ACCUMULATION OF REPLICATIVE INTERMEDIATES OF MITOCHONDRIAL DNA IN *TETRAHYMENA PYRIFORMIS* GROWN IN ETHIDIUM BROMIDE

WILLIAM B. UPHOLT and PIET BORST

From the Section for Medical Enzymology, Laboratory of Biochemistry, University of Amsterdam, Amsterdam, The Netherlands. Dr. Upholt's present address is the Carnegie Institution of Washington, Department of Embryology, Baltimore, Maryland 21210.

ABSTRACT

The effect of growth of *Tetrahymena pyriformis* in ethidium bromide (EthBr) on the structure and synthesis of mitochondrial DNA (mtDNA) has been investigated. During the first 5 h of growth in EthBr, mtDNA synthesis is inhibited 95% or more. After 10–15 h, this block is partially released and large numbers of replicating molecules accumulate, indicating that inhibition by EthBr primarily affects the rate of chain growth and not the initiation of new rounds of replication. The accumulated molecules sediment more rapidly than normal *Tetrahymena* mtDNA and do not contain enough single-strand regions to distinguish them from normal *Tetrahymena* mtDNA when banded in buoyant CsCl or NaI gradients. Electron microscopy shows that the predominant species in this rapidly sedimenting DNA is a linear molecule containing one symmetrical double-stranded replication loop of varying size located at its center. No degradation of mtDNA from cells grown in EthBr was detected in alkaline velocity gradients.

INTRODUCTION

Ethidium bromide (EthBr),[1] a phenanthridine dye, has proved to be a useful tool in the investigation of mitochondrial systems due to its highly selective effects on nucleic acid and protein synthesis within the mitochondrion (1–18). Like the acridines, EthBr intercalates between the base pairs of duplex nucleic acids, causing both an increase in the distance between neighboring base pairs and an unwinding of the duplex (19–24). In all mitochondria studied, this results in a strong inhibition of mitochondrial DNA (mtDNA) and mitochondrial RNA (mtRNA) synthesis, whereas nucleic acid synthesis in the nucleus is only little affected. Mitochondrial protein synthesis is also inhibited by EthBr, both directly (25, 26) and indirectly through the inhibition of mtRNA synthesis (2, 4).

[1] *Abbreviations used in this paper*: BND-cellulose, benzoylated-naphthoylated DEAE-cellulose; EBF, mtDNA from EthBr-grown cells sedimenting faster than 33S; EBS, mtDNA from EthBr-grown cells sedimenting at around 33S; EthBr, ethidium bromide; mtDNA, mitochondrial DNA; mtRNA, mitochondrial RNA; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole; STE buffer, 80–100 ml 0.35 M sucrose, 5 mM Tris-HCl, 0.2 mM EDTA (pH 7.2).

In addition to its inhibitory effect on nucleic acid and protein synthesis, EthBr also affects the structure of mtDNA in some systems. The most dramatic effect has been found in the yeast *Saccharomyces* (27). Addition of EthBr results in a rapid degradation of mtDNA (7, 8) and a complete conversion of the yeast cells into petite mutants, lacking functional mitochondria and containing either greatly altered mtDNA (see 28–30) or no mtDNA at all (31). This has been attributed to the presence in yeast mitochondria of a repair nuclease which attacks DNA containing intercalated EthBr (see 28). Some evidence for such a nuclease activity in yeast has been reported (15).

A better understanding of the mutagenic effect of EthBr on mtDNA would require detailed knowledge of the initial events in the EthBr-induced DNA degradation. Such a study is not possible with yeast, as long as yeast mtDNA cannot be extracted in intact form. We, therefore, turned to the protozoan *Tetrahymena pyriformis*. *Tetrahymena* has two advantages for such a study. First, its mtDNA can be quantitatively extracted as linear duplex molecules, homogeneous in size (15–17 μm) and lacking single-stranded breaks (32–34). This should allow the analysis of the nature of EthBr-induced breaks, if any. Second, there is circumstantial evidence for a DNA repair process in *Tetrahymena* mitochondria. Westergaard (35–37) has shown that a DNA polymerase associated wth the mitochondrial fraction is induced when *Tetrahymena* is exposed to EthBr, X-ray and ultraviolet light radiation, or thymine starvation. He has interpreted this as the induction of a repair polymerase in response to damage of mtDNA (36).

The results of our experiments clearly show, however, that EthBr does not induce degradation of mtDNA in *Tetrahymena*. Instead, it leads to a massive accumulation of intermediates in the replication of mtDNA.

MATERIALS AND METHODS

Cell Line and Growth

T. pyriformis strain ST was obtained from Dr. Y. Suyama, Philadelphia, Pa. Stock cultures were maintained in 5 ml of 1% proteose peptone, transferred weekly, and stored in a semisterile room in the dark at approximately 20°C.

Medium 1

2% proteose peptone (Difco Laboratories, Detroit, Mich.), 0.2% yeast extract (Difco Laboratories), and 0.2% NaCl.

Medium 2

Same as medium 1, except that yeast extract was dephosphorylated (17 g yeast extract was dissolved in 500 ml water and 7.5 g $MgSO_4 \cdot 7\,H_2O$ was added. The pH was brought to 8.0 with NH_4OH and the solution was allowed to stand 2 h or longer at 4°C. It was then centrifuged at 10,000 *g* for 10 min. The supernate was brought to pH 7.0 with HCl, the solution sterilized, and stored at 4°C until use.)

Starter cultures were made by adding 1 ml of stock culture to 50–100 ml media and allowing them to stand 4–6 h without shaking at room temperature, followed by 20–24 h shaking at 28°C. Larger volumes of media, prewarmed to 28°C, were inoculated from the starter culture. Large cultures were grown in Erlenmeyer flasks filled maximally to 20% capacity. All growth was achieved on a rotary shaking table at 28°C in the dark. Growth was followed by measuring turbidity at 546 nm in an Eppendorf photometer. Samples were put in 1-cm glass cuvettes, covered with Parafilm, inverted several times, and turbidity measurements were read immediately after placing the cuvette in the cuvette holder because the cells redistributed very rapidly. A plot of cell number (measured by counting cells in a hemocytometer) against turbidity showed a linear relationship in the range used. No deviation was found for cells grown in EthBr.

Generation time in medium 1 was 3.4 h. Growth in medium 2 was found to give variable generation times and responses to EthBr. This medium was not used in later experiments.

Over time periods of 6 mo to 1 yr, the growth characteristics of our stock of strain ST showed some drift both in generation time and in growth response to a given concentration of EthBr. Differences in response were also observed in two cultures of strain ST which had been maintained separately for 1 yr but originated from the same stock culture. Similar drift has been reported by other investigators (38, 39).

EthBr was obtained from Merck, Darmstadt, Germany. All additions of EthBr to media were made from a concentrated stock solution of 5 mg/ml sterilized by ultrafiltration. The concentration of the stock solution was determined from absorbance at λ_{max} 486 nm of the solution diluted to about 100 μg/ml using an extinction coefficient of $4.83 \times 10^3\ M^{-1}$ (23). EthBr was added to cultures at 0.9–1.6 $\times 10^5$ cells/ml unless otherwise stated.

