Volume 4 Number 8 August 1977 Nucleic Acids Research

Photoreactivation and dark repair of ultraviolet light-induced pyrimidine dimers in chloroplast DNA

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Received 10 June 1977

ABSTRACT

A UV-specific endonuclease was used to detect ultraviolet light-induced pyrimidine dimers in chloroplast DNA of *Chlamydomonas reinhardi* that was specifically labeled with tritiated thymidine. All of the dimers induced by 100 J/m^2 of 254 nm light are removed by photoreaction. Wild-type cells exposed to 50 J/m^2 of UV light removed over 80% of the dimers from chloroplast DNA after 24 h of incubation in growth medium in the dark. A UV-sensitive mutant, UVS 1, defective in the excision of pyrimidine dimers from nuclear DNA is capable of removing pyrimidine dimers from chloroplast DNA nearly as well as wild-type, suggesting that nuclear and chloroplast DNA dark-repair systems are under separate genetic control.

INTRODUCTION

The presence and biological importance of DNA in organelles of eukaryotic cells has been well documented[1]. The existence in practically all cells of mechanisms for the repair of damaged DNA is also well established. Three DNA repair systems for coping with ultraviolet light-induced pyrimidine dimers have been elucidated primarily from work with bacteria; these are (1) photoreactivation, (2) excision-repair, and (3) postreplication repair. Clayton, Doda and Friedberg[2] failed to find evidence for any of these repair mechanisms operating on pyrimidine dimers induced in mitochondrial DNA of mammalian cells. Pyrimidine dimers can be removed from yeast mitochondrial DNA by photoreactivation although excision-repair is apparently absent[3,4].

The only previous study of chloroplast DNA repair failed to find evidence for excision of dimers[5] or for repair replication[6] in *Chlamydomonas* chloroplasts. However, these workers also reported a lack of excision of pyrimidine dimers from nuclear DNA of *Chlamydomonas*. Using a more sensitive assay for pyrimidine dimers, thus permitting a smaller fluence of irradiation, we found that *Chlamydomonas* does remove pyrimidine dimers from nuclear DNA in the dark[7]. We have extended the study of DNA repair in *Chlamydomonas* to chloroplast DNA. We find evidence for repair of pyrimidine dimers in chloroplast DNA both by photoreactivation and by a dark-repair process which may be excision-repair.

MATERIALS AND METHODS

Chlamydomonas strains--Chlamydomonas strains used have been described in a previous publication[7].

Detection of pyrimidine dimers in chloroplast DNA--Chlamydomonas was grown in TAP medium[8] in the presence of 10 μCi/ml of [methyl-^{3}H]-thymidine (50 Ci/mmole, New England Nuclear or ICN) for two days to a final cell density of 5 x 10^5 to 1 x 10^6 cells/ml. The cells were washed and resuspended in fresh TAP medium to a cell density of 1 x 10^6 cells/ml. Thirteen to 15 ml were irradiated in a 15 cm petri dish under a germicidal lamp at a fluence rate of 5 J/m^2 per second. (It should be noted that fluences given in the prior publication[7] must be multiplied by a factor of 2 for proper comparison to this paper due to a faulty UV meter.) The cells were diluted to 5 x 10^5 cells/ml with TAP medium before incubation in the dark at 26 C with shaking. The extraction, treatment of the isolated DNA with the UV-specific endonuclease, and sedimentation through alkaline sucrose gradients were described in an earlier publication[7]. Centrifugation was for 210 minutes at 30,000 rpm with a SW 50.1 rotor at 20 C except where noted otherwise. Bacteriophage T7 DNA was used as a molecular weight marker.

Measurement of turnover of chloroplast DNA--Chlamydomonas was grown as described above except 0.06 to 0.2 μCi/ml of [^{14}C] adenine (53.5 mCi/mmole) was present in addition to 10 μCi/ml of [^{3}H] thymidine. Adenine labels both nuclear and chloroplast DNA[9] whereas thymidine is incorporated specifically into chloroplast DNA[10]. Irradiation, incubation in the dark and extraction of DNA were the same as described above. Nuclear and chloroplast DNA were resolved on CsCl density gradients performed as previously described[7]. Turnover of chloroplast DNA was calculated from the ratio of tritium in the chloroplast DNA to ^{14}C in the nuclear DNA.

