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The Absence of a Pyrimidine Dimer Repair Mechanism in Mammalian Mitochondria

(pyrimidine dimer endonuclease/DNA repair/DNA replication/density gradient centrifugation)

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ABSTRACT **We have investigated whether mammalian cells can repair pyrimidine dimers in their mitochondrial DNA which have been induced by ultraviolet light. The assay system is based upon the ability of the phage T4 UV endonuclease to nick covalently closed circular mitochondrial DNA that contain pyrimidine dimers. Our results show that dimers are not removed from the mitochondrial DNA of mouse L cells or human KB and HeLa cells. There is also no evidence for photoreactivation of mitochondrial DNA. Analyses of ethidium bromide-cesium chloride equilibrium density gradients of mitochondrial DNA isotopically labeled before and after exposure to ultraviolet light show that the total amount of DNA replication is depressed after exposure. In addition, an increase in the frequency of molecules banding at a position expected for intermediate replicating forms and open circular daughter molecules suggests that the rate of replication is slower (or arrested) in molecules with pyrimidine dimers. The absence of a significant amount of mixing of label incorporated before and after ultraviolet-irradiation is evidence against the occurrence of a large amount of genetic exchange between mitochondrial DNA molecules under these conditions.**

At least three modes of repair of ultraviolet light-damaged DNA exist in prokaryotic cells: photoreactivation, dimer excision repair, and postreplication repair (1–3). In placental mammalian cells, excision repair of nuclear DNA has been demonstrated unequivocally (4) and a recent report suggests the presence of a post-replication repair mechanism (5). Photoreactivation of pyrimidine dimers in nuclear DNA of placental mammals has not been reported (6).

Mammalian mitochondria contain their own distinct genetic material in the form of closed circular DNA molecules (7), which are presumably susceptible to the damaging effects of ultraviolet radiation. We were therefore interested in determining whether or not mammalian cells contain a repair mechanism(s) for removal of ultraviolet light-induced pyrimidine dimers in mitochondrial DNA (mtDNA). In addition, recent work has established that these DNA molecules replicate *in situ* and the mechanism of replication has been described (8–11). If mammalian mitochondria could be shown to carry out repair of damaged DNA, it is possible that some of the previously described presumptive replicating forms of mtDNA might in part be repair intermediates.

Abbreviations: EthBr, ethidium bromide; mtDNA, mitochondrial DNA; TK$^-$, thymidine kinase minus; nicked DNA, DNA containing one or more strand breaks; UV, ultraviolet.

We have utilized the ability of the pyrimidine dimer-specific phage T4 endonuclease to assay the disappearance of UV light-induced pyrimidine dimers in closed circular mtDNA as a function of time after exposure. This enzyme cleaves a phosphodiester bond near the pyrimidine dimer site; in the case of closed circular DNA, such a cleavage produces a form readily distinguished from the closed circular form by equilibrium centrifugation in EthBr(ethidium bromide)-CsCl density gradients (12). The results of these experiments indicate that mouse L cells, human KB and HeLa cells do not remove pyrimidine dimers from their mtDNA.

MATERIALS AND METHODS

Cells. Mouse L cells, human KB, and human HeLaTK$^-$ cells were grown on 100-mm Falcon dishes in minimal essential medium plus 10% calf serum as previously described (13). In some experiments we have utilized TK$^-$ cells in order to assay specifically for isotopically labeled mtDNA in density gradients (9, 10, 14). When the cells reached approximately 50% confluence the medium was removed and the cells were washed twice with pre-warmed TD buffer (0.133 M NaCl, 0.005 M KCl, 0.0007 M Na_2HPO_4, 0.025 M Tris, pH 7.4). Cells were exposed to UV light from a 15-W General Electric low pressure mercury vapor germicidal lamp. UV dose at 254 nm was measured with an International Light germicidal photometer, model IL 254. All UV-irradiation of cells was carried out with 3.0 ml (<1 mm solution depth) of TD buffer in each dish. All manipulations involving UV-irradiation were carried out in a 37° room under conditions which excluded photoreactivating light except for experiments in which photoreactivation was being specifically examined. In that experiment, cells were exposed for 15 min at 37° to light at a distance of 6 cm from two Westinghouse FS20 fluorescent lamps equipped with a glass filter screen to exclude wavelengths below about 310 nm. Cells designated "zero time cells" were harvested immediately; other cells were incubated in fresh prewarmed medium for the times indicated.

