THE EFFECT OF ETHIDIUM BROMIDE ON MITOCHONDRIAL DNA SYNTHESIS AND MITOCHONDRIAL DNA STRUCTURE IN HeLa CELLS

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ABSTRACT

The synthesis of mitochondrial DNA (mDNA) in HeLa cells is selectively inhibited by relatively low concentrations of ethidium bromide. After exposure of cells to strongly inhibitory concentrations of the drug, the apparent superhelix density of mDNA is rapidly increased, as judged by its buoyant density in CsCl in the presence of ethidium bromide. Mitochondrial DNA synthesized in the presence of partially inhibitory concentrations of ethidium bromide is also altered in its buoyant density in the presence of the dye, but is more heterogeneous in this respect. However, the change in buoyant density of newly synthesized mDNA may be explained by changes in structure other than a change in superhelix density, as indicated by its increased resistance to digestion by pancreatic DNase.

INTRODUCTION

The phenanthridine dye ethidium bromide (EBr)[1] has been a very useful reagent in the study of the physical properties of circular DNA. The unique aspects of the interaction of circular DNA with the dye in vitro are paralleled by selective effects in vivo on the metabolism of circular DNA. Among these effects are the loss of kinetoplast DNA in *Trypanosoma cruzi* (1) and the induction of petite mutations in yeast (2). The latter phenomenon has been shown to be accompanied by the inhibition of mitochondrial DNA (mDNA) synthesis and the degradation of mDNA present in the cell at the time the drug was administered (3). In animal cells, EBr has been shown to inhibit mitochondrial protein synthesis (4, 5) and mitochondrial RNA synthesis (6, 7).

Several effects of ethidium bromide on the metabolism of mDNA in HeLa cells are reported here. Moderate doses of EBr were found to strongly inhibit the synthesis of mDNA. In addition, the superhelix density of mDNA is apparently increased by exposure of cells to a strongly inhibitory concentration of the drug. However, the properties of mDNA newly synthesized in the presence of partially inhibitory drug levels are not fully accounted for by changes in superhelix density, and may involve some other ethidium-induced effect.

[1] *Abbreviations:* mDNA, mitochondrial DNA; NP40, Nonidet P-40; Sarkosyl, sodium dodecyl sarcosinate; EBr, ethidium bromide; CsCl/EBr, cesium chloride containing 300 μg/ml EBr.

METHODS AND MATERIALS

Methods for cell culture, cell fractionation, assay of radioactivity, and sucrose density gradient centrifugation were as described elsewhere[2].

CsCl gradients containing 300 μg/ml ethidium bromide (CsCl/EBr gradients) were prepared in TE buffer (0.02 M Tris-HCl, pH 8.0, 0.001 M ethylenediamine-tetraacetate [EDTA])to have a final density of 1.56 g/ml. Uniformly mixed solutions were centrifuged a minimum of 24 hr at 20°C in either the No. 50 fixed angle rotor at 40,000 rpm (7.0 ml), or the SW50.1 rotor at 33,000 rpm (2.0 ml). Density markers were obtained from cells grown for 1 day in the presence of thymidine-^{14}C. Closed circular mDNA was prepared by the dye-buoyant density method. Nuclear DNA was prepared by SDS-pronase digestion of nuclei.

Methyl thymidine-^{3}H (10–12 Ci/mmole) and thymidine-^{14}C (50 mCi/mmole) were purchased from Schwarz Bio Research Inc., Orangeburg, N.Y. The concentrations of cells and of exogenous thymidine were adjusted to ensure continuous incorporation at maximal rates. CsCl was purchased from Varlacoid Chemical Co., Elizabeth, N. J.; ethidium bromide from Calbiochem, Los Angeles, Calif.

RESULTS

It has been shown in a separate report[2] that lysis of mitochondrial preparations or of whole cells by the use of nonionic detergents releases DNA-containing particles which are readily separated into nuclear DNA- and mitochondrial DNA-containing fractions. In particular, it has been shown that most of the mitochondrial DNA in cytoplasmic extracts was found in structures with sedimentation coefficients in the range 100–300 S. Fig. 1 (top) shows the sucrose gradient profiles of cytoplasmic extracts fractionated in this way.