Determination of chloroplast DNA synthesis after UV-irradiation--Chlamydomonas DNA was prelabeled by growing in the presence of 0.06 to 0.2 μCi/ml [^{14}C] adenine to a cell density of 5 x 10^5 to 1 x 10^6 cells/ml. Irradiation was done as described previously. The irradiated cells, along with unirradiated controls, were incubated in the dark at 26 C with shaking for 24 h in TAP medium in the presence of 16 to 20 μCi/ml of [^{3}H] thymidine. The DNA was extracted and the nuclear and chloroplast DNA separated on CsCl density gradients. The amount of chloroplast DNA synthesized was estimated from the ratio of tritium in chloroplast DNA to ^{14}C in nuclear DNA.

RESULTS

Effect of UV on the Turnover of Chloroplast DNA--In order to specifically study the metabolism of chloroplast DNA after UV-irradiation, we have taken advantage of the observation of Swinton and Hanawalt[10] that radioactive thymidine is incorporated only into chloroplast DNA. Wild-type cells were double-labeled with [^{14}C] adenine, which is incorporated into both nuclear and chloroplast DNA, and [^{3}H] thymidine, which is incorporated specifically into chloroplast DNA. Figure 1 shows the separation of chloro-

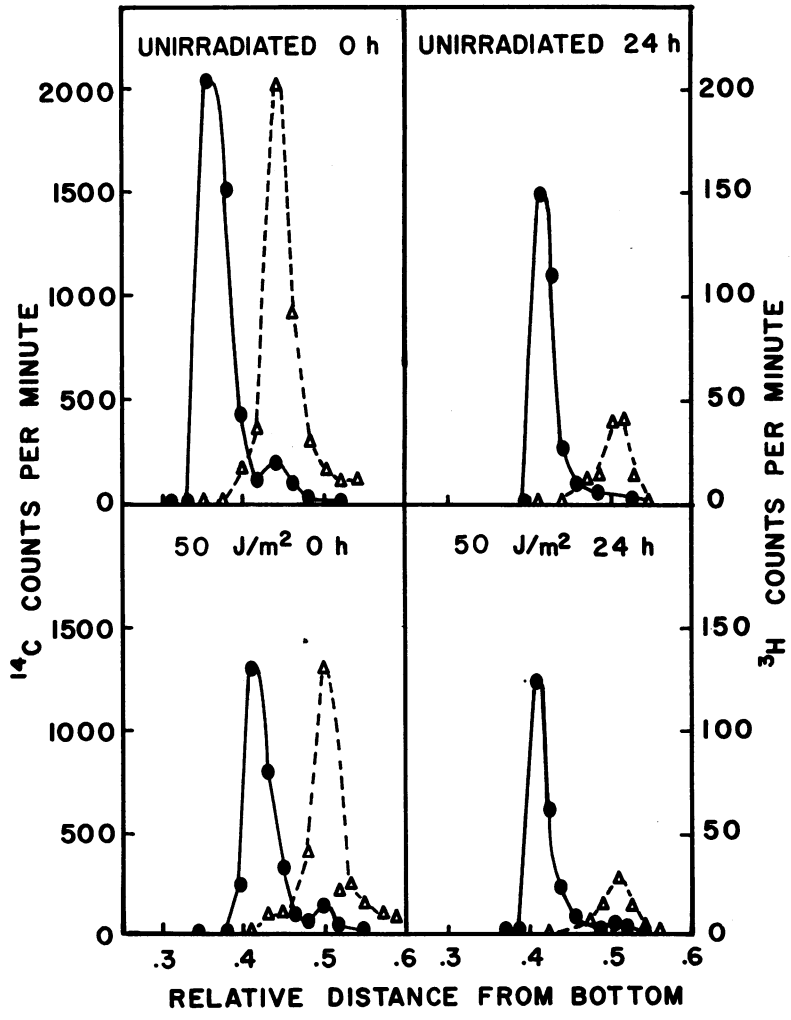

Figure 1. Separation of nuclear and chloroplast DNA on CsCl density gradients. Nuclear and chloroplast DNA was labeled with [^{14}C] adenine and [^{3}H] thymidine as described in Materials and Methods. ●——● ^{14}C radioactivity; Δ- - -Δ ^{3}H radioactivity.

plast from nuclear DNA on isopycnic CsCl density gradients. To determine the relative amount of chloroplast DNA, the ratio of tritium in the chloroplast DNA to ^{14}C in the nuclear DNA is measured at time zero and after 24 h incubation in the dark. By measuring the ratio of ^{3}H to ^{14}C, we did not have to worry