Repair of Damaged DNA in a Eucaryotic Cell: Tetrahymena pyriformis

Abstract. *Damage induced by ultraviolet light or x-rays to the DNA of a eucaryotic organism,* Tetrahymena pyriformis, *is repaired by a process similar to the repair system present in bacteria. This repair process, which involves defect excision and subsequent resynthesis of the damaged section of DNA, occurs in the dark. Photoreactivation of damage induced by ultraviolet light is also indicated by a reduction in observed repair synthesis. An improved method for detecting repair synthesis is described. Repair synthesis is measured in parental DNA strands isolated from cultures that have undergone normal DNA replication after the repair process.*

Setlow and Carrier (*1*), using *Escherichia coli,* provided the first experimental evidence for a molecular mechanism capable of repairing damaged DNA in the dark. It was shown that ultraviolet-induced intrastrand pyrimidine dimers constituted blocks to the normal replication of DNA and that in resistant bacterial strains these dimers could be excised from the DNA. Setlow and Carrier (*1*) and Boyce and Howard-Flanders (*2*) postulated a repair mechanism in which the excised region of DNA would be subsequently replaced by undamaged nucleotides. Direct physical evidence for this nonconservative (*3*) repair replication step in bacterial systems was obtained by Pettijohn and Hanawalt (*4*), who analyzed the DNA in CsCl density gradients to distinguish between the semiconservative mode of normal replication and the nonconservative mode of synthesis during repair. Repair synthesis has now been observed after treatment of *Escherichia coli* with ultraviolet light, nitrogen mustard, nitrosoguanidine, or after thymine starvation (*4, 5*). An alternative repair system capable of cleaving pyrimidine dimers enzymatically *in situ* in the presence of visible light (photoreactivation) has also been demonstrated (*6*). We now report on the question of whether such mechanisms for repairing damaged DNA are confined to bacterial systems or are also operative in the more highly evolved eucaryotic cell systems.

The ciliated protozoan *Tetrahymena pyriformis* is ideally suited to the investigation of dark repair in a eucaryotic organism because of its phylogenetic position and its natural habitat, which constantly exposes it to sunlight. *Tetrahymena pyriformis* is highly resistant to inactivation by ultraviolet or x-rays (*7, 8*). Photoreactivation of ultraviolet-induced delay of cell division and of ultraviolet-induced lethality has been shown in *Tetrahymena pyriformis* (*7, 9*). The organism is also well suited to molecular-biological studies in that it has a short generation period and can incorporate tritiated thymidine or 5-bromodeoxyuridine (an analog of thymidine) into DNA.

Normal (semiconservative) replication of DNA may be distinguished from repair (nonconservative) replication by determining whether newly incorporated nucleotides appear only in the daughter strands or in the parental strands also. In semiconservative replication, new daughter strands are synthesized with the parental strands as templates. Thus, after a complete round of normal replication, the amount of DNA has doubled, and the resulting double-stranded DNA molecules are hybrid, consisting of one parental and one daughter strand. During nonconservative repair replication, damaged regions of the parental strands are excised and then reconstructed with the complementary parental strand as a template. During repair replication there is no net increase in the amount of DNA; the resulting double-stranded DNA molecules are not hybrid but consist only of parental strands containing short repaired regions.

We used an improved method for measuring repair replication. First, a complete round of normal replication was allowed after repair to establish that all of the repaired DNA was capable of normal replication. Second, the parental strands were isolated from this hybrid DNA, and the amount of new synthesis in these parental strands was determined. The amount of new synthesis within the parental strands is a direct measure of repair replication. Photoreactivation was also measured indirectly by this means. Since photoreactivation alleviates the necessity for dark repair, it reduces the amount of label incorporated into the parental strands after irradiation.

Cultures of *Tetrahymena pyriformis* were grown in defined medium (*10*) for several generations, with thymidine-methyl-^{3}H to label the DNA uniformly. The cells were then irradiated with ultraviolet light; the average dose was 250 erg/mm^2, which is sublethal (*7*). Immediately after irradiation 5-bromodeoxyuridine and ^{32}P were added to the culture to a final concentration of 10 μg/ml and 10 μc/ml respectively. The cultures were then incubated at 28°C for more than one generation. Cells were harvested by centrifugation, washed with NET buffer (*11*) and then lysed by treatment with pronase (100