Preparation of Mitochondria

(Procedure for 800-ml culture at approximately 3×10^5 cells/ml.) All steps were carried out at 0°–4°C. Cells were chilled rapidly by addition of ice and spun down at 650 *g* for 10 min (Sorvall GSA rotor, Ivan Sorvall, Newtown, Conn.), resuspended in 80–100 ml 0.35 M sucrose, 5 mM Tris-HCl, 0.2 mM EDTA (pH 7.2) (STE buffer) and pelleted at 750 *g* for 5 min (Sorvall SS-34 rotor). The cells were then washed a second time and resuspended in STE buffer solution with added 0.2% bovine serum albumin. They were then broken by four passages through a French press (cream homogenizer). Unbroken cells and nuclei were pelleted and removed by two centrifugations for 3 min at 500 *g*. Mitochondria were pelleted at 7,700 *g* for 10 min. The mitochondrial pellet was resuspended by gentle homogenization in 80 ml STE buffer, leaving behind (if present) a small black pellet. The mitochondria were repelleted under the same conditions and, after rinsing the surface of the mitochondrial pellet with 0.15 M NaCl, 0.10 M EDTA (pH 9), they were resuspended by homogenization in 1–2 ml of this solution.

Isolation of DNA

METHOD A: Mitochondria in NaCl-EDTA were lysed by addition of a sufficient amount (approximately one-fourth volume) of detergent mix (4% Sarkosyl, 1% *p*-aminosalicylate, 1% isopropanyl-naphthalenesulfonate, 12% isobutanol) to clarify the mitochondrial suspension. The mitochondrial lysate was then gently shaken with 2 vol of phenol-cresol (500 g phenol, 170 ml cresol, 0.5 g 8-hydroxyquinoline) for 10 min. The aqueous phase was removed, reextracted with 2 vol phenol-cresol, followed by extraction two times with 2 vol chloroform-isoamyl alcohol (25:1). All steps were performed at 0°–4°C.

METHOD B: Mitochondria were lysed by addition of a 10% Sarkosyl solution to a concentration of 1–2% Sarkosyl, followed by addition of 2 mg pronase/ml (preincubated 1 h at 37°C), and were incubated at 37°C for 2 h. The DNA was then banded in 3–5 ml CsCl buoyant equilibrium gradients in SW-50 or SW-50.1 rotors at 35–43 krpm, 20°C, for 48–72 h either with 300 μg EthBr/ml (ρ = 1.56 g/ml) or without EthBr (ρ = 1.67 g/ml). The DNA was then sedimented through 5–21% neutral SW-27 sucrose gradients.

METHOD C: Mitochondria were lysed by addition of Sarkosyl to 1–2%. The mitochondrial lysate was then sedimented through a neutral sucrose gradient. Pooled fractions were incubated with 2 mg pronase/ml (preincubated 2 h at 37°C and 10 min at 80°C) for 2 h at 37°C and then banded in 5-ml buoyant CsCl equilibrium gradients (SW-50.1) at 20°C, 72 h at 35 krpm.

The exact procedure used for obtaining mitochondria and mtDNA from *Tetrahymena* grown in EthBr for extended time periods was found to be rather critical if intact DNA free of contamination in high yield was desired. Mitochondria from EthBr-treated cells form a rather poorly defined pellet when spun under standard conditions. Although increasing the pelleting forces increases the recovery of mtDNA, contamination with low molecular weight (presumably nuclear) DNA becomes troublesome (see Fig. 5). Maintaining adequate wash volumes and carefully washing the surface of the second mitochondrial pellet are important to minimize contamination with DNA of low molecular weight. Deproteinization of the mitochondrial lysate by phenol extraction gave very large losses of mtDNA from EthBr-treated cells (see Fig. 5). DNA preparation method C was found to be best in spite of the fact that poor resolution by velocity sedimentation rate was obtained with large preparations (Fig. 7). Method B gave rise to some degradation of DNA with large preparations, but with highly labeled dilute preparations either method B or C worked well.

Sucrose Gradient Sedimentation

DNA was sedimented through 5–21% sucrose gradients in a Spinco SW-27 rotor for the equivalent of 14.5 h at 22 krpm, 4°C in either neutral (1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA [pH 8]) or alkaline (0.9 M NaCl, 0.1 N NaOH, 1 mM EDTA) solutions. Sedimentation in 4.5-ml neutral 5–21% SW-50.1 gradients was carried out for 2.25–2.5 h at 4°C, 43 krpm. Gradients were dripped by gravity. When ^{32}P-labeled RNA was present, the fractions to be counted were incubated overnight at 37°C in 0.3 N KOH to hydrolyze the RNA before precipitation of DNA. 100 μg carrier bovine serum albumin was added to each fraction, and fractions were made 5–10% in trichloroacetic acid at 0°C to precipitate the DNA. The precipitated DNA was filtered onto glass filter paper. When ^{32}P-labeled phospholipids were present, the filters were washed two to four times with cold 96% ethanol (see Fig. 3). Filters were dried for a minimum of 1 h at 80°C and 5 ml scintillation fluid (10 g 2,5-diphenyloxazole (PPO), 125 mg 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) per 2.5 liter toluene) was added before counting. In some experiments ^{32}P was counted without precipitation by the Cerenkov method. In other experiments small gradient fractions were collected directly into scintillation vials, and 10 ml scintillation fluid (4 g PPO, 80 g naphthalene, 400 ml methanol, 600 ml toluene) was added and samples were counted.

Benzoylated-Naphthoylated DEAE-Cellulose (BND-Cellulose) Chromatography

1 × 1.5 cm columns of BND-cellulose (Serva) were made and washed, before use, with 50–100 ml of 1.0 M NaCl followed by 50 ml of 0.3 M NaCl, 10 mM Tris-HCl (pH 8). DNA (0.2–0.5 μg labeled DNA) was applied in 0.3 M NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH 8). The column was then eluted with 70 ml each of 0.3 M NaCl, 1.0 M NaCl (both containing the same buffer as above), 1.0 M NaCl *plus* 2% caffeine, and finally 0.1 N NaOH-0.9 M NaCl.

Protein Determinations

Protein determinations were done by the Lowry method (40).

DNA Polymerase Assays

Assay conditions were the same as those used by Westergaard (35), with the exception that linear activities with added mitochondrial supernates were obtained only after the calf-thymus DNA template was activated by treatment with pancreatic deoxyribonuclease.

Electron Microscopy

DNA from EthBr-grown cells sedimenting at around 33S (EBS) and from those sedimenting faster than 33S (EBF) (see Fig. 7 and Table III) was spread by a formamide modification of the Kleinschmidt protein monolayer technique (41). The spreading solution contained 40% formamide, 0.4 M ammonium acetate, 5 mM EDTA, and 0.015% cytochrome *c*, and the subphase consisted of distilled water, redistilled over quartz. The protein-nucleic acid film was picked up on carbon-coated Cu grids and stained with uranyl acetate. The grids were rotary shadowed with Pt and examined in a Philips EM200. Frequencies were mainly measured directly from the fluorescent screen, but doubtful molecules were photographed and measured before classification. Data for EBF resedimented (Table III) was obtained from grids prepared by R. A. Clegg, using a formamide modification of the microdiffusion technique of Lang and Mitani (42).

Radioactive Compounds

Carrier-free [^{32}P]orthophosphate was obtained from Philips Duphar, Petten, The Netherlands. [*Methyl*-^{3}H]thymidine (15,000–30,000 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Great Britain.