In this experiment, a sample of cells labeled with thymidine-^{3}H was homogenized and the cytoplasmic extract was separated into crude mitochondria and postmitochondrial supernatant (see Methods and Materials). Each of these was treated with 0.5% Nonidet P-40 (NP40, Shell Chemical Co., New York) and centrifuged through sucrose gradients. It is evident that the radioactive material from the mitochondria is more rapidly sedimenting than the material from the postmitochondrial supernatant. The tritium-labeled DNA recovered from the sucrose gradient in the case of the mitochondrial sample has been shown to be exclusively mDNA in the form of covalently closed circles. The labeled DNA in the postmitochondrial supernatant has little or no closed circular component and has been shown to be of nuclear origin, as judged by its buoyant density in CsCl[2]. A second sample of cells was lysed directly with NP40, and the total cytoplasmic extract obtained was also analyzed by sucrose gradient sedimentation (Fig. 1, top). This is seen to yield a more efficient extraction of cytoplasmic material and a sedimentation profile which is a composite of the two described above. Again, the more rapidly sedimenting material from this sample has been shown to be largely closed circular mDNA, while the slower material is largely DNA of nuclear density.

The effect of EBr on the labeling of the various DNA preparations is shown in the bottom panel of Fig. 1, which illustrates the sucrose gradient profiles of detergent-treated fractions extracted from cells which had been incubated with thymidine-^{3}H in the presence of 1 μg/ml EBr. It is clear that thymidine incorporation into mDNA-containing structures is almost completely abolished by the drug, while the labeling of the more slowly-sedimenting DNA-containing structures is essentially unaffected. The incorporation of thymidine into the bulk nuclear fraction of the cells was unaffected by the presence of EBr compared to the untreated control. Ethidium bromide, therefore, suppresses the labeling of mDNA, but not nuclear DNA.

The dose response to ethidium bromide is illustrated in Fig. 2. Here cells were labeled with thymidine-^{3}H in the presence of various concentrations of EBr. The sucrose gradient analysis of detergent-produced cytoplasmic particles reveals a profile of mDNA-containing structures qualitatively similar to that found in the untreated control, except that the extent of thymidine incorporation declines with increasing drug dose. It may be argued that EBr induces aggregation of mDNA-containing particles, so that they do not sediment in the usual fashion. However, analysis of the total cytoplasmic extract from cells labeled in the presence of 1 μg/ml EBr on CsCl/EBr gradients reveals no thymidine-labeled component distinguishable from the usual nuclear DNA contaminant.

The total radioactivity in fractions 1–16 of the gradients shown in Fig. 2 indicates that the extent of labeling of mDNA relative to the untreated control was 59%, 28% and 3% in the presence

[2] Leibowitz, R. D. 1971. Manuscript submitted.

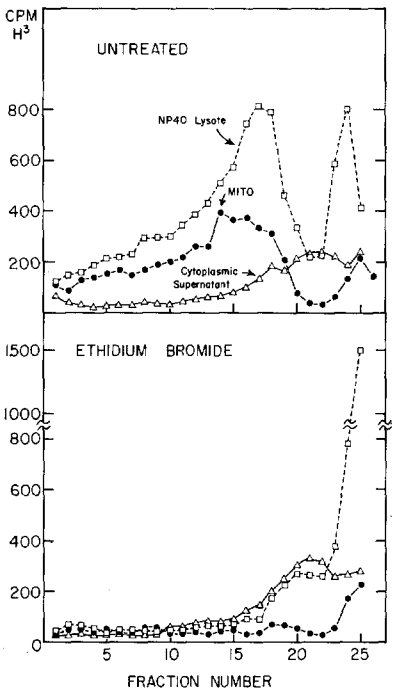

FIGURE 1 Effect of ethidium bromide on the distribution of labeled DNA in cytoplasmic extracts. A 50 ml culture (8×10^5 cells/ml) was divided into two equal parts. One half was untreated; the other half was incubated with 1 μg/ml EBr. After 5 min, 100 μCi TdR-^{3}H was added to each, and the incubation was continued for 45 min. Each culture was divided into two equal parts, and the cytoplasm was extracted either by lysis with NP40, or by homogenization. The extracts prepared by homogenization were further fractionated into crude mitochondria and postmitochondrial supernatant. NP40 was added to all samples, which were then centrifuged through 11 ml sucrose gradients (SW40, 40 krpm, 60 min, 5°C). Fractions were assayed for TCA-precipitable radioactivity on nitrocellulose filters. Total NP40 cytoplasmic extract (—□-□—); mitochondrial fraction (—●-●—); postmitochondrial supernatant (—△-△—).

of 0.04, 0.1, and 1.0 μg/ml EBr, respectively. The corresponding values for nuclear incorporation were 96%, 105%, and 112%.