Preparation of mtDNA. mtDNA was prelabeled so that radioactivity was proportional to mass by the addition of [^{14}C]thymidine (0.05 μCi/ml, 50 mCi/mmol) or [^{3}H]thymidine (0.5 μCi/ml, 3 Ci/mmol) to the cell culture medium (10 ml/dish) three generations prior to UV exposure. Double label experiments were performed by prelabeling with [^{14}C]thymidine followed by post-UV labeling with [^{3}H]thymidine as above. mtDNA was isolated and analyzed in EthBr-CsCl

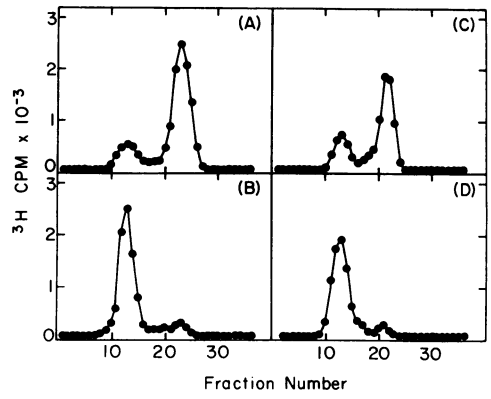

FIG. 1. EthBr-CsCl density gradient profiles of mtDNA from HeLaTK$^-$ cells prelabeled with [^{3}H]thymidine as described and exposed to 150 ergs/mm^2 UV light. Closed circular mtDNA was prepared from cells harvested at zero time and 24-hr post-UV and the mtDNA samples were incubated with and without T4 endonuclease and recentrifuged as described in *Materials and Methods*. (*A*), mtDNA from cells at zero time with enzyme treatment; (*B*), mtDNA from cells at zero time when incubated in the absence of enzyme. Less than 10% of the molecules were nicked; (*C*), mtDNA from cells harvested 24-hr post-UV with enzyme treatment; (*D*), mtDNA from cells harvested 24-hr post-UV when incubated in the absence of enzyme.

buoyant density gradients as previously described (9). Use of this isolation procedure for mtDNA is known to yield the majority of mtDNA molecules contained in isolated mitochondria (15). There is no significant difference in total yield of mtDNA from cells exposed to UV light as compared to control cells except at very high doses (>500 ergs/mm^2). Losses of mtDNA radioactivity in all subsequent experimental steps were <20% of the original isolate in two trials in which this was analyzed.

Enzymatic Nicking of mtDNA. Closed circular mtDNA was isolated as a lower band from an EthBr-CsCl gradient and dialyzed against a solution of 0.1 M NaCl, 0.005 M ethylenediaminetetraacetate (EDTA) and 0.01 M Tris, pH 8.5 which contained Dowex-50 resin for removal of EthBr (9). The DNA was then divided into two equal fractions. One fraction was incubated with 24 units of T4 endonuclease (fraction V) (16) for 30 min at 37° in 0.1 M NaCl, 0.005 M ethylenediaminetetraacetate and 0.01 M Tris, pH 8.5. The other fraction was incubated without endonuclease to serve as a control for adventitious nicking of mtDNA. Both fractions were then recentrifuged for 24 hr in an EthBr-CsCl density gradient at 38,000 rpm at 20° in a SW50.1 rotor. The gradient was dripped onto Whatman GF/A filter discs in 50-μl fractions and assayed in a toluene based solution in a Beckman LS230 scintillation counter. Conversion of the closed circular to the nicked circular form was calculated from the areas under the closed circular and nicked circular peaks in the gradient after subtraction of background. In all cases the amount of conversion was corrected for adventitious nicking by assuming that the nicking in the nonenzyme control was random. In general, this did not exceed 10% of the sample (see Fig. 1). Samples for electron microscopy were prepared and examined as previously described (9).

RESULTS

Our first approach in this investigation was to look for repair replication by an increase in the amount of isotope incorporated into mtDNA after exposure of the cells to UV light. In a series of experiments with both wild-type and TK$^-$ L cells we failed to observe any such increase. Therefore, we used a more sensitive assay procedure based upon the ability of the T4 UV endonuclease to detect a single pyrimidine dimer in a UV-irradiated closed circular DNA molecule. It has been previously shown that this enzyme attacks pyrimidine dimer sites in closed circular SV40 DNA and does not incise unirradiated closed circular SV40 DNA (17). Preliminary controls established the same result when mtDNA is used as a substrate.