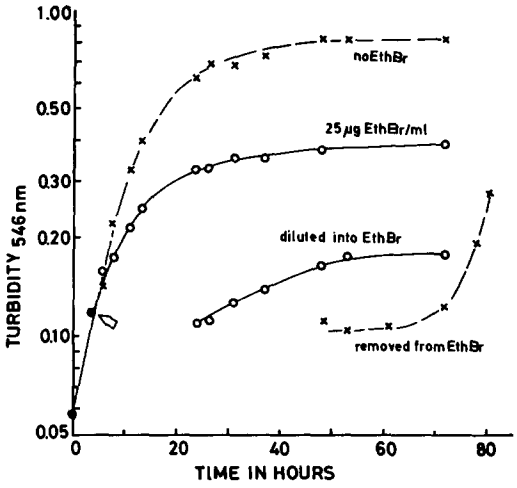


FIGURE 1 Growth of *T. pyriformis* in the presence of EthBr. Cells were grown in medium 2. At approximately 3 × 10^4 cells/ml, the culture was divided into two portions and EthBr at 25 μg/ml was added to one. After 20-h growth in EthBr, a portion of the culture was diluted into fresh medium containing the same concentration of EthBr. After 45 h, cells were pelleted from a portion of the culture, washed one time with fresh medium, and resuspended in fresh medium in the absence of EthBr.

RESULTS

Effects of EthBr on the Growth of Tetrahymena

Fig. 1 shows growth curves for *T. pyriformis* strain ST grown in medium 2 in the absence and presence of 25 μg EthBr/ml. Compared with the normally grown culture, cells grown in EthBr show an increase in the generation time and a lowering of the stationary phase cell density. The time interval before the cells reached stationary phase is not significantly shortened (cf. 43). With increasing concentrations of EthBr, increases in the generation time and decreases in the stationary phase cell density occur, maximal effects being obtained at 25 μg/ml. Cells removed from EthBr resume growth after a lag phase (Fig. 1) which is dependent on the concentration of EthBr and the length of exposure to the drug. Similar results of the effect of EthBr on the growth of *Tetrahymena* have been reported by Meyer et al. (44) and by Charret (43).

The growth response of EthBr appears to be affected by the medium used, the age of the stock culture used to inoculate the starter culture, and the cell density at which EthBr is added. Inasmuch

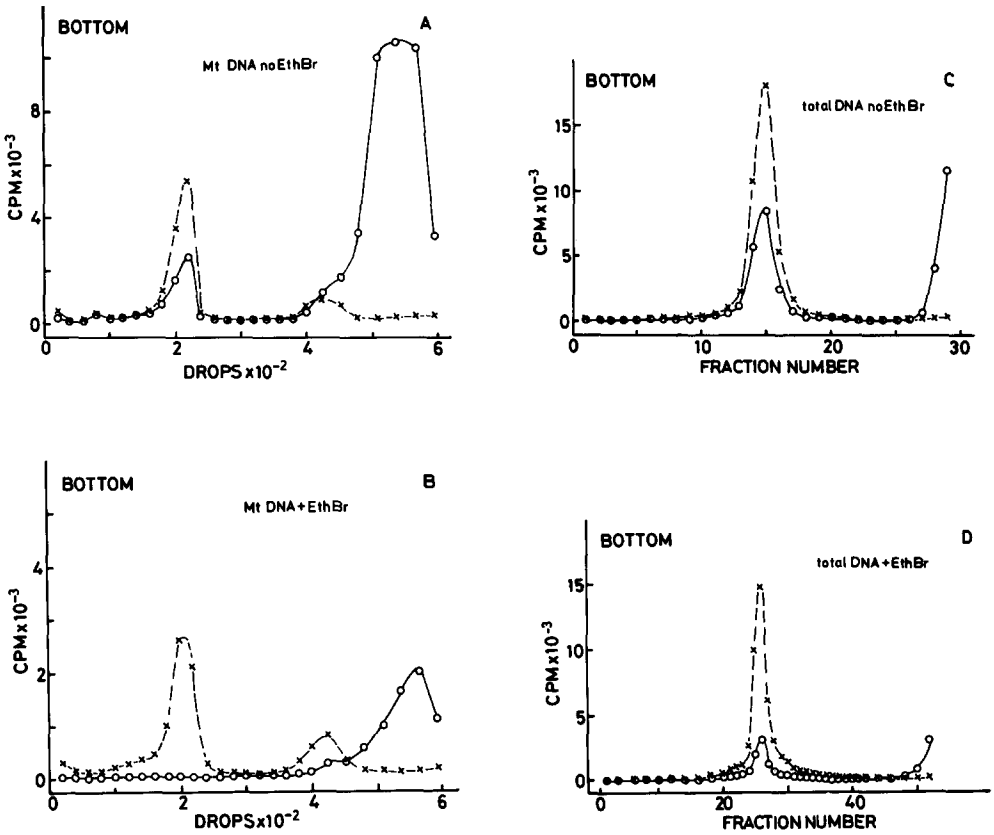


FIGURE 2 Effects of short-term growth in EthBr on total mtDNA synthesis. A 600-ml culture of *Tetrahymena* was prelabeled with 250 μCi [^{3}H]thymidine for six generations. 2.5 mCi [^{32}P]orthophosphate was then added, 250 ml of the culture was removed to a sterile flask, and EthBr added to 4 μg/ml. At 2.75 h after addition of ^{32}P, cells were harvested from both cultures. A portion of the harvested cells were lysed by addition of 2% Sarkosyl, and the total DNA was banded in CsCl buoyant gradients (ρ = 1.67) for 43 h (C) or 67 h (D) in a SW-50.1 rotor at 20°C, 35 krpm. Mitochondria were purified and lysed by addition of Sarkosyl to 1%. The mitochondrial lysate was then layered directly on SE-27 sucrose gradients without further purification. All gradient fractions were incubated in 0.3 M KOH overnight at 37°C to hydrolyze RNA before precipitation and counting. Sucrose gradients of (A) mtDNA from normally grown cells, (B) mtDNA from cells grown in EthBr. CsCl buoyant gradients of (C) total DNA from normally grown cells and (D) total DNA from cells grown in EthBr. ×---×, ^{3}H prelabel; o—— o, ^{32}P.

as *Tetrahymena*, unlike animal cells in tissue culture, does not visibly accumulate large amounts of EthBr, these differences may be primarily due to differences in permeability. A concentration of 4 μg EthBr/ml was chosen for most of the work presented here because this level of EthBr permitted sufficient growth of cells and synthesis of DNA to allow meaningful studies.

Effects of EthBr on DNA Synthesis

In order to study the comparative effect of EthBr on mitochondrial and nuclear DNA synthesis, double-label experiments were done to differentiate between DNA synthesized before and after the addition of EthBr. *Tetrahymena* cultures were prelabeled with [^{3}H]thymidine, followed by ^{32}P labeling in the presence and absence of EthBr. After a period of growth in EthBr, nuclear and mtDNA were analyzed. Nuclear DNA, assumed to be adequately represented by total DNA since mtDNA comprises only about 2% of total DNA (32), was analyzed by banding the DNA in CsCl buoyant equilibrium gradients. MtDNA was analyzed by sedimentation of the Sarkosyl lysate of purified mitochondria on neutral sucrose gradients. The mtDNA isolated from normally grown cells sediments as a homogeneous peak at 33S (Fig. 2 A). In most preparations there is a variable

TABLE I

Inhibition of DNA Synthesis by EthBr

Exp. no.	Growth in ^{32}P	Total DNA ($^{32}P/^{3}H$)		MtDNA ($^{32}P/^{3}H$)		Percent preferential inhibition mtDNA
		− EthBr	+ EthBr	− EthBr	+ EthBr	
	h					
1	2.75	0.49	0.20 (57%)	0.47	0.007 (98%)	97
	5.0	1.48	0.53 (64%)	1.15	0.062 (95%)	87
2	21.5		0.272		0.182–0.272	0–33

The table summarizes the data from Figs. 2 and 3.
Percent inhibition (in parentheses) compares DNA synthesis in the presence of EthBr with that in the absence of EthBr over the same time interval. Percent preferential inhibition compares mtDNA synthesis with total DNA synthesis, both measured in cells from one culture containing EthBr. The two values for mtDNA from cells treated with EthBr for 21.5 h were obtained from two separate gradients (Figs. 3 F and 3 D).

amount (see Materials and Methods) of low molecular weight contaminating DNA which remains at the top of the gradients. We attribute this to nuclear contamination and define mtDNA as DNA isolated from purified mitochondria and sedimenting faster than 30S.