In addition to the depression in the apparent rate of synthesis, the mDNA synthesized in the presence of EBr is aberrant in one of its properties, namely, the extent of dye binding and the consequent buoyant density in CsCl in the presence of EBr. When mDNA labeled with thymidine-^{3}H in the presence of 0.04 μg/ml EBr is recovered from a sucrose gradient (Fig. 2) and analyzed further by CsCl density gradient centrifugation in the presence of ethidium bromide (300 μg/ml), the result shown in Fig. 3 is obtained. In this case, the tritium-labeled sample was mixed with samples of ^{14}C-labeled nuclear DNA and ^{14}C-labeled native closed circular mDNA (see Methods and Materials). The mixture was then centrifuged to equilibrium in CsCl containing EBr (CsCl/EBr). As shown in Fig. 3, the mDNA synthesized in the presence of partially inhibitory levels of EBr bands at a density intermediate between that of native closed circular mDNA and that of (linear) nuclear DNA. Moreover, the newly synthesized mDNA is quite heterogeneous in its density under these conditions.

A similar alteration of the properties of preexisting closed circular mDNA is exerted by treatment of cells with higher levels of EBr. In the experiment illustrated in Fig. 4, cells were labeled with thymidine-^{3}H and the culture was divided into three equal parts. One sample was chilled without further treatment. Ethidium bromide, 1 μg/ml, was added to the second. The third was incubated 5 min with 3 mM unlabeled thymidine,

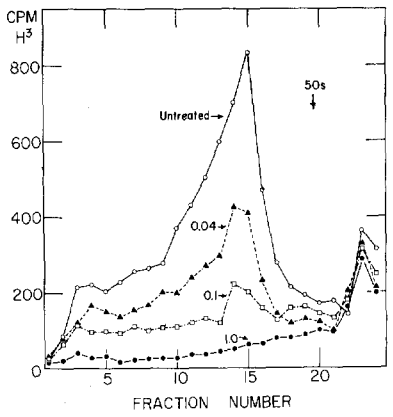

FIGURE 2 Inhibition of mDNA synthesis by ethidium bromide. A 100 ml culture (5×10^5 cells/ml) was divided into four equal parts. One sample was untreated, and EBr was added to the other three to final concentrations of 0.04, 0.1, and 1.0 μg/ml, respectively. After 30 min, 100 μCi TdR-^{3}H was added to each, and the incubation was continued for 2 hr. Cytoplasmic extracts were prepared from each sample by lysis with NP40, and were centrifuged through 11 ml sucrose gradients (SW40 rotor, 40 krpm, 90 min, 5°C). 200 μl samples of each fraction were removed and assayed for TCA-precipitable radioactivity on paper discs. Control (—○-○—); 0.04 μg/ml (—▲-▲—); 0.1 μg/ml (—□-□—); 1.0 μg/ml (—●-●—).

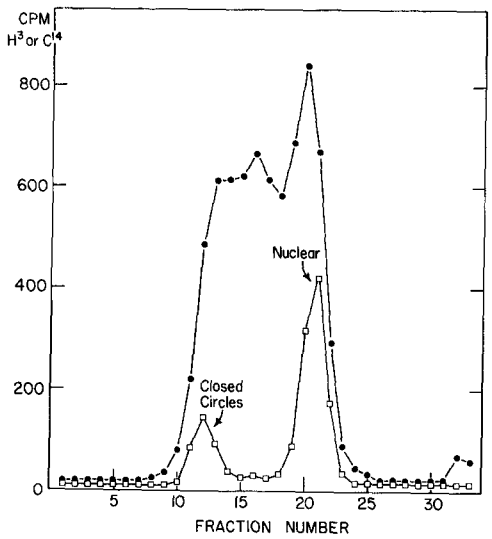

FIGURE 3 Buoyant density in CsCl/EBr of mDNA labeled in the presence of low doses of ethidium bromide. A 20 ml culture (5×10^5 cells/ml) was incubated with 0.04 μg/ml EBr for 30 min, 125μCi TdR-^{3}H was added, and the incubation was continued for 3 hr. A cytoplasmic extract was prepared by lysis with NP40, and centrifuged through a sucrose gradient as in Fig. 2. Fractions 2–16 were pooled, and the radioactivity was collected by centrifugation in the No. 40 rotor for 90 min at 40 krpm (5°C). The pelleted material was dissolved in TE buffer containing 1% Sarkosyl, and mixed with samples of ^{14}C-labeled closed circular mDNA and nuclear DNA, and centrifuged in 2 ml CsCl/EBr (SW50.1 rotor, 33 krpm, 27 hr). Fractions were collected and assayed for TCA-precipitable radioactivity on paper discs. H^3 counts/min. (—●-●—); C^{14} counts/min. (—□-□—).