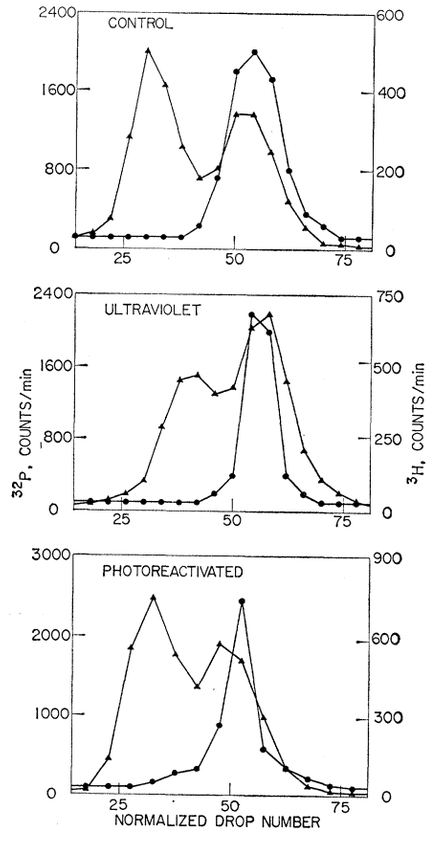

Fig. 1. Fractions from a neutral CsCl gradient of DNA labeled for 1.3 generations with ^{32}P and 5-deoxybromouridine. The control culture was not irradiated, while the ultraviolet and photoreactivated cultures each received an average dose of 250 erg/mm^2 of ultraviolet irradiation administered with a 15-watt germicidal lamp. The ultraviolet culture was grown in the dark after irradiation, whereas the photoreactivated culture was grown in bright light (5 cm from 40-watt microscope illuminator). Circles, ^{3}H label before irradiation; triangles, ^{32}P label after irradiation.

μg/ml) and 0.1 percent Sarkosyl-NL-97 at 60°C for ½ hour. The 6-ml lysate was extracted with an equal volume of a chloroform-octanol mixture (9:1) and banded at equilibrium in an isopycnic CsCl gradient (*12*). The gradient was fractionated by collecting drops through the tube bottom, and radioactivity in each fraction was determined (*13*).

Results from a typical experiment are given in Fig. 1, which shows a set of graphs of the fractions obtained from such a CsCl gradient. The peak on the right contains both ^{3}H and ^{32}P and corresponds to the hybrid DNA composed of ^{3}H-labeled parental strands and daughter strands synthesized after irradiation; containing ^{32}P and 5-bromodeoxyuridine. The peak on the left contains only ^{32}P corresponding to heavy DNA in which both strands contain 5-bromodeoxyuridine. There is no light peak to the right of the hybrid peak, which indicates that there is no unreplicated parental DNA. The absence of this peak indicates that all of the DNA has replicated normally after the irradiation.

Fractions containing the hybrid DNA were then placed in an alkaline isopycnic CsCl gradient (*14*) to separate the daughter and parental strands. In practice, it was necessary to repeat this step twice to completely isolate parental strands from the daughter strands. Having isolated the parental strands we could then measure the incorporation of ^{32}P after irradiation in these strands as an indication of repair synthesis. The results of such an alkaline CsCl gradient containing parental strands are shown in Fig. 2. In the control culture, there is no detectable ^{32}P peak associated with the parental strands (^{3}H peak), an indication that there had been no incorporation within these strands after irradiation. The culture treated with ultraviolet has a large ^{32}P component associated with the parental strands, indicating repair synthesis. The photoreactivated culture indicates less repair synthesis after the administration of photoreactivating light, as might have been expected.

If the repaired regions were distributed uniformly in the parental segments, the ^{3}H (incorporated before irradiation) and the ^{32}P (repair) peaks would be coincident in the gradient from the culture treated with ultraviolet light. This is not the case (Fig. 2); the ^{32}P peak is slightly denser than the ^{3}H peak. This figure indicates that most of the repair is contained in a relatively small fraction of the parental DNA segments. A further CsCl gradient containing repaired parental DNA strands and a density marker indicated that the position of the ^{3}H was identical to that of unirradiated single-strand DNA; this result shows that many parental DNA fragments contained little or no repair.

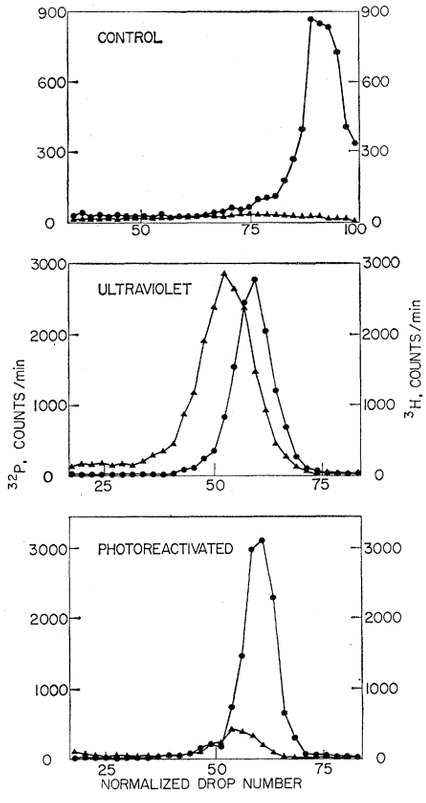

Fig. 2. Fractions from an alkaline CsCl gradient containing parental DNA strands from the gradient shown in Fig. 1. Circles, ^{3}H label before radiation; triangles, ^{32}P label after irradiation.