The results of one such double-label experiment after 2.75 h of growth in EthBr are shown in Fig. 2 and Table I. Fig. 2 B shows that hardly any ^{32}P has been incorporated into mtDNA during the first 2.75 h of growth in the presence of EthBr, whereas substantial incorporation has occurred in the absence of EthBr (Fig. 2 A). After 5-h growth in EthBr no change is seen in the shape of the sedimenting band and comparison of the $^{32}P/^{3}H$ ratios (Table I) shows that inhibition of mtDNA synthesis compared with the control culture over the same time interval is about 95%. In contrast, inhibition of nuclear DNA is only approximately 60% (Fig. 2 and Table I) which is about the same as the inhibition of growth, as the generation time increased from 3.5 h in the absence to 8 h in the presence of EthBr.

These biochemical results confirm previous results of Charret (43), who showed by autoradiography under slightly different conditions that mtDNA synthesis is strongly inhibited by growth in EthBr.

Similar experiments were done to measure the effect of EthBr on mtDNA synthesis after longer intervals of time. After 21.5 h of growth in EthBr, total DNA was prepared as above and analyzed in CsCl buoyant gradients containing EthBr. MtDNA was purified by banding in CsCl and then sedimented through neutral sucrose gradients (Fig. 3). A comparison of $^{32}P/^{3}H$ ratios for total and mtDNA from the cells grown in the presence of EthBr shows only a rather small preferential inhibition of mtDNA synthesis (Table I).

Two similar experiments were done with cells grown in medium 2 and with mtDNA prepared by method A. After growth for 26 h at 25 μg EthBr/ml, intermediate and variable amounts of inhibition of mtDNA synthesis (20–70%) were found when compared with mtDNA synthesis in cells grown to the same cell density in the absence of EthBr. The mitochondrial-associated DNA polymerase activity was checked in one of these experiments and found to be present at four to five times the level present in normally grown cells.

Taken together, these results show that preferential inhibition of mtDNA synthesis by EthBr is nearly complete during short exposures to the drug but may be partially overcome after longer exposure to EthBr. This partial release of the block in mtDNA synthesis occurs without a corresponding release of the inhibition of cell growth by EthBr.

Effects of Growth in EthBr on the Structure of mtDNA

As seen in Fig. 3 F, a large fraction of mtDNA from cells grown for 21.5 h in EthBr sediments abnormally fast. After 2.75 or 5 h this rapidly sedimenting material is not evident (Fig. 2). In order to follow the formation of this material, three consecutive mtDNA preparations were made by