about unequal sampling which was caused by the propensity of the cells to stick to the walls of the flask. Independent experiments have shown no significant turnover of nuclear DNA even after fluences of 254 nm radiation as high as 150 J/m^2 (data not shown). As shown in the top two panels of Figure 1, chloroplast DNA is significantly degraded relative to nuclear DNA in unirradiated cells after 24 h incubation in the dark. The bottom two panels show the results obtained from cells irradiated with 50 J/m^2 of 254 nm light. Table I summarizes the quantitative data from the experiment shown in Figure 1 as well as a second experiment. As can be seen from the last Column of Table I, about half of the radioactivity that was incorporated into chloroplast DNA of unirradiated cells is lost after 24 h incubation in the dark. Irradiation with 50 and 100 J/m^2 appears to increase the amount of DNA broken down upon incubation in the dark.

Removal of Pyrimidine Dimers by Photoreactivation--Figure 2 shows alkaline sucrose gradient analysis of [^{3}H] thymidine labeled (i.e., chloroplast) DNA extracted immediately from UV-irradiated cells, from cells that were exposed to photoreactivating light for 90 min immediately after UV irradiation, and from cells that were kept in the dark for 90 min after

TABLE I. EFFECT OF UV IRRADIATION ON TURNOVER OF CHLOROPLAST DNA

Treatment	^{3}H cpm in Chloroplast DNA	^{14}C cpm in Nuclear DNA	Ratio of Chloroplast to Nuclear cpm	Percent Chloroplast DNA Remaining
Experiment 1				
Unirradiated, 0 time	419	4699	.089	100
Unirradiated, 24 h	157	3409	.046	52
50 J/m^2, 0 time	289	3171	.091	100
50 J/m^2, 24 h	93	2421	.038	42
Experiment 2				
Unirradiated, 0 time	443	796	.556	100
Unirradiated, 24 h	321	1046	.307	55
50 J/m^2, 24 h	164	836	.196	35
100 J/m^2, 24 h	77	562	.137	25