The experiments reported here consisted of prelabeling the mtDNA with [^{3}H]thymidine, exposing the cells to varying doses of ultraviolet light, isolating lower band closed circular mtDNA, and analyzing this DNA for its sensitivity to the T4 UV endonuclease as a function of the time of post-UV incubation. Since the fraction of mtDNA in the upper band isolated at the time of exposure to UV light did not change with UV exposure, no unusual amount of nicking occurred at the time exposure. An enzymatic conversion of these molecules from a closed circular to a nicked circular conformation is interpreted as evidence that pyrimidine dimers are present in the DNA. A typical buoyant density gradient used to calculate these data is shown in Fig. 1. At all doses used and in all cell lines examined, the data (Table 1) show that pyrimidine dimers persist in closed circular molecules for as long as 48 hr after UV-irradiation. If molecules with pyrimidine dimers could not be repaired, undergo recombination or replicate, no decrease in sensitivity to enzyme of the prelabeled mtDNA mass should be evident. If, however, molecules are not repaired but can undergo replication, then at the doses of UV light where most molecules have only one dimer (80 ergs/

TABLE 1. *Summary of results of enzymatic nicking of prelabeled mtDNA*

Cell line	UV dose (ergs/mm^2)	%* of prelabeled molecules nicked		
		t = 0	t = 24 hr	t = 48 hr
LA9	50	30	28	—
LA9	50	—	33	30
KB	50	24	20	—
HeLaTK$^-$	50	36	30	—
HeLaTK$^-$	50	32	31	—
LA9	100	—	70	—
C2-1†	100	68	66	—
LA9	150	—	75	—
KB	150	80	73	—
HeLaTK$^-$	150	81	68	—
LA9	300	92	85	—
C2-1	300	95	90	—
LA9	500	95	89	—
LA9	1500	>99	—	—

—, not analyzed.

* The percentage of molecules in the closed and nicked circular form was calculated as described in *Materials and Methods*.

† C2-1 is a TK$^-$ mouse L cell derived from LMTK$^-$ cells by double cloning (11).

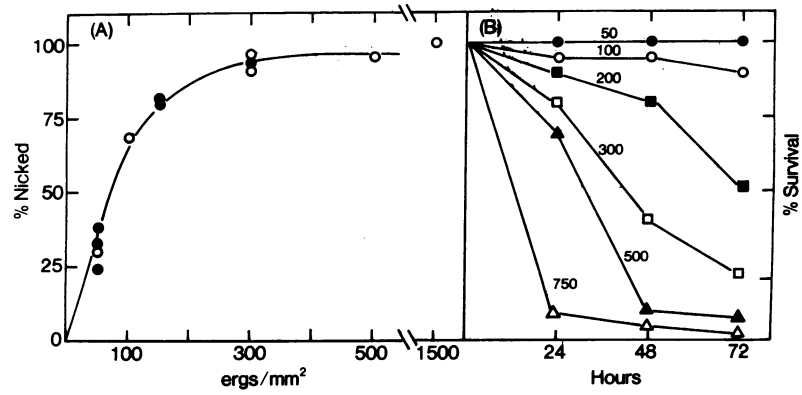

FIG. 2. Percentage of closed circular mtDNA molecules with one or more pyrimidine dimers at zero time after exposure to UV light of varying dose and cell survival as a function of time after UV dose. Cell survival was assayed by scoring as viable those cells which excluded trypan blue dye, maintained a normal appearing morphology, and gave rise to daughter cells within a circumscribed area of the tissue culture dish. Cells not meeting these criteria were presumed nonviable. (*A*), Percentage of closed circular molecules susceptible to T4 endonuclease nicking at zero time as a function of UV dose to the cells. ●, human cells; ○, mouse cells. (*B*), Percentage of cells appearing normal after exposure to UV light. The *numbers* within the figure indicate ergs/mm².