The amount of repair may be expressed as the ratio of ^{32}P (repair) to ^{3}H (incorporated before irradiation) in the parental peak, and then this ratio may be normalized to the amount of ^{32}P background in the control. The type of experiment just described has been performed with four strains of *Tetrahymena pyriformis,* two micronucleate strains HSM and 6III and two amicronucleate strains GL and ST (*15*). In these experiments the normalized ratio for the ultraviolet-treated cultures was 28 ± 6, that is, the parental strands of the ultraviolet-treated cultures had 28 times the amount of ^{32}P that appears as background in the parental strands of the control cultures. The normalized ratio for photoreactivated cultures was 2.4 ± 0.9.

The experiment described above was repeated with x-rays as the source of damage. The x-ray dose used was 80,000 rads, which is only a small fraction of the lethal dose for the cell (*8*). The analysis of the parental strands from this experiment showed approximately the same amount of repair after 80,000 rads of x-ray as after 250 erg/mm² of ultraviolet irradiation. There is some previous evidence for repair of x-ray damage to DNA in *Escherichia coli* (*16*). Unlike the ultraviolet case for x-ray irradiation there is no apparent photoreactivation. This result was anticipated since photoreactivation has been demonstrated to cleave only pyrimidine dimers (*6*).

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References And Notes

1. R. B. Setlow and W. L. Carrier, *Proc. Nat. Acad. Sci. U.S.* **51**, 226 (1964).
2. R. P. Boyce and P. Howard-Flanders, *ibid.*, p. 293.
3. The repair mode of DNA replication is termed nonconservative because the damaged portions of the parental DNA strand are excised and replaced; thus the parental strand is not completely conserved.
4. D. E. Pettijohn and P. C. Hanawalt, *J. Mol. Biol.* **9**, 395 (1964).
5. P. C. Hanawalt and R. H. Haynes, *Biochem. Biophys. Res. Commun.* **19**, 462 (1965); E. Cerda-Olmedo and P. C. Hanawalt, *Mutation Res.* **4**, 369 (1967); C. Pauling and P. C. Hanawalt, *Proc. Nat. Acad. Sci. U.S.* **54**, 1728 (1965).
6. J. K. Setlow, *Current Topics Rad. Res.* **2**, 195 (1966).
7. E. Christensen and A. C. Giese, *J. Gen. Physiol.* **39**, 513 (1956).
8. C. Wells, *J. Cell. Comp. Physiol.* **55**, 207 (1960); A. M. Elliott, E. H. Hayes, J. R. Byrd, *Biol. Bull.* **107**, 309 (1954).
9. J. Calkins, *Photochem. Photobiol.* **5**, 787 (1966).
10. Medium modified from A. M. Elliott, L. E. Brownell, J. A. Gross, *J. Protozool.* **1**, 193 (1954).
11. NET buffer: 0.5*M* NaCl, 0.05*M* sodium-EDTA, and 0.95*M* tris (hydroxymethyl) aminomethane; *p*H 8.5.
12. M. Meselson and F. W. Stahl, *Proc. Nat. Acad. Sci. U.S.* **44**, 671 (1958); Cs_8Cl was added to a final density of 1.70 g/cm³, and the solution was centrifuged in the No. 40 rotor of a Beckman Spinco Model L 2 at 37,000 rev/min and 25°C for 36 hours.
13. Portions of each fraction were precipitated with 5 ml of 5 percent trichloroacetic acid and filtered with Millipore HA filters, which were then dried and placed in vials containing 5 ml of toluene, 18 mg of 2,5-diphenyloxazole, and 0.45 mg of 1,4-bis-2-(4-methyl)-5-phenyloxazolylbenzene. The vials were then counted in a Packard Tri-carb liquid-scintillation spectrometer.
14. Gradients were made alkaline with 2 ml of 0.5*M* K_2HPO_4 buffer (*p*H 12.8).
15. Strains were obtained from the following sources: 6III and HSM (Parson), GL (Nachtwey), ST (Suyama).
16. R. A. McGrath and R. W. Williams, *Nature* **212**, 534 (1966).
17. Aided by AEC contract AT(043)326-7 and an NIH predoctoral fellowship to C.F.B.

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