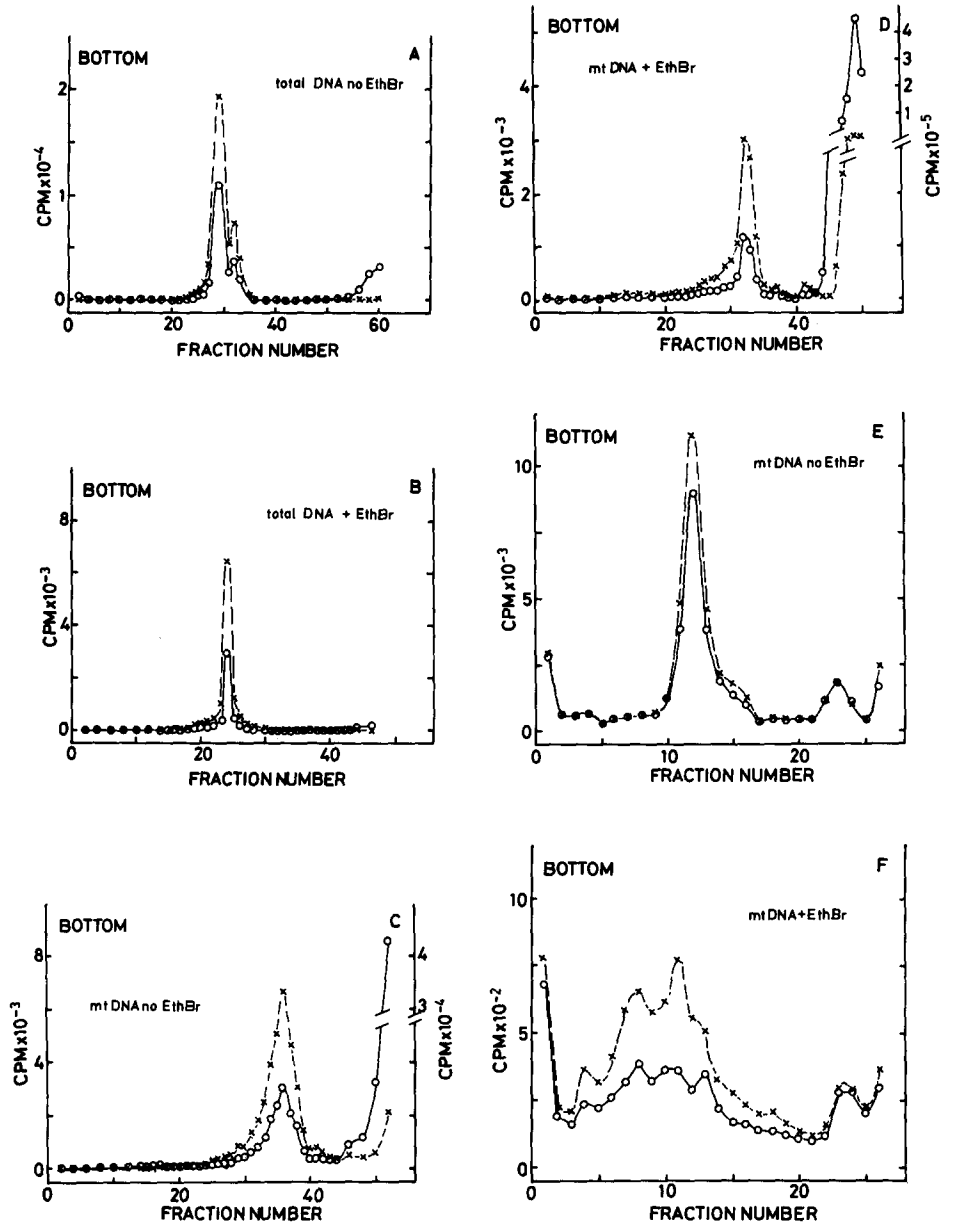


FIGURE 3 Effects of long-term growth in EthBr on synthesis of total and mtDNA. Cells were labeled in 800 ml medium 1 with 1 mCi [^{3}H]thymidine for 5.7 generations. The culture was divided into two 400-ml portions, and 60 mg thymidine was added to each. 4 μg EthBr/ml was added to one half and 30 min later 4 mCi [^{32}P]orthophosphate was added to each culture. After 3.5 h, at a cell density of approximately 3.4 $\times$ 10^5 cells/ml, the normally grown culture was harvested, the cells were washed, 1% of the washed cells were lysed with 2% Sarkosyl, and stored frozen. MtDNA was prepared by method B. After 21.5 h of growth in EthBr, the remainder of the cells were processed as above. Concentrated EthBr solution and solid CsCl were added to both cell and mitochondrial lysates for a final volume of 5 ml, 300 μg EthBr/ml (ρ = 1.55). The DNA was then banded in a SW-50.1 rotor at 40 krpm, 20°C for 48 h. Alternate fractions from the mtDNA gradients were precipitated and counted. The remaining peak fractions were pooled, dialyzed against Dowex-50 in 2 M NaCl to remove EthBr, further dialyzed into 0.15 M NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH 8), and analyzed by velocity sedimentation through neutral SW-27 sucrose gradients at 20.4 krpm for 16 h at 4°C. Buoyant CsCl gradients: (A) total DNA from normally grown cells; (B) total DNA from cells grown in EthBr; (C) mtDNA from normally grown cells; (D) mtDNA from cells grown in EthBr. Sucrose gradients: (E) mtDNA from normally grown cells; (F) mtDNA from cells grown in EthBr. $\times$---$\times$, ^{3}H prelabel; o——o, ^{32}P. The extra shoulder at fraction 32 in gradient (A) is considered to be a dripping artefact.

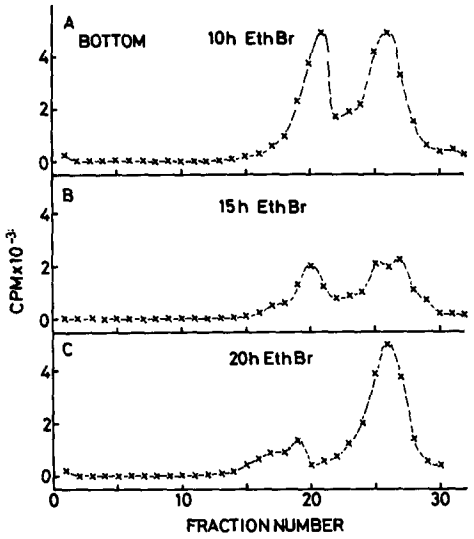


FIGURE 4 Neutral sucrose gradients of mtDNA from cultures grown for varying lengths of time in EthBr. A 1200-ml culture in medium 1 was grown for 3.8 generations with 400 μCi [^{3}H]thymidine. 4 μg EthBr/ml was then added and after 10-h growth, 400 ml of culture was removed, and mtDNA was prepared by method A. The mitochondrial lysate was temporarily stored at 0°C. At 15 and 20 h after addition of EthBr, similar preparations were made. The deproteinized mitochondrial lysates were then layered onto SW-27 sucrose gradients and sedimented at 21.1 krpm for 11 h at 4°C. MtDNA from cells grown for (A) 10, (B) 15, and (C) 20 h in 4 μg EthBr/ml.

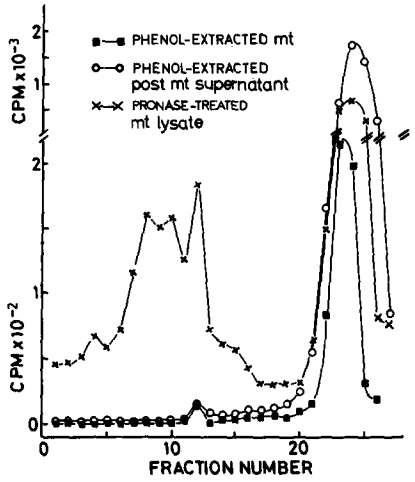


FIGURE 5 Sucrose velocity gradients comparing recovery of mtDNA from EthBr-treated cells prepared by phenol extraction and pronase treatment. Cells were labeled continuously in medium A with [^{3}H]thymidine before and during 26-h exposure to 4 μg EthBr/ml. Mitochondria were purified and divided into two equal portions. MtDNA was prepared either by method A (phenol extraction) or method B (pronase incubation). The supernates from the mitochondrial pellets were combined and spun 15 min at 35,000 *g*. The pellet was then further processed as mitochondria by method A. All three samples were banded in SW-50 CsCl-EthBr buoyant equilibrium gradients. The peak regions were pooled, dialyzed first against Dowex-50 in 4 M NaCl to remove EthBr and then dialyzed into 0.15 M NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH 8). The samples were then sedimented through SW-27 neutral sucrose gradients for 11.25 h at 24.3 krpm. ×——×, pronase-treated mitochondrial lysate; ■——■, phenol-extracted mitochondrial lysate; ○——○, phenol-extracted post-mitochondrial fraction.

method A from one culture exposed for 10, 15, and 20 h to EthBr. Neutral sucrose gradients of this DNA are shown in Fig. 4. At 15 h a clearly visible, rapidly sedimenting shoulder is present and at 20 h about 50% of the DNA is sedimenting more rapidly than normal mtDNA. This rapidly sedimenting DNA appears at approximately the same point in time at which there is some breakthrough in the EthBr inhibition of mtDNA synthesis.

Parallel to this accumulation of rapidly sedimenting material, there is a decreasing recovery of mtDNA after phenol extraction. In order to check the reason for this decrease, equal amounts of purified mitochondria from cells treated with EthBr for 26 h were deproteinized either by extraction with phenol (method A) or with a pronase treatment (method B) and, after banding in CsCl-EthBr, the DNA was sedimented through neutral sucrose gradients (Fig. 5). The recovery of mtDNA from the phenol extraction is unusually low in this experiment and only about 5% of that from the pronase treatment. Also shown is the sedimentation pattern of DNA from a phenol extraction of a high-speed pellet of the mitochondrial supernate which contains very large amounts of small DNA. This is probably the same material which is present as a contaminant in variable amounts in mtDNA preparations.

Neutral sucrose gradients (Figs. 2 and 4) show no degradation of the mtDNA from cells grown in EthBr. Alkaline gradients which allow the detection of single-strand interruptions in the DNA were used for further analysis of double-labeled mtDNA from cells exposed 26 h to EthBr (Fig. 6). The ^{3}H label incorporated before the addition of EthBr is present almost exclusively in intact strands, but about two-thirds of the newly in-

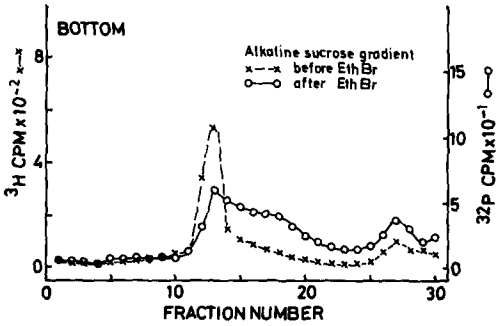


FIGURE 6 Alkaline sucrose gradient of mtDNA from cells grown in EthBr. Cells were prelabeled with [^{3}H]-thymidine in medium 2 and labeled with [^{32}P]orthophosphate for 26 h after the addition of 25 μg EthBr/ml. MtDNA was prepared by method A and sedimented on an alkaline sucrose gradient in a SW-27 rotor. x- - -x, ^{3}H prelabel; o——o, ^{32}P incorporated in the presence of EthBr.

corporated ^{32}P is present in relatively large, incomplete fragments. That fact that ^{3}H counts present in rapidly sedimenting DNA sediment in alkaline gradients at the same position as intact single strands of mtDNA or slower, provides a strong indication that the rapidly sedimenting DNA must be mtDNA. In a repeat of the experiment identical results were obtained. Thus there is no significant degradation of the preexisting DNA, and the rapidly sedimenting molecules contain incomplete daughter strands.

