Feinman, R. D., & Li, F. H. H. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 51.
Feinman, R. D., Chang, T. L., Wilson, S. M., & Li, E. H. H. (1977) in *Chemistry and Biology of Thrombin* (Lundblad, R. L., et al., Eds.) p 217, Ann Arbor Science, Ann Arbor, Mich.
Fenton, J. W., II, & Fasco, M. J. (1974) *Thromb. Res. 4*, 809.
Fenton, J. W., II, Fasco, M. J., Stackrow, A. B., Aronson, D. L., Young, A. M., & Finalyson, J. S. (1977a) *J. Biol. Chem. 252*, 3587.
Fenton, J. W., II, Landis, B. H., Walz, D. A., Finalyson, J. S. (1977b) in *Chemistry and Biology of Thrombin* (Lundblad, R. L., et al., Eds.) Ann Arbor Science, Ann Arbor, Mich.
Harpel, P. C., & Rosenberg, R. D. (1976) *Prog. Hemostasis Thromb. 3*, 145.
Kingdon, H. S., Noyes, C. M., & Lundblad, R. L. (1977) in *Chemistry and Biology of Thrombin* (Lundblad, R. L., et al., Eds.) p 91, Ann Arbor Science, Ann Arbor, Mich.
Koehler, K. A., & Magnusson, S. (1974) *Arch. Biochem. Biophys. 160*, 175.
Landis, B. H., Zabinski, M. P., Lafleur, G. J. M., Bing, D. H., & Fenton, J. W., II, (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1445.
Li, E. H. H., Orton, C., & Feinman, R. D. (1974) *Biochemistry 13*, 5012.
Li, E. H. H., Fenton, J. W., II, & Feinman, R. D. (1976) *Arch. Biochem. Biphys. 175*, 153.
Lorand, L., & Credo, R. B. (1977) in *Chemistry and Biology of Thrombin* (Lundbald, R. L., et al., Eds.) p 311, Ann Arbor Science, Ann Arbor, Mich.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Machovich, R., Blasko, G., & Borsodi, A. (1976) *Thromb. Haemostas. (Stuttgart) 36*, 503.
Machovich, R., Blasko, G., & Palos, L. O. (1975) *Biochim. Biophys. Acta 379*, 193.
Machovich, R., Borsodi, A., Blasko, G., & Orakzai, S. (1977) *Biochem. J. 167*, 393.
Magnusson, S., Petersen, T. E., Sottrup-Jensen, L., & Claeys, H. (1975) in *Proteases and Biological Control* (Reich, H., Rifkin, D. B., & Shaw, E., Eds.) p 123, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
Martin, B. M., Wasiewski, W. W., Fenton, J. W., II, & Detwiler T. C. (1976) *Biochemistry 15*, 4886.
Owen, W. G. (1975) *Biochim. Biophys. Acta 405*, 380.
Owen, W. G. (1977) *Biochim. Biophys. Acta 494*, 182.
Roden, L., Baker, J. R., Cifonelli, J. A., & Mathews, M. B. (1972) *Methods Enzymol. 28*, 73.
Rosenberg, R. D., & Damus, P. S. (1973) *J. Biol. Chem. 248*, 6490.
Scheraga, H. A. (1977) in *Chemistry and Biology of Thrombin* (Lundbald, R. L., et al., Eds.) p 145, Ann Arbor Science, Ann Arbor, Mich.
Sturzebecher, J., and Markwardt, F. (1977) *Thromb. Res. 11*, 835.
Thaler, E., & Schmer, G. (1975) *Br. J. Haematol. 31*, 233.
Thompson, O. A. Enfield, D. L., Ericsson, L. H., Legaz, M. E., & Fenton, J. W., II (1977) *Arch. Biochem. Biophys. 178*, 356.
Zetter, B. R., Chen, L. B., & Buchanan, P. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 596.

Yeast Mitochrondrial Deoxyribonuclease Stimulated by Ethidium Bromide. 1. Purification and Properties†

Hélène Jacquemin-Sablon,* Alain Jacquemin-Sablon, and Claude Paoletti

ABSTRACT: A deoxyribonuclease (EtdBr DNase), which is about 25 times more active on double-stranded DNA, in which EtdBr is intercalated has been purified from yeast isolated mitochondria. This enzyme appears to be located in the mitochondrial membrane. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the purified fraction yielded six major bands whereas, in native conditions, it migrates as a single band. In addition to the EtdBr DNase, this fraction contains two other DNase activities, both of which are inhibited by EtdBr at the same concentration which is used to measure the EtdBr DNase activity. One is detected on double-stranded DNA at acid pH and the other on single-stranded DNA at neutral pH. The three activities cosediment in a sucrose gradient and have similar rates of heat inactivation. The level of stimulation of the EtdBr DNase depends on the amount of intercalated EtdBr per nucleotide, the maximal activity being reached when all the intercalated sites are occupied. More detailed studies on the mechanism of this stimulation are described in the accompanying paper (Jacquemin-Sablon, H., et al. (1979) *Biochemistry 18* (following paper in this issue)). The enzyme, which cuts one strand at a time in a covalently closed circular PM2 DNA molecule, is classified as an endonuclease. Possible involvement of the EtdBr DNase in the process of "petite" induction by EtdBr was investigated by measuring the enzyme level in two mutants resistant to EtdBr mutagenesis. This enzyme was present at a normal level in both strains.

The DNA intercalating drug ethidium bromide[1] displays biological properties in several organisms. Smith et al. (1971) reported that, in mammalian cells, it rapidly inhibits mitochondrial DNA synthesis and induces a change in its superhelix density. However, there was no significant nicking or degradation of this DNA (Smith, 1977). Similar effects have been observed in the kinetoplastic DNA of trypanosome (Riou & Delain, 1969; J. Benard, G. Riou, & J. M. Saucier,

† From the Unité de Biochimie et Enzymologie, Institut Gustave-Roussy, 94800 Villejuif, France. *Received May 5, 1978*. Financial support was provided by the Centre National de la Recherche Scientifique (L.A. 147), the Institut National de la Santé et de la Recherche Médicale (U 140), and the Commissariat à l'Energie Atomique.

