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Abnormal DNA Patterns in Animal Mitochondria: Ethidium Bromide-Induced Breakdown of Closed Circular DNA and Conditions Leading to Oligomer Accumulation*

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Abstract. Treatment of cultured mouse fibroblasts (L-cells) and hamster kidney cells with ethidium bromide was found to inhibit the incorporation of [^{3}H]thymidine into mitochondrial closed-circular DNA, but not into nuclear DNA. The specific activity of nuclear DNA in treated cells was higher than in control cells. Treatment also led to breakdown of closed-circular mitochondrial DNA, greatly enlarged mitochondrial profiles with few or no cristae, and a higher yield of mitochondrial protein per cell.

A significant increase in the content of circular DNA dimers and oligomers of mitochondria was observed after treatment of L-cells with cycloheximide and puromycin or treatment of chick fibroblasts with cycloheximide, and in hamster kidney cells transformed with polyoma virus. Little or no effects on dimer and oligomer content were found in L-cells treated with vinblastine, colcemid, rifampicin, chloramphenicol, or mengovirus, in Rous-transformed hamster cells, or in cuprizone-induced giant mitochondria of rat liver.

In studies of the function and significance of mitochondrial DNA it is desirable to obtain conditions *in vivo* in which either certain forms of mitochondrial DNA can be concentrated[1,2] or the DNA is actually being degraded to yield mitochondria that may be deficient in certain components and enzymes. Both of these parameters have been studied in a survey of conditions that affect the structure of mitochondrial DNA *in vivo*. The first part of this report describes the effects of ethidium bromide (2,7-diamino-10-ethyl-9-phenylphenanthridiniumbromide) on mitochondria of cultured mouse fibroblasts.[3] The second part summarizes conditions leading to the accumulation of double- and multiple-length circular DNA molecules (dimers and oligomers), which normally occur as minor species in mitochondrial DNA.[1,2,4–6] This study confirms and extends our earlier observations.[1,3]

Materials and Methods. Cell culture: The L-cell line (mouse fibroblasts) used in our laboratory was obtained in 1963 from Dr. John Littlefield[7]; it was initially grown in suspension culture as described,[7] and subsequently grown in Joklik-modified medium (Grand Island Biological Co., Grand Island, N.Y.) with 10% foetal calf serum. Streptomycin (25 μg/ml) and penicillin (25 U/ml) were added to the medium in addition to the antibiotics already present. The cells were grown in the presence of chlortetracycline (50 μg/ml) for 3 days per week and examined for mycoplasma once a month; they were found to be negative.

Ten-day-old chick embryo fibroblasts were prepared and grown in monolayers as described by Rubin.[8] Medium 199 was used with 10% tryptose–phosphate broth, 5% calf serum, 1% beef-embryo extract, penicillin, streptomycin, and anti-mycoplasma agent. The same medium was used for L-cells grown in monolayers. The BHK_{21}/C_{13} cells[9] and C_{13}/B_4 cells[10] (baby-hamster kidney) were kindly supplied by Dr. I. Macpherson, and the polyoma-transformed BHK cells by Dr. R. Roosa, Wistar Institute, Philadelphia. The cells were grown in monolayers as described.[9,10] Human cells (leucocytes, mononucleosis, and Burkitt lines) were kindly furnished by Drs. G. and W. Henle, Virus Laboratory, The Children's Hospital of Philadelphia.

Mice that bore Ehrlich ascites tumors were each injected three times over a period of 40 hr with 1 mg of cycloheximide in 1 ml of saline. Control mice obtained only saline injections. Mengovirus-infected L-cells were obtained by infection with virus at a multiplicity of 22 and subsequently cultured in Joklik medium for 17 hr.

Analytical methods: The methods of isolating mitochondria, mitochondrial DNA, sedimentation analyses of DNA, and electron microscopic scoring of DNA on grids and photographic prints were essentially as described.[1,5]

Results. Mitochondrial DNA in L-cells after exposure to ethidium bromide (EB): After addition of EB to L-cells for either 1 or 2 days at 1 μg/ml, no radioactivity was observed in the region corresponding to covalently-closed mitochondrial DNA (DNA I) in cesium chloride–ethidium bromide gradients (Table 1 and Fig. 1). However, the linear DNA in region II, which in these experiments

TABLE 1. *Yield and specific activities of mitochondrial closed-circular DNA and nuclear DNA from ethidium bromide (EB) treated L-cells.*

	Hours	No. of cell divisions	ng M-DNA per 10^6 cells	μg M-DNA per mg M-protein	^3H, cpm per μg M-DNA	^3H, cpm per μg N-DNA
Control	21	1.0	7.5	0.48	2466	1985
EB	21	0.6	3.1	0.19	18	3575
Control	45	2.0	7.0	0.56	2391	2862
EB	45	0.8	2.4	0.16	15	4500

Abbreviations: M, mitochondrial. N, nuclear.

represents mainly nuclear DNA, was labeled. This nuclear DNA, as well as DNA isolated directly from nuclei of EB-treated cells, consistently had a higher specific activity than nuclear DNA of control cells (Table 1).

