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PROGRESS REPORT

To: United States Atomic Energy Commission

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Title of Project: Molecular Basis for the Mutagenic and
Lethal Effects of Ultraviolet Irradiation

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Background and Progress Report

Our laboratory during the past year of support by the Atomic Energy Commission has directed its attention toward an examination of those enzymes involved in the early steps of repair. The second aspect of the project supported by this grant had to do with the complete repair of biologically active DNA in vitro by pure enzymes. The description of the pathways to be examined are outlined in Fig. 1 and will provide a guide for a discussion of this repair cycle.

I. Incision

The former proposals and our attendant progress has been confined to examining those endonucleases specific for UV-irradiated DNA, isolated from Micrococcus luteus, and now Escherichia coli. This organism was initially chosen because it is a UV-resistant organism with low endogenous levels of non-UV-specific nucleases. The advantage, therefore, of studying excision enzymes in such an organism is that it has demonstrable levels of excision repair enzymes against a low background of non-UV-specific nucleases which allows for the isolation of those enzymes which would be in a minor population. The disadvantage, however, with M. luteus, even though it is a UV-resistant organism with high levels of UV-endonuclease, is that there is no genetic linkage map allowing one to identify the biological involvement of a specific enzyme. The ideal organism for such studies would have been E. coli, for which a very detailed genetic map has been established. Although E. coli is not as UV-resistant as M. luteus, and has a plethora of non-UV-specific nucleases, the genetic advantages have pointed to approaching this organism

for an understanding of its repair mechanisms. Although we have a very good understanding of the basic enzymology in M. luteus, we have no understanding in E. coli. For that reason, once the technology had been established, we then approached this problem, utilizing some of the techniques outlined in last year's progress report. By taking advantage of these extremely sensitive techniques for measuring UV-specific endonucleases, our lab then went ahead toward the isolation, characterization and identification of UV-endonuclease as a gene product. This project has been successful. (7).

At least two endonucleolytic activities that preferentially incise UV-irradiated DNA exist in extracts of E. coli. These two activities have been separated by phosphocellulose chromatographic fractionation and specific molecular weight fractionation techniques. One of the activities elutes from phosphocellulose with 0.25 molar potassium phosphate buffer pH 7.5. This UV-specific endonucleolytic activity is absent from partially purified extracts of the uvrA and uvrB pyrimidine dimer excision-defective mutants, but is present in normal amounts in the uvrC and uvrD excision-defective mutants. The enzyme incises UV-irradiated DNA 5'-to the dimer, leaving a 3' hydroxyl group on the DNA. In the presence of excising enzymes, the E. coli UV-specific endonuclease is required for pyrimidine dimer excision in vitro. Furthermore, the requirement for this enzyme in in vitro repair of transfecting linear and circular DNAs has also been shown. We can, therefore, show that this enzyme is biologically involved in vivo, can carry out dimer excision in vitro, and behaves

in many ways like the enzyme isolated from M. luteus. The enzyme has a molecular weight of approximately 10,000, and using graded pore size dialysis sacs, it has been shown that the enzyme can be resolved from most of the proteins through its ability to pass through such graded pores. This latter technique has also been used to show that this enzyme activity is absent from both uvrA and uvrB mutants. These data are in agreement with physiological data, showing that, in vivo, uvrA and uvrB mutants are unable to carry out the first step in the repair process. Our efforts, as outlined in the proposal section will be toward elucidating the gene products of uvrA and uvrB to determine what the requirements are for the separate gene products.

II. Biological Assay for in vitro Repair

The transfection of E. coli protoplast with UV-irradiated ϕ X174 RF DNA is subject to whole cell reactivation. The survival of the irradiated double-stranded DNA is substantially lower in UV-sensitive, hcr^- hosts than in $UV^+ hcr^+$ hosts. It was first shown by Rörsch that treatment of irradiated RF DNA in vitro, with crude extracts prepared from cells of M. luteus, resulted in a marked enhancement in the survival of the treated DNA in hcr^- hosts. These results were confirmed and extended by other authors, who reported enhancement or partial in vitro repair of irradiated ϕ X174 RF DNA treated M. luteus extracts and purified T4 endonuclease V.

The in vitro repair of irradiated phage DNA is not restricted to ϕ X174 RF, but occurs also with linear λ DNA. We have infected calcium treated cells of E. coli uvrA, B, C, D, E and F with UV-specific endonuclease from M. luteus treated DNA and found an in-

crease in biological activity of the enzyme treated DNA in all strains except *uvrF*. Since the enhancement phenomenon appears in five out of six *E. coli* *uvr* strains, one must assume that these five *hcr*⁻ mutants all share common defects, i.e., the absence of a functional UV-endonuclease. However, an examination of the characteristics of these five mutants suggests the presence of some in vivo endonuclease activity in *uvrC* and *uvrD* after cellular exposure to UV-light or mitomycin C.