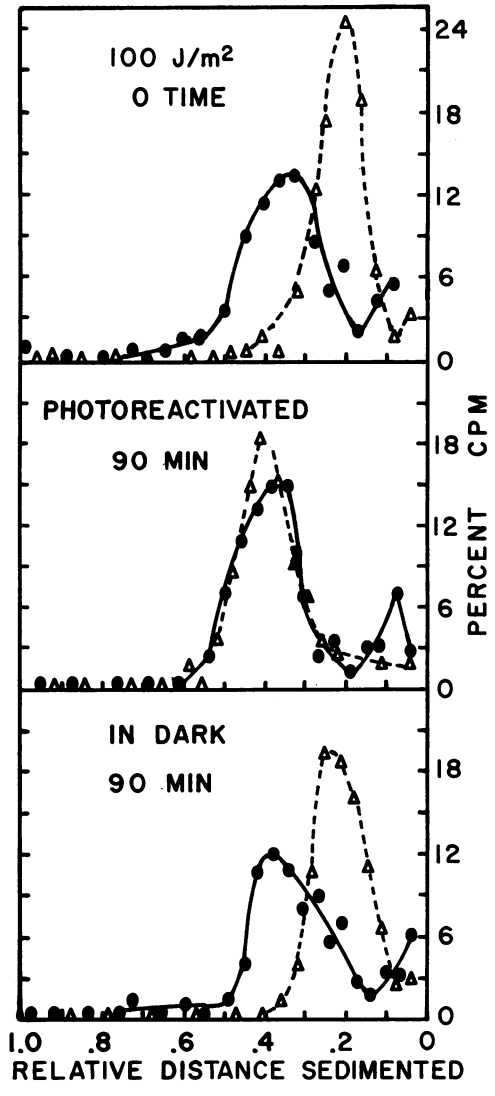

Figure 2. Removal of UV-specific endonuclease susceptible sites from chloroplast DNA by photoreactivation. Upper panel: sedimentation pattern on alkaline sucrose gradients of chloroplast DNA extracted from wild-type cells immediately after exposure to 100 J/m^2 of 254 nm light. Middle panel: sedimentation analysis of DNA extracted from cells irradiated with UV-light and exposed to photoreactivating light for 90 min. Bottom panel: sedimentation analysis of DNA extracted from cells that had been incubated in the dark for 90 min following UV-irradiation. Centrifugation was for 150 min. ●——● No UV-specific endonuclease; Δ- - -Δ Treated with UV-specific endonuclease. Approximately 250 cpm were recovered from each gradient.

UV irradiation. As calculated from the sedimentation rates shown in the top panel, the DNA had a number average molecular weight of 3.8 x 10^6 daltons before treatment with the UV-specific endonuclease and 1 x 10^6 after treatment with the UV-specific endonuclease. All of these endonuclease sensitive sites, i.e., pyrimidine dimers, were removed by exposure to photoreactivating light for 90 min.

Dark Repair of Pyrimidine Dimers--Figure 3 shows the results of alkaline sucrose gradient analysis of chloroplast DNA extracted from cells immediately after exposure to 50 J/m^2 of 254 nm light and after incubation in the dark in growth medium for 24 h. The two panels on the left in Figure 3 illustrate the results obtained using wild-type cells, whereas the two panels on the