[1] Abbreviations used: EtdBr, ethidium bromide; mtDNA, mitochondrial DNA; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin.

0006-2960/79/0418-0119$01.00/0

to be published). EtdBr is also able to cure bacterial plasmids (Bouanchaud et al., 1969). Recently, it has been shown to be a frame-shift mutagen in the *Salmonella* test system (Mc Cann et al., 1975).

The most striking effect of EtdBr is its ability to induce cytoplasmic petite mutation in yeast (Slonimski et al., 1968), either in growing or nongrowing cells. In growing cells, one of the earliest detectable events is a blockage of synthesis and transcription of mtDNA which is rapidly degraded (Fukuhara & Kujawa, 1970; Goldring et al., 1970; Perlman & Mahler, 1971). It has been shown that mtDNA is first cut into large fragments. The initial step is followed, in growing conditions, by a further degradation of these fragments into acid-soluble oligonucleotides. This second step, which is ATP dependent, does not occur in nongrowing conditions (Bastos & Mahler, 1974; Hall et al., 1976).

The initial scission of mtDNA, as well as its subsequent degradation, suggests the existence of one or several nucleases specifically active on the EtdBr–DNA complex. We previously described the identification of a mitochondrial deoxyribonuclease which was more active on DNA in which EtdBr is intercalated (Paoletti et al., 1972). In this paper we report the further purification and characterization of this enzyme, which we designate as the EtdBr DNase.

Materials and Methods

Materials

Yeast Strains. The EtdBr DNase has been purified from *Saccharomyces cerevisiae* IL-8-8C (α, *his*, *trp*, ω^+, $C_{514}{}^R$) obtained from Dr. P. P. Slonimski. *S. cerevisiae* 1945 C (*a*, *ade 6*, *his* 4, *ur* 1), a mutant resistant to the induction of "petite" mutation by EtdBr (B^R), and its parental strain, 14/I (B^S), were obtained from Dr. J. C. Mounoulou. Strains N 123-5 (α, *his* 1, *uvsρ* 5) and N 123-72 (α, *his* 1, *uvsρ* 72) and their parental strain were kindly provided by Dr. E. Moustacchi.

Bacteria and Phages. Bacteriophage T7 and its host *Escherichia coli* BB were a gift from Dr. R. Hausman. Bacteriophage ϕ X 174 was grown on *E. coli* H 502 (*F*$^-$, *endo* I, *hcr*, *uvr* A, *thy*) obtained from Dr. N. Truffaut. Bacteriophage PM2 and its host *Pseudomonas* BAL 31 were a gift from Dr. Espejo.

Media. The yeast growth medium contained, per liter, 10 g of bactopeptone (Difco), 10 g of yeast extract (Difco) and 20 g of galactose.

Nucleic Acids. Tritium-labeled T7 and ϕ X 174 DNAs were prepared respectively as described by Richardson (1966) and Francke & Ray (1971). PM2 DNA was prepared according to Espejo et al. (1969) and Le Pecq (1971). ^{32}P-labeled mtDNA was extracted from *S. cerevisiae* according to a procedure developed by A. Di Franco (to be published).

Other Materials. Ethidium bromide was purchased from Boots Pure Drug Co.; bovine serum albumin (fraction V) was from Pentex; Triton X-100 was from Calbiochem; fluorescamine was from Roche; phenylmethanesulfonyl fluoride was from Sigma. Lyophylized helicase was provided by Industrie Biologique Française. DEAE-cellulose (Cellex-D) and Biobeads SM2 were obtained from Bio-Rad, carboxymethylcellulose (CM 52) was from Whatman, Agarose (Seakem) was from Biomedical, and Sephadex G25 was from Pharmacia. DNA–agarose columns were prepared with Seakem agarose and calf thymus DNA, according to Schaller et al. (1972).

Methods

Standard Assay for the EtdBr DNase. This assay measures the release of acid-soluble products from T7 [^{3}H]DNA, in the presence of EtdBr. The standard reaction mixture (0.3 mL) contains 20 μmol of Tris-HCl, pH 7.7, 3 μmol of $MgCl_2$, 0.1 mg of BSA, 0.3 nmol of T7 [^{3}H]DNA, 6.9 nmol of EtdBr. The reaction was started by adding 0.01 mL of enzyme solution. The incubation was carried out at 28 °C for 15 min, and the reaction was stopped by the addition of 0.2 mL of a BSA solution (3 mg/mL) and 0.5 mL of 10% trichloroacetic acid. After 5 min at 0 °C, the mixture was centrifuged for 10 min, at 8000*g*. The acid-soluble radioactivity was determined in a 0.5-mL aliquot of the supernatant, counted in 10 mL of Bray scintillation fluid. Whenever necessary, the enzyme was diluted with 0.01 M Tris-HCl buffer, pH 7.7, containing 0.1% (w/v) Triton X-100. One unit of enzyme converts 1 nmol of DNA nucleotide to acid-soluble material in 30 min at 28 °C.

Other DNase Activities. As discussed in the Results section, two additional nuclease activities can be detected in the purified EtdBr DNase. One is active on double-stranded DNA, at pH 5.6 in absence of EtdBr, and is measured in the standard assay conditions, except that Tris-HCl buffer was replaced by 3 μmol of acetate buffer, pH 5.6. The other is active on single-stranded DNA and is measured in a reaction mixture (0.3 mL) containing 20 μmol of Tris–maleate, pH 7.0, 3 μmol of $MgCl_2$, 0.1 mg of BSA, and 0.15 mol of ϕ X 174 [^{3}H]DNA. The determination of the acid-soluble products was carried out as described in the standard assay.

Other Enzyme Assays. Alkaline phosphatase was assayed as described by Garen & Levinthal (1960) and phosphodiesterase according to Koerner & Sinsheimer (1965). RNase activity was assayed on poly(cytidylic acid) according to Zimmerman & Sandeen (1965). ATPase activity was assayed as described by Goffeau et al. (1974). Cytochrome oxidase was determined according to Yonetani & Ray (1965).

