RADIATION INDUCED BREAKS INCREASE THE PRIMING ACTIVITY OF RAT SARCOMA DNA IN THE DNA POLYMERASE REACTION

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SUMMARY

Radiation produces large numbers of DNA breaks which are characterized by 5' termini. The presence of these breaks is accompanied by an increase in the ability of the DNA to serve as a primer in the DNA polymerase reaction. Since DNA polymerase adds nucleotides from the 3' end of the molecule, and since a 3' OH terminus is required, our results provide additional evidence that some radiation induced DNA breaks are characterized by 5'PO_4 - 3' OH termini. DNA ligase is very likely responsible for rejoining this type of break.

INTRODUCTION

In earlier studies we used the polynucleotide kinase method to show that radiation produces DNA breaks characterized by 5'PO_4 termini (1). Because 2,4-dinitrophenol (DNP) inhibited both the appearance and the rejoining of these breaks (2,3), energy requiring enzymatic processes apparently are required. Also, we suggested that DNA ligase could be responsible for the rejoining of these breaks. This enzyme, which rejoins single-strand DNA breaks characterized by 5'PO_4 - 3' OH termini, requires a high energy cofactor, either ATP or DNP, for its function (4).

The polynucleotide kinase assay focuses upon the 5' termini; under the conditions of the assay these termini are labeled with $^{32}PO_4$. As a result, the DNA specific activity is proportional to the number of DNA breaks characterized by 5' termini.

In this paper we report the results of experiments in which the DNA from irradiated rat sarcoma cells was used both in the polynucleotide kinase reaction and as a primer in the DNA polymerase reaction. Since DNA polymerase adds nucleotides to the 3' end of the DNA molecule (5), we would be able to observe the participation of both the 5' and the 3' termini in post-irradiation rejoining.

METHODS

The rat sarcoma cells were originally obtained (in 1967) from a Fischer rat which had been infested with the ova of T. formis (6); the cells have been subsequently carried as a cell culture. Although the cells have been transferred many times during the past three years, they still maintain their malignant potential. An injection of 10^6 cells into the subcutaneous tissue of the Fischer rat will yield a solid tumor of 1 cm in diameter in approximately 45 days.

For the present experiments the cells were carried as monolayers; Eagle's medium (MEM) supplemented with "non-essential" amino acids and 10% calf serum was used. The experimental design was essentially that as described before (2). The cells were detached with dilute trypsin, pooled, washed twice with glucose free Hank's balanced salts solution (HBSS), and then divided into two portions. The cells of the first portion were suspended in 1×10^{-4}M DNP (dissolved in HBSS) while the cells of the second portion were suspended in HBSS (without DNP). The cells were suspended at a density of 5×10^6 cells/ml and maintained at 37°C. Immediately before irradiation (1000 rads; 500 rads/min; 250 kVp X-rays), 3 ml samples were taken from both suspensions and delivered into test tubes. The tubes (DNP and HBSS) were simultaneously irradiated; at the appropriate time after exposure they were quick-chilled in dry ice-acetone. The tubes were then centrifuged at 4°C and the cell pellet processed as described before (1). Non-irradiated controls were also handled in pairs.

Both polynucleotide kinase and DNA polymerase are produced in high concentration by T-even bacteriophage infected E. coli (7). For our studies E. coli B, infected with T-2 bacteriophage, was purified through step IV as described by Richardson (8). This material contained both polynucleotide kinase

activity and DNA polymerase activity. Further purification (6, 8), while greatly increasing the enzyme specific activities, reduced the total activity. We found no real differences in results using fraction IV as compared with more highly purified preparations. Measurement of DNA content and the polynucleotide kinase reaction was performed as described before (1-3). The DNA polymerase reaction was carried out as follows. The reaction mixture, 1.0 ml, contained 3.5 μg DNA, 0.1 unit DNA polymerase activity (7), $MgCl_2$ (2.4 mM), tris (30.8 mM), mercaptoethanol (1.2 mM), dCTP, dATP, dGTP, and dTTP (8.6 mM) and 0.25 μCi of ^{3}HdATP (5.9 Ci/mM--Schwartz). The DNA was in the double-stranded form. The mixture was incubated at 37°C for 30 minutes. The reaction was terminated by the addition of 0.05 ml of 10 N perchloric acid (PCA). The precipitate was collected on a Millipore filter disc and washed with ice cold 0.5N PCA. After drying, the discs were counted with a liquid scintillation counter. The results are expressed as DNA specific activity (cpm/μg). Duplicate assays were performed for each sample and the averages calculated. The range of values of the duplicates was less than the size of the plotted points.

RESULTS AND DISCUSSION

Figure 1 shows the results of a typical experiment. The upper left panel shows the results of the polynucleotide kinase assay for cells suspended in HBSS. Note the contraction of the abscissa time scale after 1 minute. The DNA specific activity (^{32}P cpm/μg) is proportional to the number of 5'PO_4 termini present. As this panel indicates, the largest number of DNA breaks appeared immediately after irradiation. With time, the number of these breaks decreased to control levels, as a consequence of rejoining. The presence of DNP (lower left panel), however, prevented the appearance of DNA breaks above control levels. These findings are similar to those previously described for L cells (1-3).