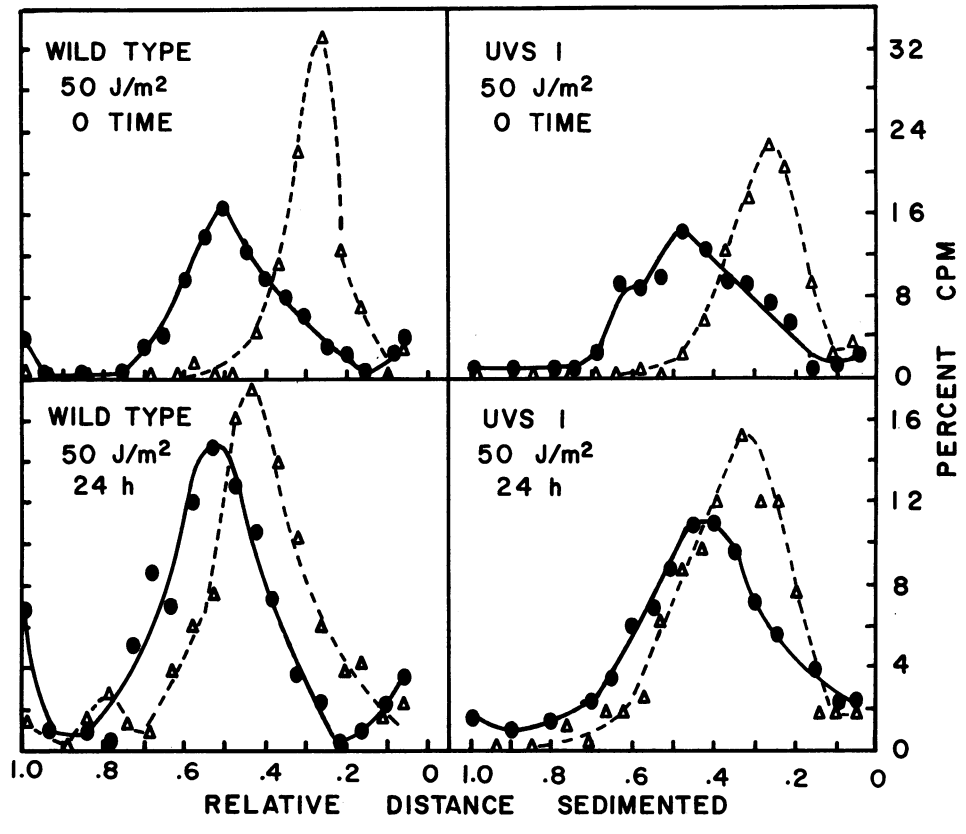

Figure 3. Removal of pyrimidine dimers from chloroplast DNA in wild-type and UVS 1 cells upon 24 h incubation in the dark. The procedure for the detection of pyrimidine dimers in chloroplast DNA is described in Materials and Methods. ●——● No UV-specific endonuclease: Δ- - -Δ Treated with UV-specific endonuclease. Between 250 and 350 cpm were recovered from each gradient.

right give the sedimentation patterns obtained when the same experiment was done using UVS 1 cells, a UV sensitive mutant that is defective in the excision of pyrimidine dimers from nuclear DNA[7]. The quantitative aspects of this experiment are summarized in Table II. A fluence of 50 J/m^2 of 254 nm light induces between 8 and 9 pyrimidine dimers per 10^7 daltons of chloroplast DNA both in the case of wild-type cells and UVS 1. After 24 h incubation in the dark, wild-type cells have only about 1.3 dimers per 10^7 daltons or 15% of the original amount of dimers and the UVS 1 cells have about 2.8 dimers per 10^7 daltons or 32% the original content. Thus, both in the case of wild-type cells and the UV sensitive strain, UVS 1, pyrimidine dimers appear to have been removed from chloroplast DNA as judged by the increasing distance between dimers after incubation in the dark for 24 h.

One possible explanation for the apparent dark removal of pyrimidine dimers from chloroplast DNA is a combination of synthesis of new DNA which undergoes recombination with parental DNA, thus increasing the distance between dimers much in the same fashion as has been described for *E. Coli*.[11] We measured the amount of chloroplast DNA synthesized after UV-irradiation by the incorporation of [^{3}H] thymidine into chloroplast DNA in both wild-type and UVS 1 cells. As summarized in Table III, after 24 h wild-type cells had

TABLE II. PYRIMIDINE DIMERS IN CHLOROPLAST DNA AFTER 24 h INCUBATION IN THE DARK

Strain	Treatment	UV-Endonuclease Added	Number Average Molecular Weight (x 10^{-6})	Dimers per 10^7 Daltons DNA
Wild-type	50 J/m^2, 0 time	-	5.15	8.3
		+	0.97	
Wild-type	50 J/m^2, 24 h	-	5.68	1.27
		+	3.3	
UVS 1	50 J/m^2, 0 time	-	4.15	8.7
		+	0.9	
UVS 1	50 J/m^2, 24 h	-	3.1	2.8
		+	1.65	

TABLE III. CHLOROPLAST DNA SYNTHESIS AFTER UV-IRRADIATION

The amount of chloroplast DNA synthesis that occurred in 24 h was determined by the incorporation of [^{3}H] thymidine into chloroplast DNA in cells prelabeled with [^{14}C] adenine as described in Materials and Methods. In the case of wild-type cells, 0.2 μCi/ml of [^{14}C] adenine was used for the prelabel and 16 μCi/ml of [^{3}H] thymidine for the 24 h labeling of chloroplast DNA. For UVS 1 strain, 0.05 μCi/ml of [^{14}C] adenine was used for the prelabel followed by 20 μCi/ml of [^{3}H] thymidine.

