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FATE DURING CELL GROWTH OF YEAST MITOCHONDRIAL AND NUCLEAR DNA AFTER PHOTOLYTIC ATTACHMENT OF THE MONOAZIDE ANALOG OF ETHIDIUM*

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SUMMARY: The ^{14}C-labeled photosensitive monoazide analog of ethidium, 3-amino-8-azido-5-ethyl-6-phenylphenanthridinium chloride, produced covalent adducts in yeast cells with both nuclear and mitochondrial DNA on photolysis by visible light. With subsequent cultivation in nutrient medium, drug molecules on mitochondrial DNA were removed only through extensive mitochondrial DNA degradation. In contrast, drug attached to nuclear DNA was eliminated with conservation of DNA, presumably through a repair process.

INTRODUCTION:

Induction of petite mutations in yeast has served as an important general model for mitochondrial mutations. A remarkable feature of the process is that virtually 100% of a population of yeast cells can be converted to petites with accompanying loss of mt DNA[1], but without loss of nuclear functions. The special vulnerability of mtDNA has not been explained, although it has been suggested that mitochondria do not perform excision repair, and furthermore mtDNA may be especially susceptible to complete nuclease digestion after damage.

The question of mtDNA repair has not been settled entirely since some sort of recovery process appears to occur following UV as shown by liquid holding recovery (1). However, no evidence has been obtained for

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Abbreviations: [1]mtDNA - mitochondrial DNA; [2]EB - ethidium bromide; [3]EA - ethidium monoazide (3-amino-8-azido-5-ethyl-6-phenyl-phenanthridinium chloride).

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dimer excision either in yeast or animal cells using UV specific endonucleases to detect persistent dimers.

Ethidium bromide (EB)[2] is a potent inducer of petite mutants in Saccharomyces and causes degradation of mitochondrial DNA (2,3). EB binds reversibly to nucleic acids (4) and stimulates nuclease actions on DNA in vitro (5), but the in vivo mechanism for insult to mitochondrial DNA and the production of petite mutations is still not clear. Bastos and Mahler (6,7,8) proposed that metabolic activation of EB in mitochondria results in conversion of a reversible complex between EB and mitochondrial DNA (mit DNA) to a covalent adduct, which is then responsible for the degradation of mit DNA by the mitochondrial nuclease, resulting in formation of petites.

A photosensitive analog of ethidium, ethidium azide (EA)[3] synthesized in this laboratory, was shown to bind non-covalently like EB, but on photolysis was bound to both nuclear and mitochondrial DNA (9) with enhancement of petite mutagenesis (10), and production of nuclear mutations in yeast (11),and frameshift mutations in Salmonella (12). These results indicated the importance of covalent attachment of drug with resulting changes in mit DNA as a mechanism for petite induction in yeast.

The efficient labeling of nuclear and mitochondrial DNA with the ethidium photoaffinity probe also provided the opportunity of following simultaneously the fates of covalent lesions in both classes of DNA to distinguish between selective repair and simple DNA turnover. We have, therefore, studied the fates of drug-DNA adducts during growth after EA mutagenesis.

MATERIALS AND METHODS:

Saccharomyces cerevisiae var. ellipsoideus ATCC 4098 was used. Yeast cells were cultivated to exponential phase (about 5 x 10^7 cells/ml) in yeast nitrogen base medium (DIFCO) supplemented with 1% glucose at 30°C. They were then labeled for 2 hours with 5 μCi/ml 6-[^{3}H] uracil (New England Nuclear, 21.9 Ci/mmol). Cycloheximide (Sigma) was added prior to the labeling to inhibit nuclear replication (13). Cells were washed in 0.067M phosphate buffer (pH 7.0) and resuspended in the same buffer at a cell density of 5 x 10^8 cells/ml.

Samples (10 ml) were treated with 100 μM of 6-[^{14}C]-EA (17.7 Ci/mol) (14) for one hour in the dark and then exposed to light for one hour as described previously (15). After EA treatment, cells were washed and

resuspended in 1% glucose yeast nitrogen base medium and incubated at 30°C. After growth as indicated, cells were harvested, washed and converted to spheroplasts by the method of Mahler and Bastos (8). Spheroplasts were lysed in 0.5 ml of 2% sarcosyl (K and K Lab) and treated with 50 µg/ml of RNase (Sigma, Bovine Pancreas heated at 80° for 20 min) and with 100 µg/ml of pronase (Calbiochem) for 30 min at 37°C, and then subjected to cesium chloride density gradient centrifugation analysis.