followed by the addition of EBr to 1 μg/ml. Both EBr-treated cultures were incubated for an additional 3 hr. The CsCl/EBr gradient profiles of mitochondrial extracts of each are shown in Fig. 4. ^{14}C-labeled nuclear DNA was included as a density marker in each gradient. The arrows indicate the fraction of the gradient corresponding to the density of native closed circular mDNA. The direct analysis of mitochondrial material in CsCl without a preliminary purification by sucrose gradient centrifugation results in a considerable contamination by nuclear DNA. Nevertheless, it is clear that exposure of the cells to EBr at a concentration sufficient to almost completely suppress the labeling of mDNA, leads to conversion of preexisting mDNA to a new form of intermediate density in CsCl/EBr gradients. This conversion is not prevented by the general inhibition of DNA synthesis exerted by high concentrations of thymidine.

Further characterization of this DNA in CsCl/EBr gradients containing appropriate density markers indicates that the newly appearing mDNA form bands at a density very nearly the average of linear and closed circular forms (Fig. 5, bottom). It is also much more homogeneous in its density than the mDNA synthesized in the presence of low concentrations of EBr. If this mDNA form is isolated by banding in a CsCl/EBr gradient, freed of the dye, and rebanded in CsCl along with a density marker, it is found to have the density expected of mDNA. Finally, the loss of native mDNA after EBr treatment of cells is not due to leakage of the mDNA from the mitochondrion. This was shown by analysis of the total cytoplasmic extract from cells treated with EBr as described in Fig. 4, which was found to contain DNA with the buoyant density of mDNA only in the "altered" form shown in Fig. 4 (my unpublished observations).

The characteristic density of native closed circular mDNA in CsCl/EBr is determined by its base composition and its degree of supercoiling, as well as by the limited capacity to bind the dye that is common to all closed circular DNA. A change in the buoyant density of a closed circular molecule in CsCl/EBr gradients, without a change in density in the absence of the dye, indicates that the molecule has undergone an alteration in its superhelix density, i.e., the number of superhelical turns per unit length of DNA (8). However, there are several other possible explanations for the unusual density in CsCl/EBr gradients of mDNA from the drug-treated cells. Among these are: (*a*) The mDNA has become tightly bound to some other substance and the complex has the observed average density; (*b*) The mDNA is partly single stranded. An experiment which shows that the drug-induced mDNA forms contain closed circular components, and which provides evidence against the other two explanations, is shown in Fig. 5.

In this experiment, each of the new mDNA forms, labeled with thymidine-^{3}H, was isolated from CsCl/EBr gradients similar to those described in Figs. 3 and 4, respectively. After removal of the dye, each was mixed with ^{14}C-labeled native closed circular mDNA, and incubated with a very low concentration of pancreatic DNase. After incubation for the times shown, the products were analyzed on CsCl/EBr gradients. It is clear that very low levels of phosphodiester cleavage convert