Protein Determinations. Proteins were assayed by the fluorometric method of Böhlen et al. (1973). This method allows for the determination of protein concentrations in the presence of Triton X-100, without any interference from the detergent in the assay (Simms & Carnegie, 1975).

Removal of Triton X-100. A column containing 5 g of moist SM2 Biobeads, prepared as described by Holloway (1973), was equilibrated with 0.01 M Tris-HCl buffer, pH 7.4, at 4 °C. A 2-mL sample (fraction I) was layered on the top of the column and washed with 0.01 M Tris-HCl buffer, pH 7.4. EtdBr DNase activity was assayed in the standard conditions, and detergent concentration was estimated from its absorbance at 275 nm ($E_{1cm}{}^{1\%}$ in 0.01 M Tris-HCl buffer = 21.0).

Iodination with ^{125}I. EtdBr DNase (fraction IV) was reacted with ^{125}I (4 mCi/mL of reaction mixture of Na^{125}I, New England Nuclear), in denaturing conditions, using Chloramine-T as oxidative agent, as described by Gibson (1974). For iodination in native conditions, urea and $NaDodSO_4$ were omitted, and 0.1% Triton X-100 was present throughout the procedure. In both cases, the labeling was carried out in the presence of 1 mM $PhCH_2SO_2F$, in order to prevent a possible proteolysis (Pringle, 1975). The mixture was then passed through a Sephadex G-25 column, equilibrated with 0.05 M Tris-HCl buffer, pH 7.5. The iodinated proteins eluted at the void volume separated from the excess of unbound ^{125}I.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. After heating, for 3 min, at 100 °C in the presence of 1% $NaDodSO_4$, 4 M urea, the denatured iodinated proteins were analyzed by electrophoresis on a 12.5% polyacrylamide–$NaDodSO_4$ slab gel (Studier, 1973). Electrophoresis was carried out with a voltage of 90 V for 150 min. Gels were

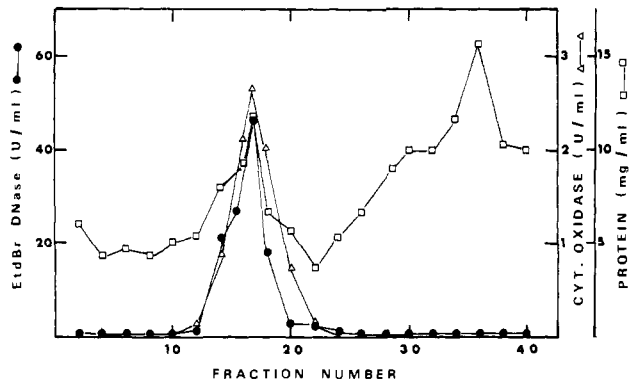

FIGURE 1: Subcellular localization of EtdBr DNase. A yeast homogenate (10 mL) was layered onto a 30–70% sucrose gradient and centrifuged at 15 °C for 10 h at 24 000 rpm, in a SW 25.2 rotor. Fractions (1.5 mL) were collected from the bottom of the tube. After determination of the protein concentration, Triton X-100 was added to each fraction at a final concentration of 2% (w/v). Cytochrome oxidase and EtdBr DNase activities were then measured after appropriate dilution. (□—□) Proteins; (●—●) EtdBr DNase; (△—△) cytochrome oxidase.

stained in 0.25% Coomassie blue for 25 min and then destained in methanol:water:acetic acid (5:5:1) for 1 h and in 7.5% acetic acid, 5% methanol for 15 h at room temperature. ^{125}I was detected by scintillation autoradiography (Bonner & Laskey, 1974) using a Kodak RPRO film for radioautography. After 15-h exposure at −70 °C, the film was scanned at 600 nm, in a Joyce Loebl microdensitometer. Molecular weights were determined from a standard plot using BSA (68 000), ovalbumin (43 000), and α-chymotrypsinogen (26 000) as standard proteins.

Polyacrylamide Gel Electrophoresis of Native Proteins. Proteins, iodinated in nondenaturing conditions, were analyzed by electrophoresis on 5% polyacrylamide disc gels in the presence of 0.1% Triton X-100 (Dewald et al., 1974). The current was 3 mA per tube for 6 h. The gels were longitudinally sliced and treated for scintillation autoradiography as described above.

Results

Subcellular Localization of the Enzyme

The mitochondrial localization of the EtdBr DNase was ascertained by cell fractionation experiments. Subcellular components from homogenized yeast spheroplasts, prepared as described below, were separated by sedimentation in sucrose density gradients. The total recovery of enzyme activity cannot be evaluated, since the enzyme is poorly detected in the cell homogenate. However, as shown in Figure 1, the distribution pattern of the EtdBr DNase activity is identical with the pattern of the mitochondrial enzyme cytochrome oxidase. No EtdBr DNase activity could be detected in other nuclear or cytoplasmic fractions. An extensive washing of the isolated mitochondria, which completely eliminates the D-lactate dehydrogenase, a cytoplasmic enzyme adsorbed on the external surface of the mitochondria, did not change the EtdBr DNase activity. Therefore, the purification was pursued from isolated mitochondria.

Purification of the Enzyme

During the purification procedure, nuclease activity is determined using the acid-soluble assay in the presence of EtdBr, as described in Materials and Methods. The results of a typical purification are given in Table I.

Growth of Yeast. Six liters of medium was inoculated with 40 mL of a *Saccharomyces cerevisiae* IL-8-8C stationary phase preculture. The culture was grown at 28 °C with aeration. At mid-exponential phase (OD_{600nm} = 15), the cells were harvested by centrifugation for 10 min at 4000*g*. The pellet was resuspended in distilled water and centrifuged again in the same conditions.

Table I: Purification of the EtdBr DNase

fraction	act. (total units)	protein (mg/mL)	sp act. (units/mg of protein)	yield (%)
I, mitochondrial extract	20 200	10.6	120	100
II, DEAE-cellulose	11 040	1.03	368	56
III, CM-cellulose	8 200	0.04	5175	40
IV, DNA-agarose	6 700	<0.01	ND[a]	32

[a] ND, not determined.