mm²) one would expect a maximum of 50% "repair" after one cell generation because of dilution of pyrimidine dimers in newly formed daughter molecules (11). Since a decrease of <10% in the fraction of molecules with pyrimidine dimers was observed, our results suggest that dimer-containing molecules did not replicate (Table 1). This small decrease in the percentage of molecules susceptible to enzymatic nicking is most likely due to the presence of molecules which were in the process of replication at the time of UV irradiation. It is known that approximately 10% of the mtDNA population is in the process of replication at any one time in an exponentially growing population of L cells (9). At the UV doses used, there is a high probability that no pyrimidine dimers were formed in the unreplicated portion of a majority of the replicating molecules. Thus, completion of replication by such molecules would produce two daughter molecules, one of which would contain prelabel in one strand but no pyrimidine dimers. Experiments designed to directly test this hypothesis are described below. To determine whether cell viability was seriously affected in these experiments we exposed cells to increasing amounts of UV light and assayed cell survival by determining the ability of cells to divide and maintain normal morphology. These results show that cell survival decreases sharply at doses ≥200 ergs/mm² (Fig. 2). At a dose of 50 ergs/mm², no cell death is obvious at 72-hr post-UV. It is therefore reasonable to assume that the cell does not require a full complement of undamaged mtDNA.

When HeLa cells were exposed to 100 or 300 ergs/mm² of UV light followed by exposure to >310 nm light, the results were identical to experiments without photoreactivation conditions. Thus, there is no evidence for a photoreactivation system operative on mtDNA under our experimental conditions.

In order to determine the fate of molecules containing pyrimidine dimers, we performed a series of experiments with cells labeled with [^{14}C]thymidine prior to UV irradiation and then exposed to [^{3}H]thymidine after irradiation. We analyzed both the buoyant distribution of *total* mtDNA in EthBr-CsCl gradients at zero and 24-hr post-UV and the distribution of the

TABLE 2. *Summary of properties of mtDNA labeled for 24 hr post-UV exposure*

Cell line	UV dose (ergs/mm²)	Total amount of post-UV incorporation of [^{3}H]thymidine* (%)	Enzyme treated post-UV radioactivity (%) in molecules	
			Remaining closed circular	Nicked
HeLaTK⁻	50	—	89	11
HeLaTK⁻	50	—	92	8
LA9	100	—	80	20
C2-1	0	100	100	0
C2-1	100	42	82	18
C2-1	300	13	51	49

—, not analyzed.

* Expressed as zero UV dose incorporation of [^{3}H]thymidine/[^{14}C]thymidine into mtDNA × 100 (Fig. 3).

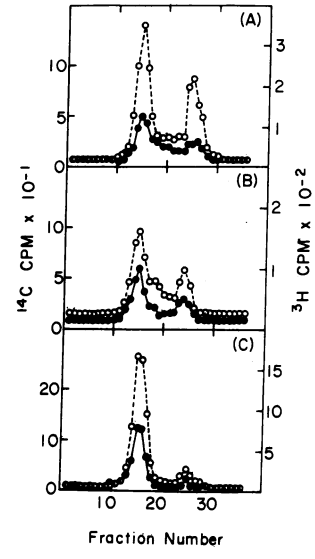

FIG. 3. EthBr-CsCl density gradient profiles of *total* mtDNA from C2-1 cells prelabeled with [^{14}C]thymidine, exposed to UV light and post-UV labeled for 24 hr with [^{3}H]thymidine. mtDNA from cells exposed to (*A*), 100; (*B*), 300; (*C*), zero ergs/mm² UV light. ●, ^{14}C; ○, ^{3}H.

two isotopes contained in closed circular mtDNA molecules sensitive to the UV endonuclease. Analyses of the pre-UV label yields some of the results reported in Table 1. There is a small, but consistent, reduction in the fraction of closed circular mtDNA molecules sensitive to the T4 endonuclease as a function of post-UV incubation time.