RESULTS AND DISCUSSION:

Exposure of yeast cells to EA in the light for one hour resulted in binding of [^{14}C] EA to DNA (Fig. 1A) as reported previously (9). In the

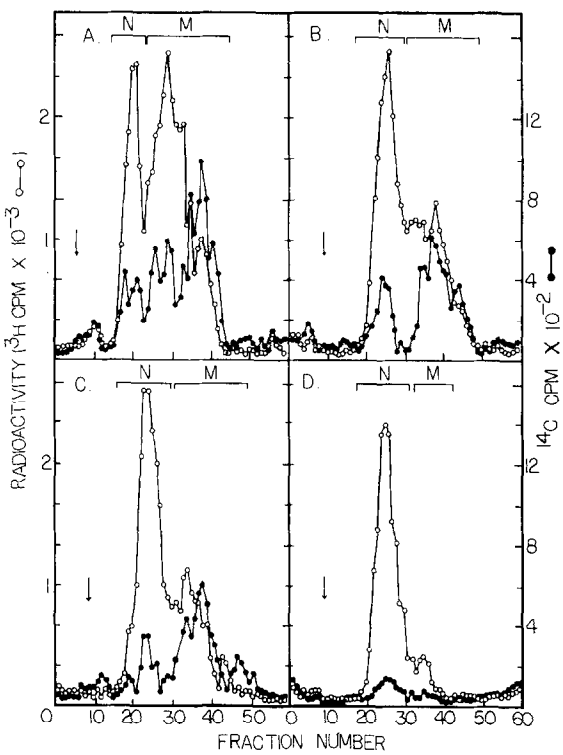

Fig. 1 CsCl density gradient analysis of binding of EA and degradation of DNA.

Yeast cell lysates were loaded in tubes containing CsCl (Gallard Schlesinger) and Micrococcus lysodeikticus DNA (Sigma, density 1.731 g/ml) as a density marker reference (final 8 ml in 0.15M NaCl 0.015M sodium citrate, pH 7.0, 1.690 g/ml). Samples were centrifuged in a Beckman L2-65B preparative ultracentrifuge using a type 65 fixed angle rotor at 35,000 rpm for 65 hours at 25°C. Fractions (60-65 samples) were collected from the bottoms of the tubes and densities were determined by weighing 100 µl of every 5th fraction and then adjusted by comparison with the density marker. All samples were digested with 0.5M NaOH and collected on glass fiber filters (Whatman CF/C) by the method of Grossman, et al (13). Filters were dried and counted in Isocap 300 liquid scintillation counter (Searle Analytic Co.). Spillover of counts between 3H and ^{14}C was corrected by a standard curve.

Gradient is from left (bottom) to right (top). The arrow indicates the point of M. lysodeikticus DNA. (A): control, no cultivation after mutagenesis with EA. Sample cultivated (B) 2.5 hours (C) 5 hours or (D) 15 hours in yeast nitrogen base medium after EA mutagenesis. N, nuclear DNA; and M, mitochondrial DNA.

Table 1. FATES OF [^{3}H] DNA AND ATTACHED [^{14}C] ETHIDIUM IN GROWING CELLS

	Experiment I ($EA^{14}C/DNA^{3}H$)		Experiment II ($EA^{14}C/DNA^{3}H$)	
	Nuclear	Mitochondria	Nuclear	Mitochondria
Control no replication	0.181	0.438	0.189	0.426
2.5 h growth	0.178	0.487	0.164	0.417
5 h growth	0.089	0.420	0.077	0.513

The experiments were done as in Figure 1 and the total CPM for nuclei and mitochondria used from the fractions indicated to calculate the ratios $EA^{14}C/DNA^{3}H$.

present experiments, drug bound to proteins and RNA was removed by the pronase and RNase treatments, and the cycloheximide treated cells showed more ^{14}C counts in mitochondrial than in nuclear DNA. Under the conditions of labeling, petite induction was greater than 98% at a survival level of more than 80%. As observed previously (15), the density of mit DNA was shifted and heterogeneous (1.685 - 1.670 g/ml), resulting from the binding of drugs. The [^{14}C] counts for DNA were decreased with subsequent growth of EA treated cells (Fig. 1B, C, D). In the case of the nuclear DNA fractions, [^{14}C] counts were removed, but the pre-existing DNA was unchanged in density and concentration. In contrast, mit DNA containing the drug was degraded by further cultivation, and almost all mit DNA was degraded after 15 hours of cultivation (Fig. 1D).

The mitochondrial and nuclear fractions were pooled for each of 2 identical experiments and the data were summarized in Table I as ratios of DNA (^{3}H) to covalently bound drug (^{14}C). The EA/DNA ratios were decreased during 5 hours of growth for nuclear DNA, but not for mit DNA. Longer periods of growth resulted in virtual removal of drug labeling in mit DNA through degradation while the nuclear genome was essentially intact. These results indicated that excision repair was provoked by the binding of EA to

nuclear DNA. EA causes nuclear gene conversion, mitotic-crossing over and mutations in yeast (11), as well as nuclear petite mutations (unpublished data).

Repair was not distinguishable from the degradation provoked by EA binding in mitochondria. This emphasizes that mitochondria do not have the same DNA repair capabilities as the nuclear genome since excision was not a simple localized process but involved extensive DNA degradation.

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