For further characterization of this rapidly sedimenting DNA, large-scale preparations were made by method C. This method gives a very broad mtDNA band (Fig. 7 A) with poor resolution of DNA by sedimentation coefficient but adequate separation from nuclear DNA fragments and mitochondrial protein at the top of the gradient. The poor resolution is apparently due to overloading the gradient with protein because, when the DNA-protein concentration is reduced by a factor of 20, normal mtDNA sedimentation profiles are obtained. The peak of mtDNA from cells grown in the presence of EthBr was divided into two pooled fractions, as shown, and further purified by method C.

To obtain better separation of the DNA as a function of sedimentation coefficient, the concentrated fast fraction of DNA from banding in CsCl was rerun on a SW-50.1 sucrose gradient (Fig. 7 B). Individual fractions from this gradient were rerun on SW-50 sucrose gradients with ^{32}P-marker normal mtDNA for characterization (Figs. 7 C and 7 D). The DNA resedimented as discrete peaks characteristic of the fractions from which they were obtained. This indicates that the rapidly sedimenting material is not a nonspecific aggregation of molecules.

Two types of experiments were done to verify whether the rapidly sedimenting DNA contains single-stranded regions. First, it was sedimented to equilibrium in neutral CsCl (Fig. 8). No significant displacement was observed of the ^{3}H-labeled, rapidly sedimenting DNA in relation to ^{32}P-labeled normal 33S mtDNA, showing that less than 10% of this DNA occurs in single-stranded regions. Similar results were obtained with NaI gradients in which the resolution between double- and single-stranded DNA is even greater (45). Second, ^{3}H-labeled, fast-sedimenting DNA was fractionated on BND-cellulose columns, again using ^{32}P-labeled DNA from normal cells as internal marker. Fig. 9 A and Table II show that mtDNA from normally grown cells elutes mainly in the duplex DNA fraction, but the mtDNA from EthBr-treated cells does not elute under normal conditions for either native DNA (1.0 M NaCl) or for denatured DNA (2% caffeine) but remains trapped on the column until it is denatured by 0.1 M NaOH. This trapping appears to be a peculiarity of the rapidly sedimenting DNA and it is not solely due to single-stranded regions in this DNA, because it does not occur with either native or denatured normal *Tetrahymena* mtDNA and does occur irrespective of the presence of cold carrier DNA (Fig. 9 B). Similar problems with retention of replicating chick embryo lethal orphan viral DNA have been reported by Bellett and Younghusband (46).

The combined results of the BND-cellulose column and the buoyant banding in CsCl and NaI indicate that the rapid rate of sedimentation is not the result of collapsed single-stranded regions of the DNA.

Electron Microscopy of Rapidly Sedimenting DNA

Table III presents a rough frequency distribution of molecules observed in three different types of preparations, the EBF and EBS fractions of Fig. 7 A and fraction 8 of the resedimented EBF fraction in Fig. 7 B. For reasons that are unclear, spreading of these (and similar) fractions by the

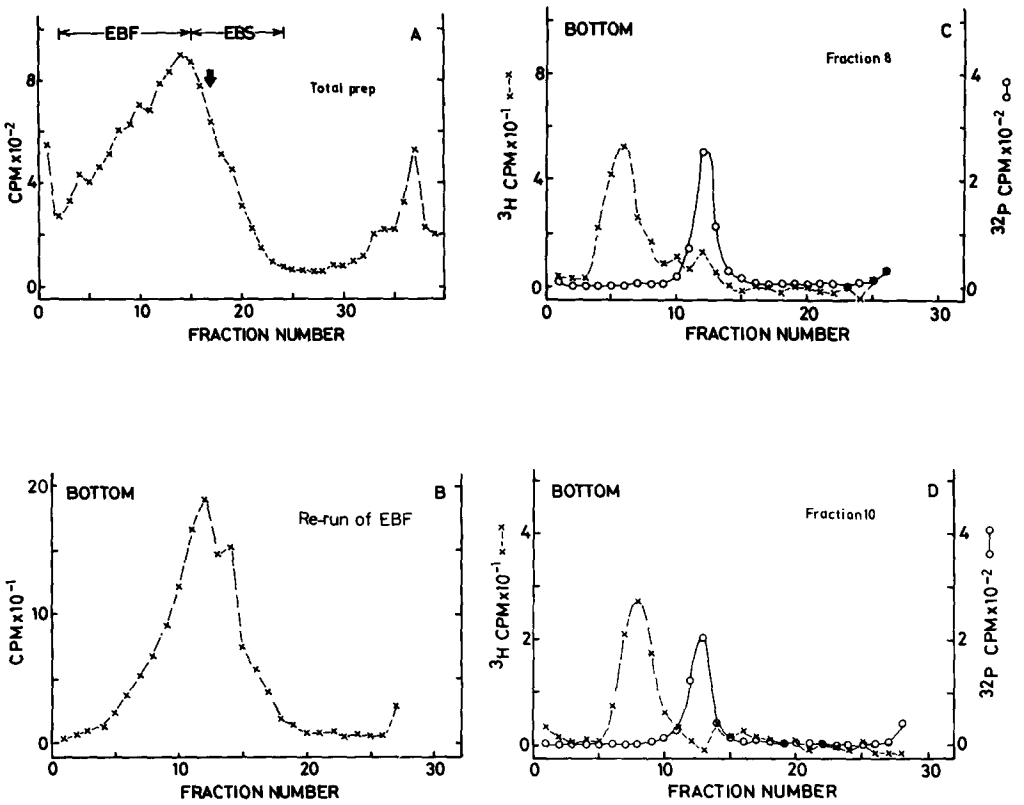


FIGURE 7 Sucrose velocity sedimentation of fast-sedimenting mtDNA from cells grown in EthBr. Cells were grown in 800 ml medium 1 for five to six generations with 1.0 mCi [^{3}H]thymidine, 4 μg EthBr/ml was added to the culture and the cells were allowed to continue growing for 20 h. MtDNA was prepared by method C. (A) SW-27 sucrose gradient of mitochondrial lysate. The arrow indicates the position of mtDNA from normally grown cells. Alternating fractions of 30 and 1 drops were collected. The 1-drop fractions were precipitated and counted. The remaining DNA was pooled into fractions EBF and EBS as indicated, pronase treated, and banded in CsCl buoyant gradients. After dialysis, fraction EBF was resedimented through a SW-50.1 sucrose gradient (B), and individual fractions were used for further experiments. (C) Resedimentation of fraction 8 from EBF in SW-50 rotor; (D) resedimentation of fraction 10 from EBF as in (C). X- - -X, ^{3}H-labeled mtDNA from cells grown in EthBr; o——o, ^{32}P marker mtDNA from normally grown cells.

formamide version of the protein monolayer technique was difficult and about 60% of the molecules were in "flowers", incompletely traceable or unclassifiable for other reasons. Well-spread, full-length molecules consisted predominantly of two types:

CLASS 1: Linear molecules without branches.

CLASS 2: Linear molecules with duplex eyes. Often these molecules have short, single-stranded sections in one arm of one or both forks (Fig. 10). Included in this class are a small number of full-length molecules containing a short internal double-stranded branch. These comprised about 10–20% of the class 2 molecules. We assume that these are broken eyes since the position and length of the short branch are consistent with this hypothesis.[2,3]

The frequency of linear molecules without branches was low in rapidly sedimenting material purified by resedimentation (last column of Table III). We attribute their presence in EBF to overloading of the gradient presented in Fig. 7 A. The

[2] Clegg, R. A., P. Borst, and P. J. Weijers. 1974. In preparation.