The upper right panel shows the results of the DNA polymerase reaction for cells suspended in HBSS. The DNA specific activity (^{3}H cpm/μg) is proportional to the number of nucleotides added, and presumably, to the number of 3' termini present. The similarity between the results of these measurements

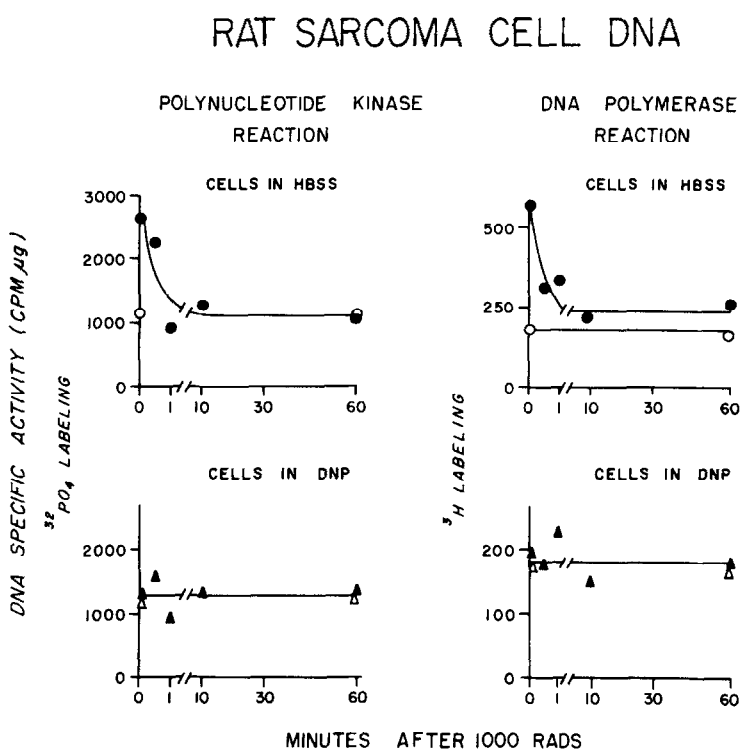

Fig. 1. The left panels (upper and lower) show the results of the polynucleotide kinase assay, while the right panels indicate the effectiveness of the DNA to prime in the DNA polymerase reaction. The results are expressed as DNA specific activities; ^{32}P cpm/μg for the polynucleotide kinase assay and ^{3}H cpm/μg for the DNA polymerase reaction. Notice that DNP prevented both the ^{32}P and ^{3}H increased labeling seen for the cells suspended in HBSS. The open symbols represent the unirradiated cells while the closed symbols show the response of the irradiated populations.

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and the polynucleotide kinase assay is apparent. The increased ^{32}P labeling parallels an increased ^{3}H labeling immediately after irradiation. Also, the post-irradiation period is characterized by a decrease in both ^{32}P and ^{3}H labeling toward control. In a similar manner, the DNP treatment prevented the appearance of breaks characterized by $5'PO_4$ termini and it prevented the appearance of the increased ^{3}H labeling. We have also observed this parallelism between the polynucleotide kinase and DNA polymerase assay results for solid rat tumors irradiated in vivo.

We interpret these results to mean that radiation induced DNA breaks

characterized by 5' termini are accompanied by 3' OH termini; the appearance and the disappearance of the 5' termini is associated with the appearance and disappearance of 3' termini. Consequently, DNA ligase seems even more implicated in the processes responsible for the rejoining of this specific type of DNA break.

We should again re-emphasize that we do not believe this type of DNA break to represent the only type of DNA break caused by radiation (1-3, 9). In a recent publication by Swada and Okada (10), these workers, using sucrose gradient sedimentation analysis, reported that dinitrophenol did not prevent the appearance or the rejoining of radiation induced DNA breaks by L5178 mouse leukemia cells. These results are somewhat at variance with the experience of Humphrey, et. al, who found that cyanide prevented the rejoining of DNA breaks by Chinese hamster cells (11). Since Swada and Okada added their DNP to complete medium, possibly the effectiveness of the DNP to uncouple oxidative phosphorylation could have been compromised. We have found that the presence of protein (such as calf serum proteins) to cause an erratic response in DNP experiments (12), perhaps due to complex formation between the DNP and the protein. Consequently, we have always used DNP dissolved in a protein-free medium such as HBSS. In spite of these factors, though, the available data suggest that more than one type of DNA break occurs after irradiation with ionizing radiations.

As a final point, a comment should be made about the results reported by Harrington (13). She found irradiated DNA to be less effective than non-irradiated DNA to serve as a primer in the DNA and RNA polymerase reactions. Since she used DNA from cultured mammalian cells (L5178Y) frozen immediately after irradiation, a large number of DNA breaks would be anticipated. At present, we have no explanation for the differences in her results and ours.

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