Furthermore, only trace amounts of covalently-closed mitochondrial DNA were observed by fluorescence measurements of the fractions, or by spectrophotometric and electron microscopic analysis of the material collected and concentrated from the region of the gradient corresponding to DNA I (Table 1). Electron microscopy showed a few circular DNA monomers and dimers of standard size (5 μm), as well as smaller linear fragments that may have been overlapping from the broadened peak of DNA II. It appears that mitochondrial DNA was nicked or degraded to some degree since covalently-closed DNA was undetectable even when mitochondrial DNA was labeled for 24 hr prior to exposure to ethidium bromide (Fig. 1). The same results were obtained with BHK cells. Isolated mitochondria from EB-treated cells spread by osmotic shock[1] showed fewer circular and more linear DNA molecules than control mitochondria.

In ultra-thin sections of ethidium bromide-treated cells, most mitochondrial profiles were enlarged and had a few or incomplete cristae (Fig. 2). Longer

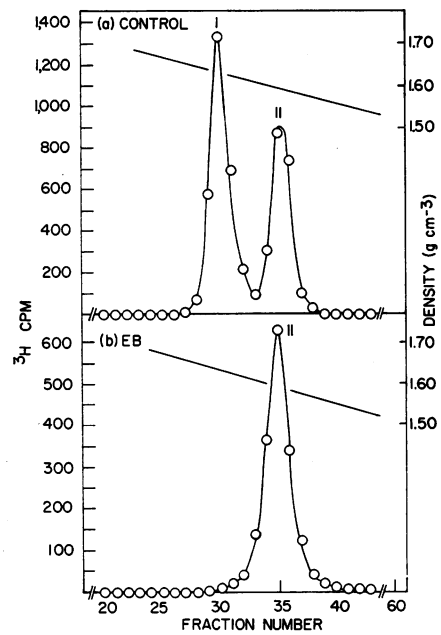

FIG. 1. Equilibrium centrifugation of mitochondrial DNA in cesium chloride–ethidium bromide gradients after treatment of L-cells with the mutagenic dye ethidium bromide. (*a*) Control cells, growing logarithmically for 45 hr; the DNA was isolated from 7.9 mg mitochondrial protein and 7.4×10^8 cells. (*b*) Cells grown in the presence of 1 μg ethidium bromide/ml for 45 hr (see Table 1, last line); the DNA was derived from 7.9 mg mitochondrial protein and 4.8×10^8 cells. Both cultures were prelabeled for 1 day, and then continuously for 45 hr with 0.1 μCi [^{3}H]thymidine/ml. The buoyant densities of DNA II in (*a*) and (*b*) were identical (1.592 g/cm^{-3}).

treatment resulted in even larger profiles, frequently lacking cristae. The yield of protein in isolated mitochondrial fractions from EB-treated cells, however, was consistently 30–50% higher than in control preparations. Further studies are in progress.

After exposure of cells to EB (1 μg/ml) for 72 hr, cell growth could be restored by transferring cells to normal medium. However, the cells did not recover after exposure to 5 μg EB/ml.

Species of mitochondrial DNA in L-cells after exposure to various metabolic inhibitors: In Table 2 are presented the summarized results of treating L-cells with metabolic inhibitors that affect the mitotic apparatus (vinblastine, colcemid), protein synthesis (cycloheximide, puromycin, chloramphenicol), RNA synthesis (rifampicin), and DNA synthesis (hydroxyurea). At the end of each treatment the cells were tested for their capacity to incorporate precursors into DNA, RNA, and protein (see legend to Table 2) to assure that the inhibitors worked in the anticipated manner. Mitochondrial DNA was isolated and examined in the electron microscope for the occurrence of multiple-length species. There are two types of dimers in L-cells, a unicircular molecule with a perimeter of 10 μm (molecular weight 20×10^6), or two interlocked monomers of 5 μm each.[1,5,6] The most pronounced increase in oligomer concentration was obtained with cycloheximide and puromycin when L-cells were allowed to grow from monolayers into confluency. The cells may actually become enriched with DNA oligomers before they become confluent, presumably because these cells, which are not contact-inhibited, tend to grow in clumps.