[3] Arnberg, A. C., E. F. J. Van Bruggen, R. A. Clegg, W. B. Upholt, and P. Borst. 1974. In preparation.

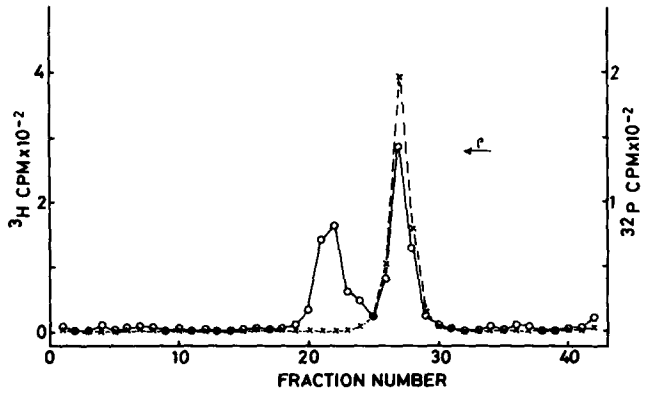


FIGURE 8 CsCl equilibrium centrifugation of fast-sedimenting mtDNA from cells grown in EthBr. 0.25 μg of fraction EBF of the gradient in Fig. 7 A was mixed with 0.1 μg each of ^{32}P-labeled native and heat-denatured mtDNA from normal control cells and run in a neutral CsCl gradient (ρ = 1.70) at 35 krpm for 60 h at 20°C in a Spinco 40-angle rotor, using polyallomer tubes.

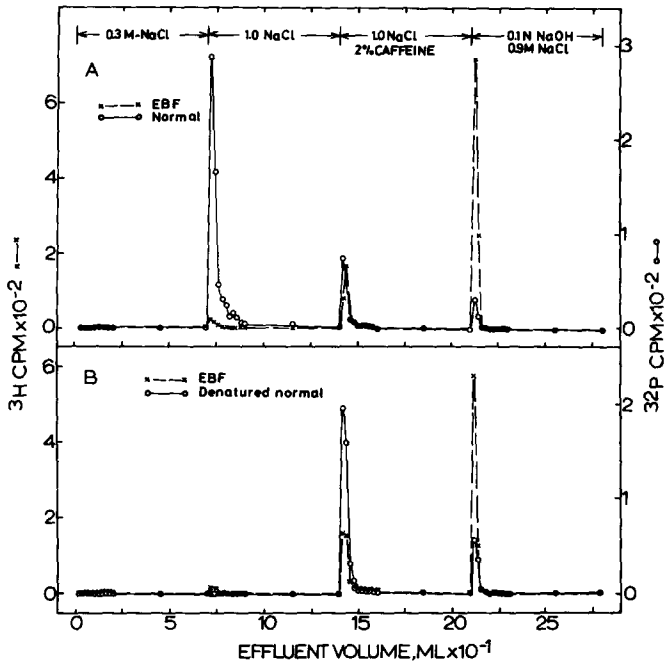


FIGURE 9 BND-cellulose chromatography of mtDNA from cells grown in EthBr. (A) X- - -X, ^{3}H-labeled DNA from the fast-sedimenting fraction (Fig. 7 A) of mtDNA from cells grown in EthBr; O—— O, ^{32}P-labeled normally grown mtDNA. (B) X- - -X, ^{3}H-labeled mtDNA as in (A); O—— O, ^{32}P-labeled normal mtDNA denatured by heating for 10 min at 100°C after addition of 10 μg of unlabeled calf-thymus DNA.

average eye size was 16% of the length of the molecules in EBS and 25% in EBF. This shows the expected positive correlation between eye size and sedimentation coefficient.

In addition, two other classes of molecules were scored.

CLASS 3: Full-length Y-type molecules with two daughter branches equal within 20%. These molecules were found only in rapidly sedimenting DNA of normal cells.[2,3]

CLASS 4: Unusual molecules. One subclass of these, containing an eye within an eye (class 4a),

TABLE II

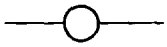
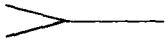
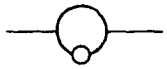
Fractionation of Fast-Sedimenting and Normal MtDNA on BND-Cellulose Columns

Exp.	MtDNA	Percent of total DNA recovered in: 0.3 M Nacl	1.0 M NaCl	1.0 M NaCl + 2% caffeine	0.1 M NaOH
1*	EBF	0	4	27	69
	Native normal	0	74	19	7
2‡	EBF	1	3	37	59
	Denatured normal	0	0	78	22

See legend of Fig. 9 for experimental details.
* See Fig. 9 A.
‡ See Fig. 9 B.

TABLE III

Frequency Distribution of Different Molecular Species in MtDNA Preparations from EthBr-Grown Cells

Class	DNA species	Frequency (% of classified molecules): EBS	EBF	EBF recentrifuged
1		55.3	33.6	21.7
2		40.5	58.2	69.8
3		0	0	0
4a		2.8	5.2	2.3
4b, c	Unusual molecules	1.4	3.0	6.2
	No. of molecules classified	141	134	129
	No. of unclassifiable molecules	44	86	42
	No. of flowers	185	209	0
	Total no. of molecules analyzed	370	429	171

EBS and EBF are the corresponding fractions of the gradient in Fig. 7 A; EBF recentrifuged is fraction 4 of the gradient in Fig. 7 B.

probably results from the initiation of a new round of replication before the previous one is finished.

No circles and no molecules larger than unit-length *Tetrahymena* mtDNA were found. The latter observation provides additional evidence for the conclusion that EBF does not contain significant amounts of nuclear DNA.

The molecules of classes 1–3 have been found in the rapidly sedimenting DNA of normal cells,[2,3] whereas class 4 is peculiar to EthBr-treated cells. Additional electron micrographs and a more detailed comparison with replicating molecules from normal cells will be presented in a subsequent paper.[3]

DISCUSSION

When moderate levels of EthBr are added to a culture of *T. pyriformis*, an immediate and essentially complete block in mtDNA synthesis occurs. After 5–15 h this block is partially overcome and the slow synthesis of mtDNA at that time leads to the accumulation of molecules with properties very similar to those of the pulse-labeled replicative intermediates, identified in parallel experiments in normal cells.[2,3] This similarity includes sedimentation behavior in neutral and alkaline gradients, absence of large single-stranded regions, retention on BND-cellulose in 1 M NaCl, and appearance in electron micrographs. In both cases the predominant replicative intermediate is a linear molecule with one duplex eye. In both cases the position of the eye is compatible with a model in which replication starts at a unique site near the middle of the molecule and then proceeds bidirectionally to both ends (Fig. 11). In addition, we have found unusual molecules in mtDNA from EthBr-treated cells, not seen in normal cells. These were present at low frequency, and with three exceptions they are readily accounted for by the model in Fig. 11 (see footnote 3). These may not have been found in normal cells, because of the low concentration of replicating molecules in these cells. Therefore, all or nearly all of the forked molecules accumulating in EthBr-treated cells appear to be normal replicative intermediates of *Tetrahymena* mtDNA rather than abnormal structures induced by EthBr treatment.

