected as blue or light blue plaques. As shown by Kunkel and his

collaborators, reversion can take place either by deletion of a T at the position of the extra T or by base deletions which restore the reading frame anywhere in a region surrounding the original change. We have found it desirable to use hybridizing fragment prepared from RF from strains with the deletion to eliminate a background of blue plaques due to undigested or singly digested RF molecules. In a small sample of 15 revertants sequenced we found that four reverted by change at the position of the extra T. Interestingly, all but one of the other revertants occurred by loss of a base at a position where (in studying forward mutation) we had previously observed deletion either spontaneously or after γ -ray irradiation. We can not yet tell whether the changes are radiation induced but it does seem that the DNA polymerase, Sequenase, has a preference for particular sites. This makes it particularly important to carry out these experiments with different enzymes.

PLANS FOR THE COMING YEAR

We are now in a position to exploit our methodology for study of the questions originally asked. We plan to:

- 1) Determine the correlation between loss of transfection activity, DNA strand interruptions and the production of alkali-labile damage after ^{60}Co γ ray irradiation and after exposure to hydroxyl radicals produced by chemical means to ascertain the nature of the lethal lesions produced by ionizing irradiation. We are particularly interested in learning whether the yield of products is different when double and single stranded molecules are irradiated.
- 2) Determine the yield of gapped hybrid as a function of radiation dose and to carry out mutation frequency experiments and determine reversion specificity after synthesis using different DNA polymerases starting with the same amount of hybrid. In particular we plan to use enzymes with and without proofreading exonuclease and from prokaryotic and eukaryotic sources for these studies.
- 3) Given our ability to isolate hybrid, we plan to carry out biochemical termination studies on these substrates to determine the site of the blocking lesions with different polymerases and to compare these with the site and type of mutations produced.

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