Preparation of Mitochondria. The cells, suspended in 100 mL of a solution containing 0.3 M β-mercaptoethanol, 0.25 M EDTA, pH 7, were incubated for 30 min at 28 °C. They were then centrifuged at 4000*g* and washed twice with 200 mL of buffer S (1.2 M sorbitol, 0.05 M citric acid, 0.15 M potassium phosphate, pH 5.8). The cells were resuspended in 100 mL of helicase solution (1 × 10^6 U β-glucuronidase, 15 × 10^6 U sulfatase in 100 mL of buffer S) and incubated at 28 °C. Spheroplasts formation was monitored by the decrease of the turbidity of the cells suspended in water, measured by adsorption at 600 nm. The spheroplasts were collected by centrifugation at 2000*g* and washed twice with buffer S. They were then suspended in 200 mL of sorbitol buffer (0.9 M sorbitol, 0.1% BSA, 0.15 M Tris-HCl, pH 7.4) and homogenized for 1 min in the cold with a Waring Blendor homogenizer. All subsequent steps were carried out at 4 °C. The homogenate was centrifuged twice at 2500*g* for 10 min to remove unbroken cells and debris. The supernatant solution was centrifuged at 27 000*g* for 20 min, and the pellet was resuspended in 200 mL of 0.9 M sorbitol, 0.01 M Tris-HCl, pH 7.5. The mitochondria were pelleted down and resuspended twice, and finally suspended in 10 mL of 0.6 M sorbitol, 0.15% BSA, 1 mM EDTA, 0.05 M Tris-HCl, pH 7.4.

Solubilization of the Enzyme. After ultrasonic treatment of the mitochondria, there was no detectable activity in the soluble fraction. The solubilization of the enzyme requires the dissociation of phospholipids from proteins, obtained by combination of high salt concentration (1 M KCl) and Triton X-100 (1.5 mg per mg of mitochondrial proteins). The mixture was kept at 0 °C for 2 h and then centrifuged at 105 000*g* for 30 min. The supernatant, which contains 98% of the lysate activity, was dialyzed overnight against buffer A (0.1% Triton X-100, 1 mM β-mercaptoethanol, 0.01 M Tris-HCl, pH 7.4) to remove KCl, which inhibits the EtdBr DNase (fraction I).

DEAE-Cellulose Chromatography. Fraction I, containing 117 mg of proteins, was applied to a column of DEAE-cellulose (15 cm × 2.5 cm^2) previously equilibrated with buffer A. The column was washed with buffer A, and the enzymatic activity appeared in a single peak, just after the void volume of the column. The active fractions, about 20 mL, were pooled (fraction II).

Carboxymethylcellulose Chromatography. Fraction II was applied to a column of carboxymethylcellulose (10 cm × 2 cm^2) previously equilibrated with buffer A. The column was washed with 20 mL of buffer A. Then a linear gradient (400 mL total volume) from 0 to 0.3 M NaCl in buffer A was applied. The active fractions (0.05–0.06 M NaCl) were pooled (fraction III) and dialyzed 3 h against buffer A containing 25 mM NaCl.

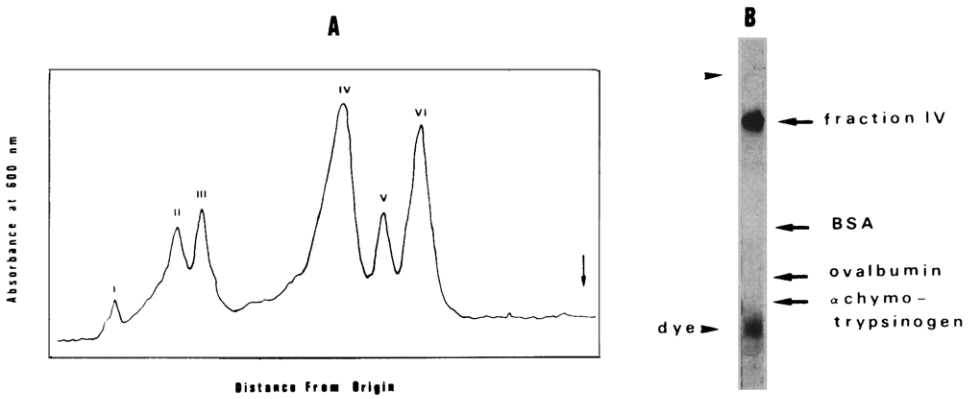

FIGURE 2: Analysis of ^{125}I-labeled fraction IV by polyacrylamide gel electrophoresis. Labeling of the proteins, electrophoresis, autoradiography, and scanning were carried out as described in Materials and Methods. (A) Electrophoresis in 12.5% polyacrylamide–$NaDodSO_4$ slab gel. In order to clarify the presentation of the results, the scanning of the upper part of the autoradiogram, which did not display any band, is not shown on the picture. The origin of migration would be located 4.2 cm on the left side of the graph. The arrow denotes the position of the front of migration. The roman numerals mark the major protein bands. (B) Disc electrophoresis on 5% polyacrylamide gel. Prior to autoradiography, the marker proteins were identified by staining as described in Materials and Methods, and their positions are indicated. The arrows on the left side show the origin and the front of migration.

DNA–Agarose Chromatography. Fraction III (1 mg of proteins) was applied to a column of DNA–agarose (5 cm × 0.64 cm^2), previously equilibrated with buffer A containing 25 mM NaCl. The column was washed with 10 mL of the same buffer. A linear gradient (100 mL total volume), from 0.025 to 0.5 M NaCl in buffer A, was applied. The major portion of the enzyme activity was eluted at about 0.06 M NaCl. The fractions containing the enzymatic activity (about 20 mL) were pooled and concentrated tenfold, using a dialysis bag covered with Sephadex G-50 (fraction IV). Glycerol was added at a final concentration of 50% and the enzyme was stored at −20 °C. This fraction was used as the enzyme source in the following experiments.