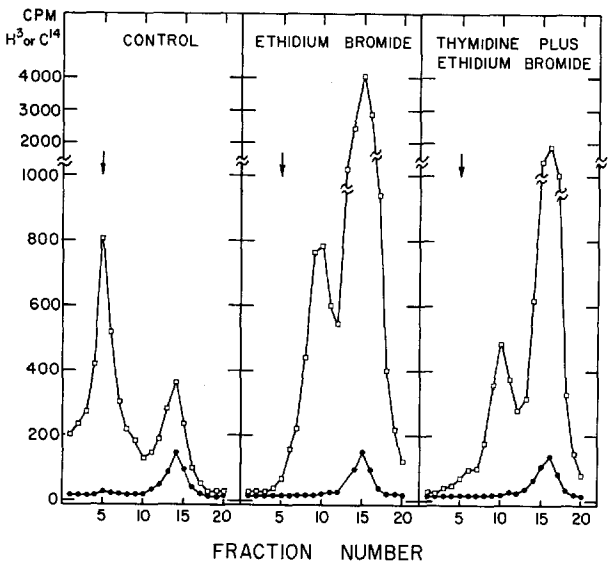

FIGURE 4 Conversion of intracellular closed circular mDNA to a form with altered density on CsCl/EBr gradients. A 150 ml culture (4×10^5 cells/ml) was incubated with 375 μCi TdR-^{3}H for 75 min. A sample of 50 ml was chilled without further treatment. EBr was added to a second 50 ml sample, to a final concentration of 1 μg/ml. Unlabeled thymidine (3 mM) was added to a third 50 ml sample, and then EBr (1 μg/ml) was added after 5 min. Both EBr-treated cultures were incubated for 3 hr. Each sample was homogenized and a crude mitochondrial fraction prepared (Methods and Materials). The mitochondrial fractions were lysed in TE with NP40, and centrifuged at 10 krpm for 10 min to pellet part of the contaminating nuclear DNA. The supernatants were decanted, made 1% in Sarkosyl, mixed with samples of ^{14}C-labeled nuclear DNA, and added to CsCl/EBr in a final volume of 7 ml. After centrifugation in the No. 50 rotor at 40 krpm for 39 hr, fractions were collected and samples were assayed for TCA-precipitable radioactivity on paper discs. The topmost fractions are not shown. The arrows indicate the position of native closed circular mDNA, determined by index of refraction measurements. ^{3}H counts/min (—□-□—); C^{14} counts/min (—●-●—).

the unusual mDNA forms to a form with the buoyant density characteristic of linear and open circular DNA. In the case of mDNA from cells exposed to the high level of EBr (Fig. 5, bottom), the conversion occurs with the same efficiency as that observed for the native mDNA, suggesting that one single-strand break per molecule is sufficient to bring about the change in buoyant density. The first of the alternative hypotheses given above is rendered unlikely by the finding that the product of this limited digestion has the buoyant density identical to that of nicked mDNA derived from native circles. The ^{14}C-labeled mDNA also provides a reliable recovery marker. Since no preferential loss of tritium-labeled DNA is observed (±5%), the excision of single-stranded DNA by the enzyme cannot account for the density shift. Hence the second explanation is also unlikely. This mDNA form, then, appears to consist of closed circular molecules with increased superhelix density.

The mDNA synthesized in the presence of the low concentration of EBr is similarly affected by DNase, with respect to its density in these gradients, but is apparently more resistant to the enzyme (Fig. 5, top). Again the parallel recoveries of the two isotopes argues against the loss of single-stranded DNA as the basis for the enzymatic conversion. The tritiated "nicked" form also has the same density as that derived from the marker, although this is somewhat obscured by the limited conversion. Hence, the qualitative aspects of the effect of DNase suggest the presence of covalently closed circles in the drug-induced mDNA form. However, an increase in superhelix density does not fully explain the observation that approximately 50% of the tritium-labeled DNA is not converted to the fully light form after