Fig. 3 shows a double-labeling experiment in which mitochondrial DNA of control and cycloheximide-treated cells were cosedimented in a sucrose gradient. The corresponding electron microscopic counts are listed in Table 2 as 15.2%

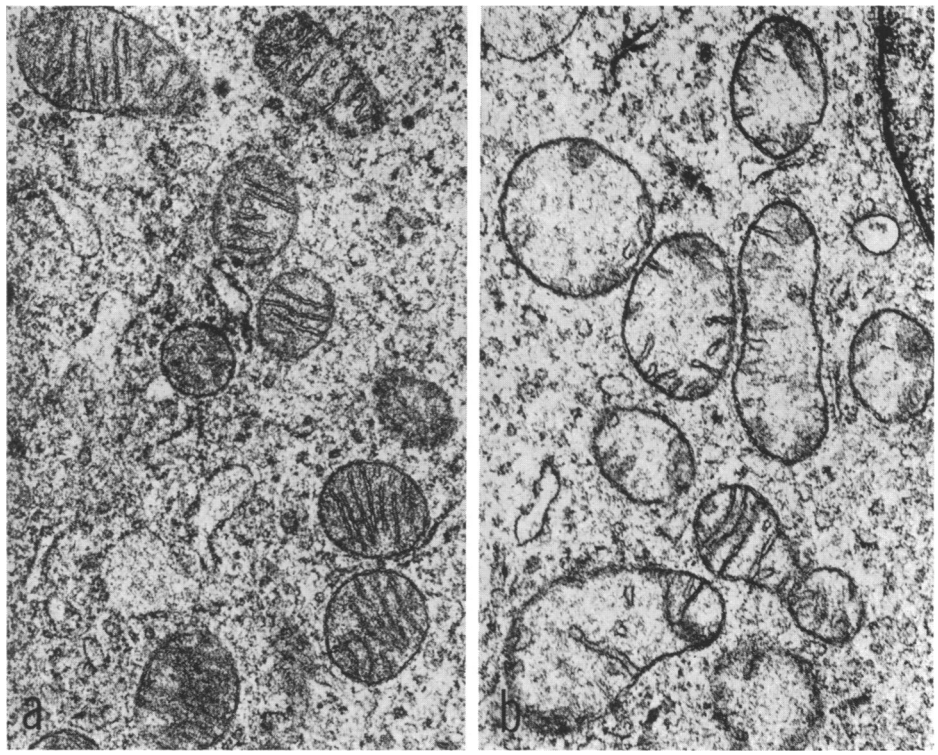

FIG. 2. Electron micrographs of L-cells: (*a*) control and (*b*) treated with 1 μg ethidium bromide/ml for 45 hr. Fixation in 2% OsO_4–0.1 M phosphate buffer (pH 7.4) for 1 hr, then in 10% paraformaldehyde in the same buffer for 20 hr, at 4°C. Further treatment as described.[15] ×23,040.

and 36.1% (dimers plus oligomers). In agreement with the visual scoring method, cycloheximide-treated cells contain more DNA dimers (54 S) than control cells and a relatively large percentage of fast-sedimenting DNA oligomers (fraction 1). The slightly displaced 39 S peak may reflect an enrichment of certain nicked circular dimers in this sample. Such nicked molecules sediment only slightly faster than covalently closed DNA monomers.[4]

A certain degree of variability in the effects resulting from these treatments was observed (see cycloheximide experiments in Table 2). Similarly, the response of L-cells to stationary phase conditions may vary between a 3- to 8-fold increase of dimers and oligomers as compared with control cells. The reason for such variations is not clear but may relate to the age of cultures,[11] handling, and other yet unknown factors. Longer treatments with the inhibitors resulted in less DNA dimer and oligomer accumulation than a shorter treatment, which may be due to a decrease of cell viability.

Species of mitochondrial DNA in other cell types: Attempts to build up the content of mitochondrial dimers and oligomers in other cell types have not been as effective as in L-cells (Table 3). Chick-embryo fibroblasts or BHK cells grown to confluency had only slightly, if at all, raised dimer and oligomer con-

tents. However, cycloheximide was effective in increasing DNA oligomers in chick cells.

Some tumor cell lines have also been examined (Table 3) and the DNA oligomer concentration in mitochondria was not found to be abnormally high.