Utilizing a variety of mutants which we have obtained in this laboratory, which are enzyme negative UV-sensitive strains, it is now possible to increase the level of transfection of DNA which is externally UV-endonuclease treated. Taking advantage of this provides for a very sensitive assay of UV-endonuclease in vitro, and is the basis for many of the experiments which are routinely carried out with any UV-specific endonuclease we have worked with, both from *M. luteus* and now from *E. coli*.

III. Reinsertion Mechanisms Carried Out in vitro

The DNA polymerase has been purified to homogeneity from *M. luteus* with a molecular weight of 100,000 daltons. In common with the *E. coli* DNA polymerase I, it has associated 3'→5' (single-stranded DNA) and 5'→3' (double-stranded DNA) directed exonuclease activities. We have shown that this enzyme is capable of carrying out both the removal of pyrimidine dimers from incised UV-irradiated DNA (5'→3' activity) and the controlled reinsertion of nucleotides into cavities in a DNA strand created by excision. Controllable biochemical conditions for these steps have been established using UV-irradi-

ated DNA incised with the UV-endonuclease from M. luteus and model p^{32} DNA templates excised with exonuclease III to form such cavities. The UV-endonuclease introduced a "nick" with a 3' hydroxyl terminus into the UV-irradiated DNA adjacent to the pyrimidine dimer. The latter was removed by the DNA polymerase in the absence of synthesis (which restricted the 5'→3' nuclease activity, and hence the size of the gap) in 15 minutes at 30° C with 2 mM magnesium. Repair synthesis was carried out at 10° C in the presence of 0.5 mM magnesium and polynucleotide ligase. Biochemical repair of UV-irradiated B. subtilis transforming DNA resulted in restoration of the single-stranded molecular weight and the biological activity up to doses of 6.6×10^2 ergs/mm², in which 50% inactivation had been observed. At higher doses, the level of biological activity which could be recovered was decreased.

There are two important features to these experiments. One is that a complete stoichiometric balance between loss of pyrimidine dimers, loss of total nucleotides and reinsertion of nucleotides can be made. Furthermore, sealing of the final phosphodiester bond by ligase and the restoration of the molecular weight of the inactivated DNA can be achieved. These experiments were monitored by restoration of the transforming activity, as well as the transfecting activity of phage DNAs, to show that the in vitro, enzymatic mechanisms have all the competency necessary for complete and faithful repair of DNA when measured at the biochemical, hydrodynamic and biological levels.

An analysis of the viability of this in vitro system to substantially increase the level of biological repair to transforming DNA irradiated at doses above 50% inactivation has been made. That maximum repair is not seen is attributed to the formation of double strand breaks, effected during repair and understood in terms of the 5'→3' unidirectionality of repair on strands of opposite polarity which leads to the effective double strand breaks during the excision process. At those doses in excess of 60% inactivation, there is a rapid loss of the double strand molecular weight of the transforming DNA which can be specifically prevented by polyamines. Both spermine and spermidine $[\text{NH}_3^+ - (\text{CH}_2)_3 - \text{N}^+\text{H}_2 - (\text{CH}_2)_4 - \text{NH}_3^+]$ and $\text{NH}_3^+ - (\text{CH}_2)_3 - \text{N}^+\text{H}_2 - (\text{CH}_2)_4 - \text{NH}_2^+ - (\text{CH}_2)_3 - \text{NH}_3^+]$ at in vivo concentrations (2-4 mM) stabilize the double strand molecular weight of the transforming DNA during repair conditions. These polyamines present in all cells and some bacteriophages may serve to prevent such effective double strand breaks as a consequence of the repair process itself. These results are currently being extended to an examination of photoproduct removal and restoration of biological activity to irradiated transforming DNA at high doses.

Excision Mechanisms - Progress report.

In vitro pyrimidine dimer removal by either polI of M. luteus or the independent exonuclease from UV irradiated incised DNA is additive. Either the polymerase alone or the exonuclease alone lead to only 50% dimer excision under saturating enzyme conditions. Quantitative removal of photoproducts is dependent on the combined activities of both excision enzymes. Since the 5'→3' exonuclease component of the polymerase is double strand specific, whereas single stranded DNA is required for the independent exonuclease, it is assumed that their functioning is interdependent. Our current working hypothesis is that the independent exonuclease excises pyrimidine dimers in AT rich regions -- regions of hydrogen bond fragility, whereas the 5'→3' exonuclease of pol I is more functional in GC rich regions -- regions of hydrogen bond stability. From our earlier publications with the independent UV exonuclease of M. luteus (Kushner, Kaplan, Ono & Grossman, Biochemistry, 1971), it was found that the nucleotides removed with the dimers (3'-to the [^]TT) were AT rich. We are currently examining the nature of those nucleotides removed with dimers by the polymerase, the nucleotides removed by the independent exonuclease, the temperature effects on excision of dimers, and the nature of the nucleotides inserted after excision by these two enzymes.

Publications Supported by this Contract

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11. Grossman, L. Sessions Summary, Enzymatic Repair of DNA in Bacteria.

Submitted for Publication

12. Braun, A., and Grossman, L. Anal. Biochemistry. The ϕ X174 RFI Filter Assay for Endonucleolytic Activity.