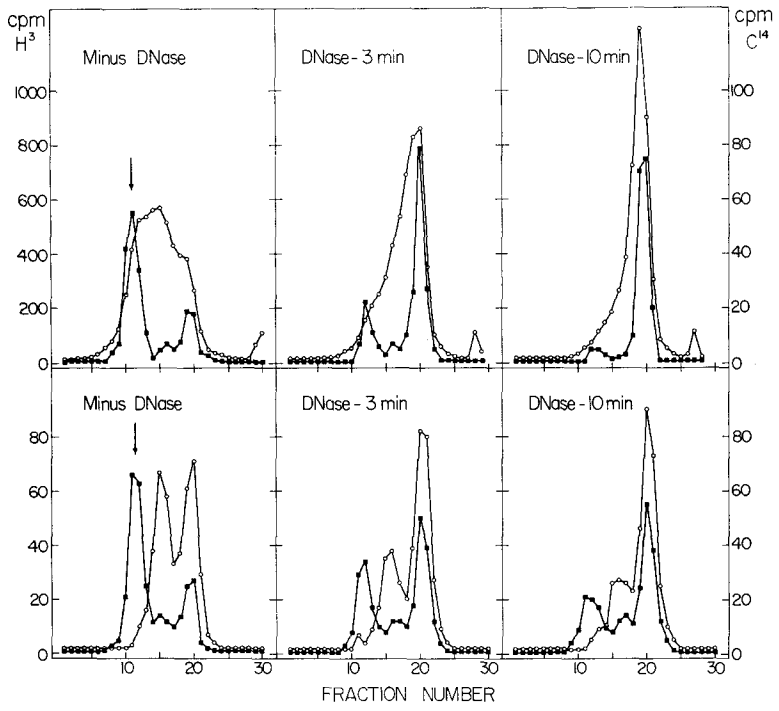

FIGURE 5 Effect of DNase on the buoyant density of the unusual mDNA forms in CsCl/EBr. mDNA was prepared from cells treated as described in Figs. 3 and 4, and recovered from preparative CsCl/EBr gradients by ethanol precipitation of all material denser than the ^{14}C-labeled nuclear DNA marker. Top, mDNA from cells labeled with TdR-^{3}H in the presence of 0.04 μg/ml EBr. Bottom, mDNA from cells labeled with TdR-^{3}H then treated with 1 μg/ml EBr. Each ^{3}H-labeled sample was mixed with TdR-^{14}C labeled mDNA prepared from untreated cells, in a final volume of 0.60 ml of 0.02 M Tris, pH 8.0, 0.005 M $MgCl_2$. 200 μl samples of each were removed and pancreatic DNase (electrophoretically purified) was added to the remaining solutions to a final concentration of 5×10^{-4} μg/ml. The DNA was digested at 37°C and 200 μl samples were removed after 3 min and 10 min, respectively. The reaction was stopped by adding each sample to a solution of CsCl prepared to give a final concentration of 300 μg/ml EBr, 0.005 M EDTA, and 0.15% Sarkosyl in a final volume of 2.0 ml. The samples were centrifuged to equilibrium and fractions were assayed for TCA-precipitable radioactivity on paper discs. H^3 counts/min (—○-○—); C^{14} counts/min (—■-■—).

90–95% of the native circles are converted. The results in Fig. 5 (bottom) indicate that an increase in superhelix density does not make the mDNA a poorer substrate for the enzyme. Possible explanations will be considered in the Discussion.

DISCUSSION

The results reported here extend the known range of effects of ethidium bromide on mitochondrial macromolecular metabolism. In particular, mDNA synthesis in HeLa cells is rapidly inhibited by the drug. A striking aspect of this effect is the similarity of the levels of inhibition at each drug dose to that previously reported for the inhibition of mitochondrial RNA synthesis (6). This similarity of dose responses suggests a common molecular basis, which is at present unknown. Ethidium bromide also inhibits mitochondrial protein synthesis (4, 5). Experiments on cellular DNA polymerases in vitro (9) have suggested that the mitochondrial enzyme is strongly inhibited by EBr, whereas the nuclear enzyme is relatively insensitive. This selectivity was shown using linear DNA template, and does not require invocation of the unique aspects of the interaction of closed circular DNA with the dye. No study has yet appeared that would indicate whether EBr can exert a similar selective effect on other mitochondrial polymerases. Hence, it is not yet known whether the pleiotropic action of the drug is due to the inhibition of several

mitochondrial enzymes, to an alteration of mitochondrial structure, or to some other effect.

The inhibition of mDNA synthesis by EBr in yeast has been shown to be accompanied by the degradation of mDNA (3). This is not the case in HeLa cells, at least over the course of several hours. Preliminary results indicate that HeLa cells can survive brief exposure to EBr (1 μg/ml for 3 hr), recovering their normal growth rate after a lag period following removal of the drug. The fate of mDNA after such treatment has not been investigated.

However, after exposure to EBr, mDNA in HeLa cells is altered in at least one respect, its buoyant density in CsCl/EBr gradients. The present data indicate that in the case of high levels of EBr, this is due to an increase in the superhelix density of the mDNA. Such a change is consistent with the expected effect of ring closure in the presence of an intercalating dye like EBr, since binding of the dye results in underwinding of the DNA duplex. If this is indeed the basis of the drug action, it is interesting that the effect occurs rapidly in the apparent absence of mDNA synthesis.

The explanation in the case of low concentrations of EBr is less clear. While the effect of DNase suggests that the mDNA synthesized in the presence of the drug contains closed circular DNA, the observed resistance to the enzyme suggests that more than one nick per molecule is required to produce the expected change in density. A possible explanation, consistent with the results reported here, might be the stimulation of catenane formation. An increase in the proportion of higher oligomers of mDNA due to various drug treatments has been previously reported (10). However, no direct evidence for such an effect exists in the case of EBr, and the clarification of the action of ethidium must await further study of the drug-induced mDNA forms.

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