An analysis of the post-UV isotope incorporation in the *total* mtDNA population in TK$^-$ cells shows that the amount of incorporation is depressed to 42% and 13% of the control amount at 100 and 300 ergs/mm^2, respectively (Table 2, Fig. 3). The reduced post-UV isotope incorporation can be accounted for by a combination of the following events: (*a*) Normal replication in the fraction of molecules with no dimers. (*b*) Completion of replication in those molecules with no dimers present in a parental strand ahead of the replicating fork. (*c*) A low level of replication in those molecules with dimers ahead of the replicating fork. Thus, we interpret our results to indicate that mtDNA molecules that contain pyrimidine dimers cannot complete the replication cycle if a pyrimidine dimer is present in an unreplicated region of the parental molecule. This hypothesis is supported by the following evidence. We were unable to demonstrate any significant level of incorporation of BrdUrd into mtDNA of TK$^-$ cells exposed to 500 ergs/mm^2 of UV light and exposed to 200 μg/ml of BrdUrd for 18 hr after irradiation. In the original EthBr-CsCl density gradient profiles of mtDNA isolated from cells labeled for 24-hr post-UV, there is an increase in the amount of isotope, both pre- and post-UV, in intermediate and upper band molecules, relative to the amount of total isotope present (Fig. 3). These results are consistent with an accumulation of expanded D-loop and gapped circular molecules in a state of arrested replication (9). There is a higher proportion of intermediate band counts at 300 ergs/mm^2 than at 100 ergs/mm^2. This could be due to the increased probability of arresting replication early in the replication process when there are more pyrimidine dimers per molecule; i.e., there is a greater probability of a pyrimidine dimer occurring nearer the origin of replication as the total number of pyrimidine dimers/molecule increases. Thus, the most reasonable explanation for all these data is that mtDNA molecules that contain pyrimidine dimers are not repaired but replicate until a dimer is encountered on a parental strand and then replication ceases. If this occurs early in replication [before synthesis of 0.6 genome lengths (8)], the molecule will be arrested as an expanded D-loop molecule. If this occurs late in replication, then the daughter molecules can separate and are isolated as gapped circular molecules that cannot complete duplex synthesis and subsequent closure. As the number of pyrimidine dimers/molecule increases there is an expected increase in expanded replicating molecules relative to the rest of the population (Fig. 3*B*).

An analysis of the enzyme sensitivity of post-UV label in closed circular mtDNA shows that the fraction nicked is dependent on UV dose (Table 2, Fig. 4). At a UV dose of 50 ergs/mm^2, a small fraction (<10%) of the post-UV isotope has been incorporated into mtDNA which is sensitive to endonuclease. At higher doses, a larger fraction of the total isotope incorporated post-UV is in molecules containing pyrimidine dimers. The amount of post-UV label in molecules susceptible to the UV endonuclease can be attributed both to a low level of incorporation in the majority of these molecules and to molecules which were in the process of replication at the time

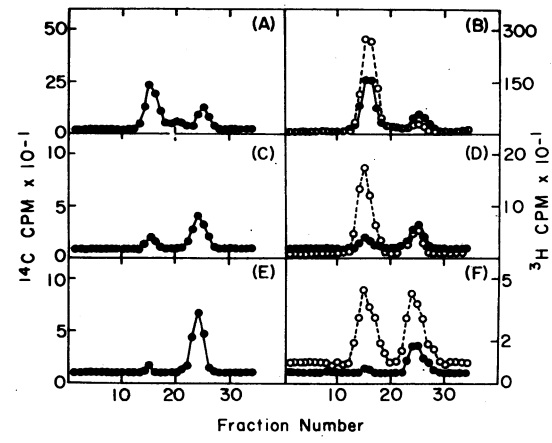

FIG. 4. EthBr-CsCl density gradient profiles of closed circular mtDNA from HeLaTK$^-$ and C2-1 cells prelabeled with [^{14}C]thymidine, exposed to UV light, and post-UV labeled with [^{3}H]thymidine. mtDNA after enzymatic nicking at zero time (*A*), and at 24 hr (*B*), post-UV exposure of 50 ergs/mm^2 to HeLa-TK$^-$ cells. mtDNA after enzymatic nicking at zero time (*C*), and at 24 hr (*D*), post-UV exposure of 100 ergs/mm^2 to C2-1 cells. mtDNA after enzymatic nicking at zero time (*E*), and at 24 hr (*F*), post-UV exposure of 300 ergs/mm^2 to C2-1 cells. ●, ^{14}C; ○, ^{3}H.

of UV-irradiation but which could complete synthesis due to the absence of a pyrimidine dimer in a parental strand ahead of the growing fork. As noted above, the total incorporation (Fig. 3) is consistent with this amount of isotope plus isotope in the fraction of molecules which have no pyrimidine dimers and can complete the replication cycle. It should be noted that the fraction of post-UV radioactivity susceptible to enzyme would be greater (for 300 ergs/mm^2, approximately double the amount shown in Fig. 4*F*) if all intermediate and upper band molecules (Fig. 3*B*) could be shown to be enzyme sensitive. Only lower band closed circular molecules were isolated for enzyme treatment since this fraction represents fully replicated mtDNA and upper band molecules are, by definition, already in the open circular conformation. Therefore, the amount of mtDNA containing post-UV radioactivity and shown to be enzyme sensitive (Fig. 4*F*) is a minimum value. We interpret all these results to indicate that mtDNA molecules that contain pyrimidine dimers cannot complete the replication cycle.