Effect of Triton X-100. Attempts to purify the enzyme in absence of Triton X-100 were unsuccessful. After filtration of fraction I through a column of Biobeads SM2, the detergent concentration was lowered to 0.04% without loss of activity. However, after chromatography of this fraction on DEAE-cellulose column, in absence of Triton X-100, we were unable to recover the DNase activity. Therefore the presence of the detergent is required throughout the purification procedure. We also examined the effect of Triton X-100 on the activity of the purified enzyme. When fraction IV was filtered through the Biobeads column, the detergent concentration could be lowered to 0.01%, a concentration which is very close to the critical micellar concentration (Helenius & Simons, 1975). In these conditions, 80% of the enzyme activity was irreversibly lost. This indicates that Triton X-100 is probably necessary for maintaining the enzyme in a proper physical state. In addition, as discussed in the accompanying paper (Jacquemin-Sablon et al., 1979), Triton X-100 is also involved in the mechanism of EtdBr DNase activation by EtdBr.

Stability. A great instability of the enzyme activity was observed in the course of the purification, especially after the second step (DEAE-cellulose chromatography). Overnight storage at 0 °C of fraction II resulted in the complete loss of the activity. As already shown for many other yeast proteins (Pringle, 1975), this inactivation could be due to proteolytic degradation. In order to reduce the enzyme inactivation, the following precautions were taken: (a) the growth of the cells was stopped at least one generation before stationary phase, since it is known that the level of proteases in *S. cerevisiae* is higher in this phase (Pringle, 1975); (b) the purification procedure was carried out as fast as possible to avoid the loss of activity during the storage of the enzyme fractions between the purification steps; (c) the protease inhibitor $PhCH_2SO_2F$ was added to all fractions.

Fraction IV, made 50% with glycerol, was stored at −20 °C for 6 months with less than 10% decrease of enzymatic activity.

Purity. Fraction IV was analyzed by electrophoresis on polyacrylamide gels, either in the presence of $NaDodSO_4$ or in nondenaturing conditions. Because of the low protein concentration, there was no detectable band after staining of the gels either with Coomassie blue or fluorescamine. Therefore, the experiment was carried out with ^{125}I-labeled proteins. In nondenaturing conditions, fraction IV migrates in 5% polyacrylamide gels as a single band (Figure 2). The short distance of migration suggests a high molecular weight structure. Electrophoresis of the same fraction in a 12.5% polyacrylamide–$NaDodSO_4$ slab gel yielded six major protein bands (Figure 2). According to their mobility, the following apparent molecular weights were calculated: (I) 60 000; (II) 49 000; (III) 46 000; (IV) 32 000; (V) 28 000; (VI) 26 000. According to the distribution of the label, low molecular weight subunits (bands IV, V, VI) appear to be in excess as compared with high molecular weight proteins (bands I, II, III). However, in order to interpret these results, the following problems should be taken into account: (1) only tyrosine residues are labeled by iodination, and there is no evidence that the tyrosine content is similar for all of the polypeptides; (2) as shown by Gibson (1974), exposure to Chloramine-T prior to $NaDodSO_4$ gel electrophoresis may result in the formation of oxidation products; (3) although the purification and the labeling of fraction IV were carried out in the presence of 1 mM $PhCH_2SO_2F$, a potent proteases inhibitor, one cannot exclude some partial proteolysis of high molecular weight polypeptides.

Other Enzyme Activities. After incubation of about 2 units of EtdBr DNase for 1 h, in the optimal conditions of each reaction, fraction IV was found to be free of any of the following enzyme activities: phosphatase, phosphodiesterase, RNase, and ATPase.

Other DNase Activities. In addition to the EtdBr DNase, which is active on double-stranded DNA in the presence of EtdBr, at neutral pH, fraction IV contains two other nuclease activities which are (a) a DNase activity which hydrolyzes double-stranded DNA at pH 5.6; (b) a DNase activity which hydrolyzes single-stranded DNA at neutral pH. As shown in Figure 3, at the same concentration (10 μg/mL), EtdBr inhibits the two latter activities, while it activates the EtdBr

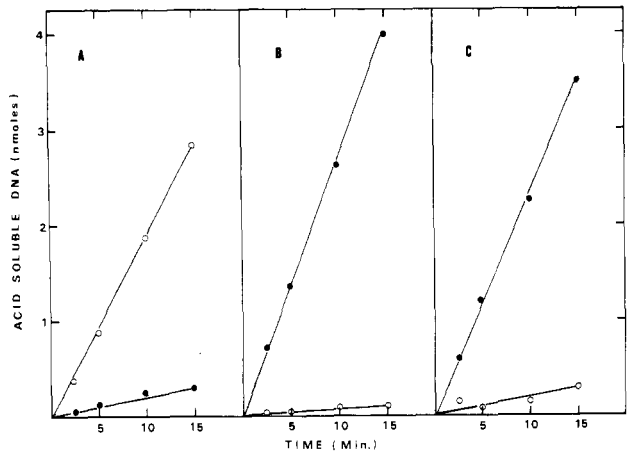

FIGURE 3: Effect of EtdBr on the different DNase activities of fraction IV. The reaction mixture (1.8 mL) contained, in the appropriate buffer (Materials and Methods), 18 μmol of $MgCl_2$, 0.6 mg of BSA, and 1.8 nmol of native or denatured ^{3}H-labeled T7 DNA. Enzyme (fraction IV) was added at the appropriate dilution. The mixture was incubated at 28 °C, and, at the indicated time intervals, a 0.3-mL aliquot was removed and acid-soluble material determined as described in Materials and Methods. (A) EtdBr DNase (2 U/mL); (B) DNase activity on double-stranded DNA, pH 5.6 (0.2 U/mL); (C) DNase activity on single-stranded DNA, pH 7.4 (0.2 U/mL); (●—●) no EtdBr; (○—○) 10 μg/mL of EtdBr.