TABLE 2. *Proportions of circular dimers and oligomers in mitochondrial DNA of L-cells after treatment with various inhibitors.*

	Experimental condition	Hours	Cell divisions	Total dimers and oligomers (%)*	Ratio†
(1)	Monolayer—log	60		39.3	
	Monolayer—confluent	36		76.0	
(2)	Control	21	1.1	25.0‡	
	Vinblastine (0.12 μg/ml) (50% metaphases)	21	0.1	32.0	1.3
(3)	Control (0.4% metaphases 2.6% multinucleated cells)	16	0.6	32.9‡	
	Colcemid (0.05 μg/ml) (21.0% metaphases 10.3% multinucleated cells)	16	0.2	31.7	0.96
(4)	Control	18	0.8	10.0	
	Cycloheximide (25 μg/ml)	18	0.02	68.4	3.5§
	Control	22	1.0	20.0	
	Cycloheximide	22	0.05	55.3	
	Control	41	2.0	10.0	
	Cycloheximide	41	0.2	30.2	
	Control	45	2.1	15.2	
	Cycloheximide	45	0.2	36.1	
(5)	Control	17	0.8	20.0	
	Puromycin (15 μg/ml)	17	0.3	57.1	2.8
(6)	Control	40	1.5	16.5	
	Chloramphenicol (150 μg/ml)	40	0.3	13.4	
(7)	Control	20	0.9	25.0‡	2.3**
	Rifampicin	20	0.3	37.0	
	Control	41	1.7	15.0	
	Rifampicin	41	0.7	34.0	
(8)	Control	18	0.5	34.0‡	
	Hydroxyurea (76 μg/ml)	18	0	51.6	1.5

The inhibition of DNA, RNA, and protein synthesis was checked after the treatments by pulse-labeling cells for 20 min with radioactive thymidine, uridine, and phenylalanine, respectively. Vinblastine: 62, 13, and 26% inhibition; rifampicin: 95, 45, and 46%; hydroxyurea: 96, 0, and 0%; cycloheximide: 98, 79, and 96%; chloramphenicol: 3, 3, and 4%; puromycin: 67% inhibition of protein synthesis.

* The count includes unicircular and interlocked dimers and higher oligomers from the lower and intermediate band of cesium chloride–ethidium bromide gradients (50–70% of total mitochondrial DNA). At least 200–500 molecules were scored with standard deviations of ±5%.

† Ratio of % dimers + oligomers in experimental/control.

‡ These experiments were performed during the same month; the high oligomer content seen in these control cells was not observed on subsequent cultures started from frozen stock cells.

§ Average of 6 experiments.

** Average of 2 experiments.

Unusually large liver mitochondria, which were 2–10 times the normal size, were prepared by adding cuprizone[12] to the diet of mice. This condition, however, did not significantly affect the oligomer content of mouse liver mitochondria (Table 3). Also, the yield of mitochondrial DNA was the same as in controls (0.39 μg/mg mitochondrial protein). A 5–20% higher yield than in controls was found in most other conditions studied.

TABLE 3. *The frequency of circular dimers and higher oligomers in mitochondrial DNA of various cell types.*

	Cell type and experimental condition	Total dimers + oligomers (%)*	Ratio*
(1)	Chick embryo fibroblasts (monolayers)		
	Log	10.7	
	Confluent	17.0	1.6
	Log, cycloheximide (25 μg/ml, 21 hr)	31.5	2.9†
(2)	Hamster kidney (BHK_{21}/C_{13}, monolayers)		
	Log	10.6	
	Confluent	17.0	1.6
(3)	Mouse liver		
	Control	4.3	
	Cuprizone (giant mitochondria)	7.8	1.8
(4)	Ascites tumor (*in vivo*)		
	Control	9.4	
	Cycloheximide	18.7	2.0
(5)	Human leucocytes (normal)	4.2	
	Human mononucleosis	7.3	
	Human Burkitt lymphoma	6.7	

* See footnotes to Table 1. Dimers and oligomers were of the interlocked type.
† Average of 3 experiments.

In all cases listed in Table 3, the species of dimer observed was the interlocked rather than the unicircular form. The unicircular species, thus, could not be generated when it was undetectable in control cells.

Mitochondrial DNA in virus-infected and transformed cells: L-cells were infected with mengovirus, an RNA virus that shuts off host RNA and protein synthesis. This situation was of interest because of the effects observed with cycloheximide. The DNA dimer and oligomer content of mitochondria, however, only doubled (Table 4). The isolation of DNA after infection for longer time periods was not convenient because of cell degeneration.