These results indicate that moderate levels of EthBr lead to the following sequence of events in *Tetrahymena*. Initially, less than 3% of all mtDNA molecules are replicating; addition of EthBr slows down the replication of these molecules by about 95%, resulting in a nearly complete block in mtDNA synthesis. In the course of hours an increasing number of molecules inititate replication (which is apparently not strongly affected by EthBr), and, after 15–20 h, more than 50% of all DNA molecules are slowly replicating. The in-

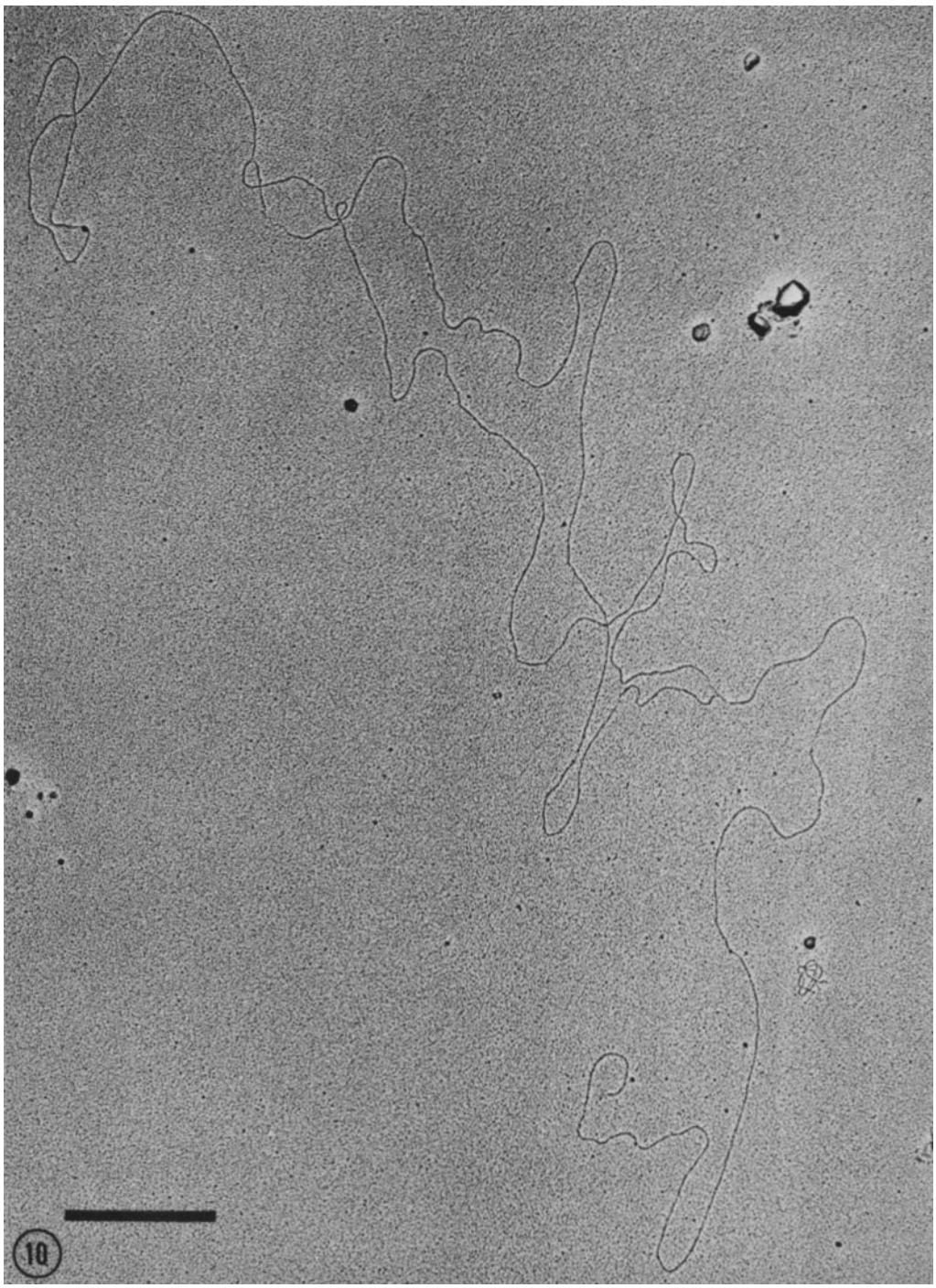


FIGURE 10 Electron micrograph of *Tetrahymena* mtDNA with a duplex eye present in the EBF fraction of Fig. 7 A. The bar is 0.5 μm. × 43,920.

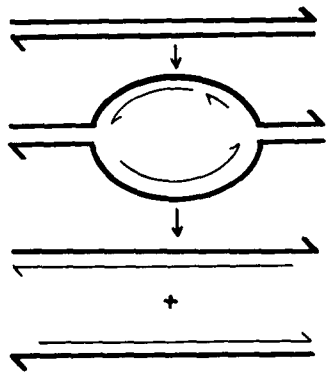


FIGURE 11 Model for the bidirectional synthesis of *Tetrahymena* mtDNA (from footnote 3).

duced polymerase may play a role in this recruitment, but the connection is not obvious. The large increase in the number of molecules at intermediate stages of replication leads to a partial recovery of the overall rate of mtDNA synthesis, although the rate per molecule may still be inhibited by more than 95%. Finally, cell division stops, possibly because the inhibition of mtRNA and protein synthesis interferes with mitochondrial function.

Westergaard and co-workers (35–37) have shown that irradiation with ultraviolet light or X rays, starvation for thymine, or treatment with EthBr all lead to induction of a mtDNA polymerase in *Tetrahymena*, and we have confirmed this for EthBr. Westergaard has suggested that these treatments result in damage of mtDNA and that the induced polymerase is a repair enzyme involved in the repair of this damage. Our results show, however, that no repairable damage (breaks or gaps) is induced by EthBr in *Tetrahymena* mtDNA, as essentially all recovered prelabeled DNA is present in material sedimenting as intact single strands in alkaline sucrose gradients. The induction of the polymerase must, therefore, have another explanation, and three possibilities can be envisaged:

(*a*) Recognition by the cell of the altered winding of the DNA double helix induced by EthBr or thymine dimers and the induction of a repair enzyme to repair the apparent damage. It is not obvious how the cell would recognize this damage; there is no biochemical evidence for repair of mtDNA in any cell type and it is difficult to see how thymine starvation would lead to repairable damage.

(*b*) The block in transcription brought about by EthBr leads to a general overproduction of nuclear gene products involved in mitochondrial biogenesis because these genes are controlled by a mitochondrial repressor that cannot be synthesized in the presence of EthBr. Evidence for such general overproduction has been obtained for *Neurospora* (see 47), although DNA polymerase was not measured in that case.

(*c*) The inhibition of mtDNA synthesis itself leads to the induction of a higher level of a replication polymerase to increase the rate of replication. This implies a direct and specific control of the level of mtDNA by the nuclear genome.

We prefer the last explanation because it provides the best explanation for the induction of the polymerase by thymine starvation. Moreover, a nuclear control of mtDNA replication independent of mitochondrial protein synthesis is suggested by the existence of mtDNA in yeast petite mutants in which mitochondrial protein synthesis is absent (see 28, 29). Further experiments on the induction of DNA polymerase in *Tetrahymena* may provide information on the mechanism of this nuclear control.

The accidental finding that intermediate levels of EthBr lead to a massive accumulation of apparently normal replicative intermediates of mtDNA has been rather useful in the analysis of mtDNA replication in *Tetrahymena*. This trick might also work in other cells, because EthBr appears to be a general inhibitor of mtDNA synthesis (cf. 5, 6, 9–14, 16–18), whereas only in *Saccharomyces* strains has it been clearly demonstrated to induce degradation of mtDNA (7, 8).

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