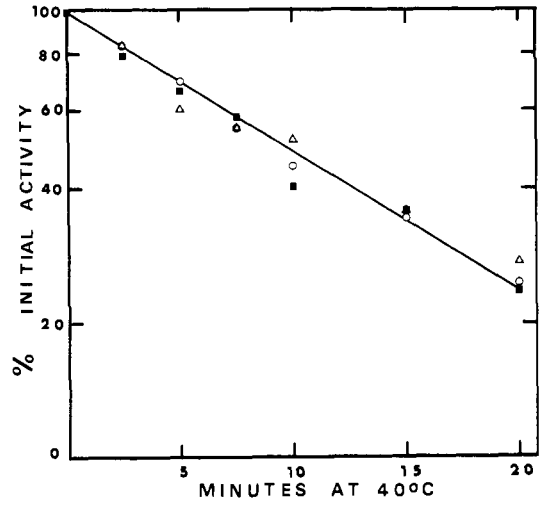

FIGURE 4: Heat inactivation of the DNase activities of fraction IV. Fraction IV diluted in 0.07 M Tris-HCl, pH 7.7, 0.01 M $MgCl_2$, 0.3 mg/mL of BSA, was incubated at 40 °C. At the indicated times, aliquots were removed and the enzyme activities were determined in the standard assay conditions (Materials and Methods). (■—■) EtdBr DNase; (○—○) DNase activity on single-stranded DNA; (△—△) DNase activity on double-stranded DNA at pH 5.6.

DNase activity. However, since there is a tenfold difference in the enzyme concentrations used in the experiments shown on panels A and C (Figure 3), one can calculate that, in the assay conditions of the EtdBr DNase, the activity toward single-stranded DNA, although about 80–90% inhibited by EtdBr, is still almost equal to the EtdBr DNase activity. Therefore one may wonder whether this activity could be involved in the EtdBr DNase activation. Although intercalation of ethidium between adjacent base pairs is known to stabilize the DNA helix, as shown, for example, by the enhancement of the melting temperature (T_M) (Le Pecq & Paoletti, 1967), one might imagine that the presence of Triton X-100 could promote a destabilization of the DNA molecule which would make it sensitive to the activity on single-stranded DNA. In order to test this hypothesis, thermal denaturation of T7 DNA was studied at various ionic strengths, in the presence of different EtdBr and Triton X-100 concentrations. Because of the very high UV light absorption of Triton X-100, the DNA melting was followed by measuring the fluorescence change of the DNA–EtdBr complex at increasing temperatures, in the conditions described by Le Pecq & Paoletti (1967). However, as discussed by Helenius & Simons (1975), Triton X-100 solutions become turbid at a critical temperature (cloud point) which depends on the experimental conditions. Beyond that point, fluorescence measurements are not significant. The T_M of T7 DNA, determined in the conditions of the EtdBr DNase assay, in the presence of EtdBr (8×10^{-5} M DNA-P, 2×10^{-6} M EtdBr), was equal to 85 °C. When Triton X-100 was added at final concentrations of 1 or 5%, the fluorescence of the DNA–EtdBr complex was exactly superimposed on the fluorescence of the complex in absence of Triton up to 66 °C, which represents the cloud point of the detergent in these conditions. In order to observe the complete melting curve, these experiments were carried out with poly[d(A-T)] at low ionic strength. In solution containing 10^{-3} M EDTA, 0.5 mM NaCl, poly[d(A-T)] (8×10^{-5} M) melted at 26.5 °C. In the presence of EtdBr (1×10^{-6} M), the T_M value is 66.5 °C, which demonstrates a remarkable stabilization of the DNA structure. In the presence of 1% Triton X-100, the T_M of the poly[d(A-T)]–EtdBr complex is 64.5 °C. This shows that the EtdBr induced stabilization is not significantly modified. As discussed in the accompanying paper, the detergent and the DNA compete for the binding of the drug. Knowing the affinity constants of ethidium for DNA and Triton X-100, this 2 °C difference may be explained by a slightly lower amount of intercalated drug.

Finally, in the presence of Triton X-100, we also tested on the T7 DNA–EtdBr complex the activity of endonucleases that selectively degrade single-stranded polynucleotides, such as the *Neurospora* endonuclease (Linn & Lehman, 1965) and the S_1 nuclease from *Aspergillus oryzae* (Vogt, 1973). We found that there was no detectable hydrolysis of the T7 DNA in the presence of various concentrations of EtdBr and Triton X-100, by any of these enzymes, whereas, under the same conditions, denatured T7 DNA was entirely hydrolyzed.

From all these experiments, we conclude that the activity toward single-stranded DNA does not interfere with the EtdBr DNase activity and cannot account for the observed activation by EtdBr.

Although the three enzyme activities present in fraction IV can be distinguished by their optimal pH, their substrate requirements, and the effect of EtdBr, they have some common properties. All of them require divalent cations (0.01 M $MgCl_2$ or $MnCl_2$) and are about 90% inhibited in the presence of 0.1 M NaCl. They copurify with EtdBr DNase, and their rates of heat inactivation at 40 °C are identical (Figure 4). Figure 5 shows that the three activities cosediment together in a 5–20% sucrose gradient in the presence of 0.1% Triton X-100.

These results suggest that the three nuclease activities are associated in the protein complex which was detected by polyacrylamide gel analysis. However, at present, because of the difficulties resulting from the membranous origin of this enzyme preparation, the assignment of these activities to a single or different polypeptides, in this complex, has not been ascertained.

Properties of the EtdBr DNase

Substrate. Double-stranded DNAs from phage T7, *Escherichia coli*, and *S. cerevisiae* mitochondria have been used in the standard assay to measure the EtdBr DNase activity. The specific activity of the enzyme and the level of stimulation by EtdBr were the same with any of these substrates.