Experimental Condition	^{3}H cpm in Chloroplast DNA	^{14}C cpm in Nuclear DNA	Ratio of Chloroplast to Nuclear cpm	Chloroplast DNA Synthesis: % of Unirradiated cells
WILD-TYPE				
Unirradiated	1287	8397	0.153	
50 J/m^{2}	833	6868	0.121	79
UVS 1				
Unirradiated	6029	2113	3.23	
50 J/m^{2}	522	1400	0.41	12.6

synthesized chloroplast DNA at about 80% the extent of unirradiated cells whereas UVS 1 cells synthesized only about 12% the amount of unirradiated control. However, UVS 1 cells removed nearly as many pyrimidine dimers in 24 h as wild-type cells (68% compared to 85%). Thus, it would not seem that "dilution" of dimers by a process of DNA synthesis plus recombination can explain our results.

DISCUSSION

The probability that chloroplasts have the ability to remove ultraviolet light-induced pyrimidine dimers from their DNA by photoreactivation has been hinted in earlier studies. Thus, Lyman et al.[12] showed that non-lethal fluences of UV light prevented the formation of chloroplasts in *Euglena gracilis*. This inhibition of chloroplast formation can be completely reversed by exposure of the irradiated cells to photoreactivating light. However, as far as we are aware the present work is the first direct demonstration of the removal of pyrimidine dimers from chloroplast DNA upon exposure of irradiated cells to photoreactivating light.

The observation that nuclear DNA is metabolically stable whereas chloroplast DNA is unstable has been reported earlier by Manning and Richards[13] in *Euglena*. They found that about 50% of the chloroplast DNA turned over after 2 cell doublings. We found about 50% turnover of *Chlamydomonas* chloro-

plast DNA after 24 h in the dark. Since the generation time for *Chlamydomonas* in TAP medium in the dark is about 14 h[14], the rates of chloroplast DNA turnover per generation in *Euglena* and *Chlamydomonas* are about the same. Irradiation of *Chlamydomonas* with fluences of 50 and 100 J/m^2 of 254 nm light appears to stimulate the rate of turnover (Table I).

When the labeled chloroplast DNA remaining at the end of 24 h incubation in the dark was examined for the presence of pyrimidine dimers, we found in wild-type cells only slightly more than one per 10^7 daltons DNA compared to more than 8 immediately after UV irradiation (Table 2), indicating the occurrence of a dark repair process. It should be emphasized that this disappearance of pyrimidine dimers cannot be explained by the non-specific turnover of the DNA. We have looked at the average distance between pyrimidine dimers in the DNA remaining after incubation in the dark. One would not expect the distance between dimers to increase as a result of non-specific degradation of the DNA. The UV-sensitive mutant, UVS 1, is completely deficient in the removal of pyrimidine dimers from nuclear DNA[7] but has the ability to remove pyrimidine dimers from chloroplast DNA nearly as well as wild-type. This indicates that the dark repair systems in chloroplasts and nuclei are under separate genetic control.

Excision-repair of damaged chloroplast DNA would appear to be the simplest interpretation of our results. Dilution of the pyrimidine dimers by a combination of DNA synthesis plus recombination would appear to be ruled out by the finding that wild-type cells synthesized 7-8 times as much chloroplast DNA as did UVS 1 (Table III) yet both strains removed nearly the same amount of pyrimidine dimers from chloroplast DNA. The demonstration of UV-stimulated repair replication in chloroplast DNA would strengthen considerably the case for excision-repair. The previous reported absence of repair replication for either nuclear or chloroplast DNA of *Chlamydomonas*[6] may well have been because of the large fluences of UV radiation used as we have previously shown that few pyrimidine dimers are removed from nuclear DNA after such large fluences.

ACKNOWLEDGMENT

This investigation was supported by NIH research grant number GM 21095 from the National Institute of General Medical Sciences.

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