From these data we also conclude that a significant amount of exchange of DNA by recombination between mtDNA molecules does not occur under these conditions. If such recombination were present, then exchange of pyrimidine dimers between molecules would follow; i.e., one should find that molecules with pyrimidine dimers would contain both pre- and post-UV isotope. This does not happen (Fig. 4, Table 2). Furthermore, electron microscopic examination of the total mtDNA population reveals no new or unusual structures and no increase in oligomeric forms after exposure to UV light.

DISCUSSION

Studies related to repair of mtDNA have been reported in two systems. When the eukaryotic organism, *Tetrahymena pyriformis*, is irradiated with UV light, a several-fold increase in mtDNA polymerase activity has been reported (18). The authors conclude that this increase in polymerase activity may represent a repair activity. There is also evidence that

Xenopus laevis cells are able to repair their mtDNA by a photoreactivation mechanism but not by excision repair (19). The present study reports an investigation of mtDNA repair in mammalian cells.

These experiments have exploited the specificity of the T4 UV endonuclease for pyrimidine dimers in UV-irradiated DNA. Our data show that L, HeLa, and KB cells do not remove UV light-induced pyrimidine dimers from their mtDNA. From this we conclude that there is no evidence for an excision repair mechanism for mtDNA in these cells. This conclusion is consistent with the results of recent studies on the deoxyribonuclease activities present in the mitochondria isolated from rabbit kidney and heart, as well as from KB cells grown in tissue culture (20); the studies failed to reveal the presence of any deoxyribonuclease activity that preferentially degrades UV-irradiated DNA compared to unirradiated DNA.

This method of analyzing mtDNA repair specifically examines that population of mtDNA molecules that are in the covalently closed conformation. mtDNA molecules that are in the late stages of DNA synthesis and hence in an expanded D-loop, gapped circular or open circular conformation at the time of UV-irradiation would not be among the population that is being examined for sensitivity to endonuclease (9). It is therefore possible, though we believe it unlikely, that UV damage to such replicating molecules is repaired. If mtDNA molecules contained more than one pyrimidine dimer/molecule, a significant extent of repair could go undetected, since a single remaining dimer would render a closed circular molecule susceptible to endonucleolytically induced cleavage. However, in most of these experiments the highest UV dose used was 300 ergs/mm^2 which should produce an average of only 5 dimers/mtDNA molecule (17).

Our data also indicate that mtDNA replication is significantly depressed in cells exposed to UV-irradiation. The results of double label experiments show only a small degree of incorporation of the post-UV label compatible with either initiation or completion of replication. We suggest that the mtDNA polymerase is unable to negotiate a pyrimidine dimer in the template strand, which would cause an arrest of DNA synthesis at such points. The decrease in total replication and the magnitude of the increased fraction of partially replicated molecules are quantitatively consistent with this hypothesis. Since there is no evidence for any mechanism that removes pyrimidine dimers from mtDNA in these cells, the question of cell survival after exposure to UV is relevant. One may argue that a repair system is expendable in most mammalian cells since there is no reason to expect that they will be subjected to UV light. It is clear (Fig. 2) that the dose required to form one or more pyrimidine dimers in every mtDNA molecule is well over that required to kill the cell. Thus, the lack of a mtDNA repair system would become a problem only under conditions where the cell was subject to large doses of UV light and had a nuclear DNA repair system that was sufficient to cope with such damage. Finally, since the mtDNA molecules which contain pyrimidine dimers are unable to complete replication, they provide no problem to a surviving cell since they will simply be diluted out with subsequent cell divisions.

In summary, all our data are consistent with the proposition that mammalian mitochondria do not have a UV excision repair system. It is reasonable to argue that the lack of such a system does not pose any problems to the cell. The absence of a mtDNA repair mechanism indicates that mammalian mitochondria offer an attractively simple system in which to analyze the detailed mechanism of DNA replication (11).

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