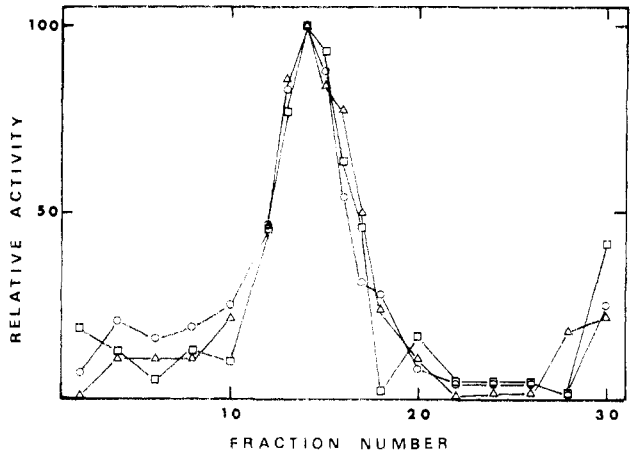

FIGURE 5: Sucrose gradient sedimentation of fraction IV. Fraction IV (0.3 mL; 0.7 unit of EtdBr DNase) was layered onto a 5–20% sucrose gradient containing 0.01 M Tris-HCl, pH 7.4, and 0.1% Triton X-100. Centrifugation was for 13 h at 45 000 rpm in the Spinco SW 65 type rotor. Fractions (0.13 mL) were collected from the bottom of the tube. The three enzyme activities were determined as described in Materials and Methods. For each enzyme activity, the fraction containing maximum activity is expressed as 100%. (O—O) EtdBr DNase; (Δ—Δ) DNase activity on double-stranded DNA at pH 5.6; (□--□) DNase activity on single-stranded DNA.

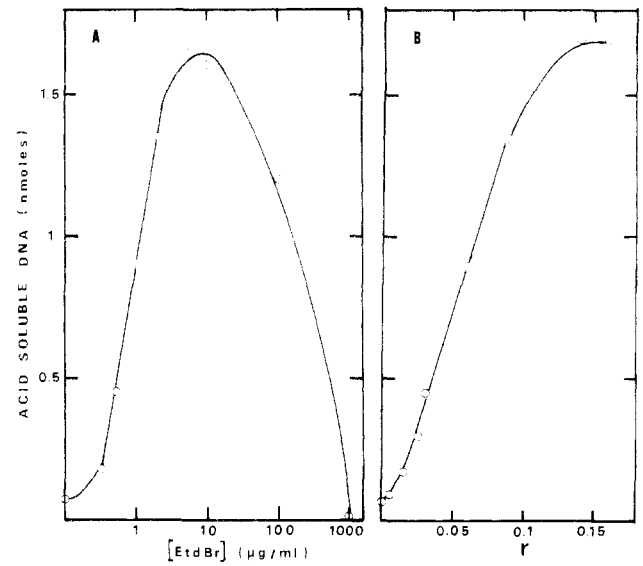

FIGURE 6: Activity of the EtdBr DNase as a function of EtdBr concentration. EtdBr activity (0.15 unit) was measured in the standard assay conditions, in the presence of the indicated EtdBr concentrations. (A) Activity as a function of the logarithm of the EtdBr concentration. (B) Activity as a function of r (number of intercalated EtdBr per nucleotide).

pH Optimum. Optimal enzymatic activity, in the presence of EtdBr, was observed over a wide pH range between 6.2 and 7.8, using Tris–maleate and Tris-HCl buffers.

Effects of Ionic Strength. The EtdBr DNase activity is inhibited at relatively low ionic strengths. The enzymatic activity in the presence of 0.02, 0.05, and 0.1 M NaCl is only 70%, 30%, and 5%, respectively, of the activity in the absence of NaCl. This inhibition does not result from a decrease in the intrinsic binding constant of EtdBr to DNA. Under the conditions of the enzyme assay, the EtdBr concentration is such that the number of intercalated drug molecules per nucleotides does not change in this range of ionic strength variations (Le Pecq & Paoletti, 1967).

Effect of ATP. Bastos & Mahler (1974) have proposed that the extensive degradation of mtDNA after exposure of isolated mitochondria to EtdBr is an ATP-dependent process, presumably involving an ATP-dependent nuclease. ATP has no effect on the EtdBr DNase in the range of concentrations from 10^{-8} to 10^{-4} M.

Requirement for Divalent Cations. The EtdBr DNase requires the presence of $MgCl_2$ or $MnCl_2$ at concentrations between 2 and 10 mM. $CaCl_2$ or $ZnCl_2$ cannot substitute $MgCl_2$ in this reaction.

Effect of Ethidium Bromide. The EtdBr DNase activity as a function of EtdBr concentration is shown in Figure 6A. In the conditions of the standard assay, the enzyme activity in absence of EtdBr is about 4% of the maximal activity. When EtdBr is added at increasing concentrations, the enzyme activity reaches a maximum at about 5 μg/mL. At higher EtdBr concentrations, the activity decreases and is completely inhibited at 1000 μg/mL.

At low concentrations, EtdBr intercalates between the base pairs of the DNA. The ratio r of intercalated EtdBr per nucleotide can be computed through the following equation (Le Pecq & Paoletti, 1967):

$$r = \frac{K^{-1} + E_t + nP - \sqrt{(K^{-1} + E_t + nP)^2 - 4nPE_t}}{2P}$$

where K^{-1} is the apparent dissociation constant (K^{-1} was determined by the fluorometric method (Le Pecq & Paoletti, 1967) under the conditions of the assay and found equal to 0.5×10^{-5} M), n is the number of binding sites per nucleotide ($n = 0.2$), E_t is the total EtdBr concentration, and P is the nucleotide concentration. When EtdBr DNase activity is plotted as a function of r (Figure 6B), the maximal activity is reached when about all the intercalated sites are occupied ($r = 0.16$). The subsequent enzyme inhibition, which is observed in the presence of high EtdBr concentrations, might result from the possible binding of EtdBr to external sites on the DNA.

These results indicate that the stimulation of the enzyme activity is related to the intercalation of EtdBr into the DNA and does not involve a direct interaction between the drug and the protein.

Effect of Substrate Concentration. The EtdBr DNase activity was measured at different DNA concentrations. Since the enzyme activity depends on the amount of intercalated EtdBr, at each DNA concentration, EtdBr concentration was adjusted in order to maintain a constant r value of 0.16. As shown on Figure 7, the curve deviates from the expected hyperbolic shape, showing a partial inhibition of the reaction at high substrate concentrations. Therefore an accurate determination of the kinetic parameters of the reaction (K_m and V) was difficult. This inhibition, which results from the interference of the detergent Triton X-100 with this enzymatic system, will be discussed in details in the accompanying paper (Jacquemin-Sablon et al., 1979).