BHK cells were transformed by polyoma virus, a DNA virus, or by Rous sarcoma virus, an RNA virus. Only the mitochondria of polyoma-transformed

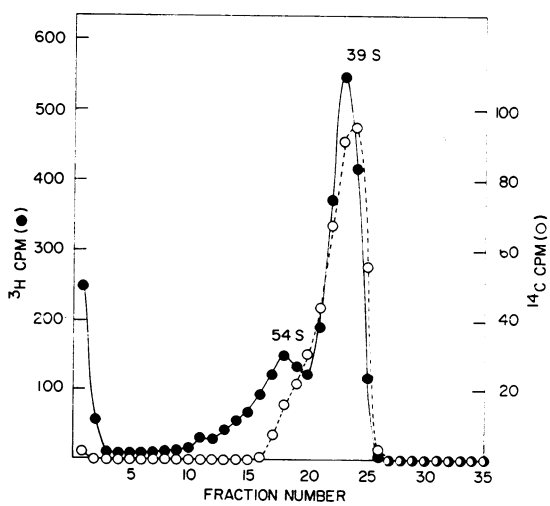

FIG. 3. Band sedimentation of mitochondrial DNA isolated from L-cells treated with cycloheximide for 2 days. ○, Control cells, growing logarithmically for 50 hr in 0.01 μCi of [^{14}C]thymidine/ml. ●, Cells incubated for 45 hr in the presence of 25 μg cycloheximide/ml and 0.2 μCi of [^{3}H]thymidine/ml. They were also prelabeled for 5 hr with the isotope. The isolated mitochondrial DNA samples were combined and cosedimented in a sucrose gradient.[9]

TABLE 4. *The proportions of circular dimers and higher oligomers in mitochondrial DNA of virus-infected and virus-transformed cells.*

	Experimental condition	Total dimers + oligomers (%)*	Ratio*
(1)	Mengovirus-infected (L-cells)		
	Control (17 hr, 0.8 cell divisions)	12.0	
	Mengo-infected (17 hr, 0 cell divisions)	24.2	2.0
(2)	Polyoma-transformed (BHK cells)		
	Control (log)	10.0	
	Control (confluent)	17.4	
	Transformed by polyoma (confluent)	33.6	1.9 or 3.2 (versus log)
(3)	Rous-transformed (BHK cells)		
	Control (log)	10.0	
	Control (confluent)	17.0	
	Transformed by Rous (confluent)	15.0	0.9 or 1.4 (versus log)

* See footnotes to Table 1.

cells were substantially enriched with DNA dimers and oligomers (Table 4). About equal numbers of dimers and higher oligomers were observed, and these were of the interlocked type. More virus-infected and transformed cells will have to be examined, however, before any generalizations can be made.

Discussion. The experiments with ethidium bromide show that in mammalian cells the synthesis and integrity of covalently-closed mitochondrial DNA, which is the predominant form of DNA in animal mitochondria, can be disrupted while nuclear DNA continues to be synthesized at an apparently higher rate than in control cells. The fate of the nicked DNA, cytochromes, and other components of EB-treated mitochondria is under study. Since mammalian cells can survive the consequences of selected EB treatments, the use of this dye promises to be an effective tool in studies of the informational role of mitochondrial DNA and the control of mitochondriogenesis.

Ethidium bromide is known to intercalate in double-stranded nucleic acids. EB has mutagenic effects on mitochondria of yeast[13,14] and the kinetoplast of trypanosomes.[15–17] In mammals, EB has a preferential inhibitory effect *in vitro* on the DNA polymerase of rat-liver mitochondria compared with the nuclear enzyme.[18] The synthesis of certain species of mitochondrial RNA is also inhibited by EB.[19,20]

The data presented here show that a few selected conditions stimulate the accumulation of multiple-length mitochondrial DNA. The highest oligomer content was observed after treatment with cycloheximide and puromycin, and in polyoma-transformed cells. In eukaryotes, cycloheximide is known to inhibit cytoplasmic but not mitochondrial (or bacterial) protein synthesis,[21] whereas the reverse if found with chloramphenicol. No simple correlations or answers are now known about the significance and mechanism of oligomer accumulation. Whether such changes are of any importance in relation to the biogenesis and normal functions of mitochondria remains to be seen. The effects of cycloheximide would suggest that an enzyme manufactured in the cytoplasm and transferred into mitochondria is involved in the control of DNA forms made in these organelles.

In *Proteus mirabilis*, the frequency of multiple-length plasmid (Col E_1) DNA has been shown to increase upon treatment with chloramphenicol or after amino

acid starvation, but these treatments did not induce multiple DNA forms of Col E_1 in an *E. coli* strain.[22] Oligomeric viral DNA molecules accumulated after temperature shifts of mammalian cells that had been transformed by temperature-sensitive mutants of polyoma virus.[23] If a common mechanism underlies all these instances, it may involve the synthesis of one or more enzymes that control DNA monomer formation.

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Abbreviations: EB, ethidium bromide; BHK, baby hamster kidney.

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