Activity on Covalently Closed Circular Double-Stranded DNA. In order to investigate if the EtdBr DNase is able to act as an endonuclease, we tested its activity on covalently closed circular DNA. Phage PM2 DNA was used as a substrate, and the enzyme activity was measured by two methods.

(a) The fluorometric method developed by Paoletti et al. (1971). By this very sensitive method, it is possible to follow, during the enzymatic reaction, the appearance of DNA molecules which contain at least one single-strand break. Cleavage of a single phosphodiester bond in a circular DNA leads to an increase in fluorescence. As shown in Figure 8, at very low enzyme concentration (0.08 U/mL), one observes

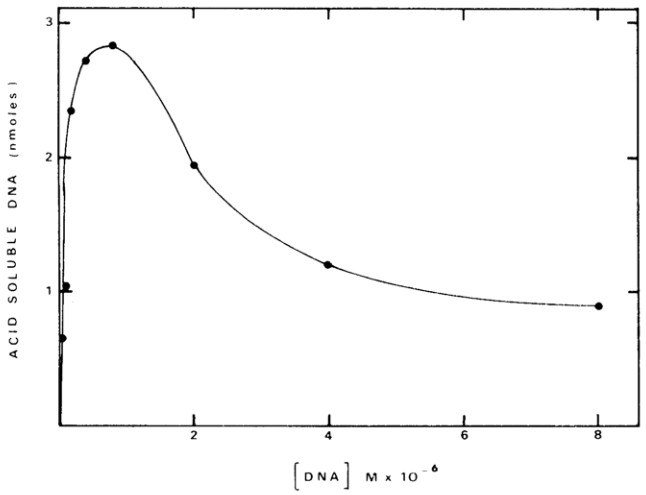

FIGURE 7: Effect of substrate concentration on the EtdBr DNase activity. EtdBr DNase activity (0.15 unit) was measured in the standard assay conditions at different DNA concentrations. For each DNA concentration, EtdBr concentration was adjusted to maintain r constant ($r = 0.16$).

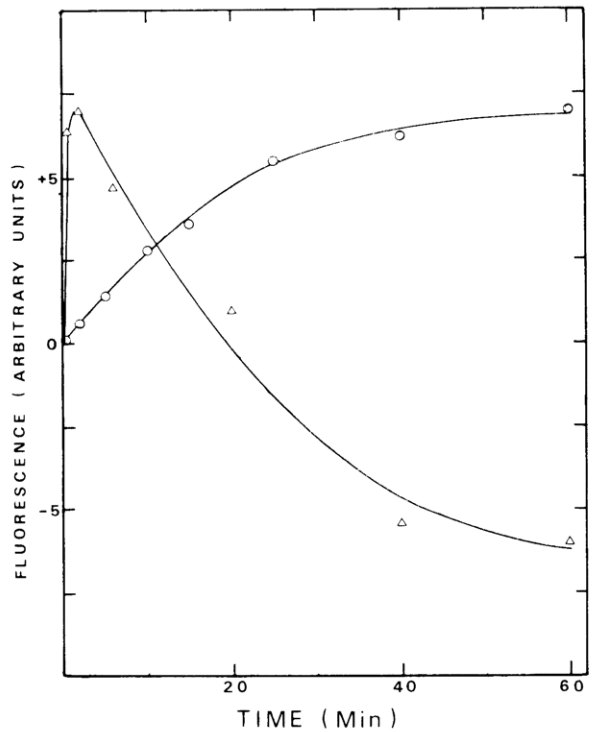

FIGURE 8: Change in fluorescence of PM2 DNA–EtdBr complex after incubation with EtdBr DNase. The reaction mixture (1 mL) contained 70 μmol of Tris-HCl buffer, pH 7.7, 10 μmol of $MgCl_2$, 6.5 μmol of PM2 DNA, and 10 μg of EtdBr; 10 μL of EtdBr DNase at the appropriate dilution was added, and the incubation was carried out at 28 °C. At the indicated times, 0.1-mL samples of the reaction mixture were withdrawn and added to 0.8 mL of 0.01 M Tris-HCl buffer, pH 7.8, containing 0.02 M NaCl, 0.005 M EDTA, and 1 μg/mL of EtdBr. The fluorescence was then measured in the conditions described by Paoletti et al. (1971). (O—O) EtdBr DNase, 0.08 U/mL; (Δ—Δ) EtdBr DNase, 2 U/mL.

the progressive opening of the covalently closed circular DNA as a function of time. The fluorescence reached a plateau, when all the substrate was converted into open circles or linear molecules. At higher enzyme concentration (2 U/mL), the very rapid fluorescence increase (maximum reached in about 30 s) is followed by a decrease due to the loss of secondary structure of the DNA. These results indicate that the EtdBr DNase does not need any termini to degrade DNA and can act as an endonuclease.

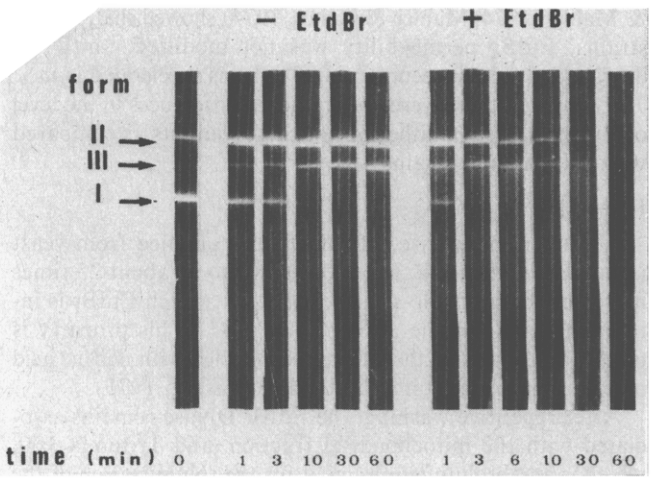

FIGURE 9: Agarose gel electrophoretic analysis of PM2 DNA after incubation with the EtdBr DNase. PM2 DNA was incubated with EtdBr DNase (2 U/mL) in the conditions described in the legend of Figure 8, either with EtdBr (10 μg/mL) or without EtdBr. In order to slow the reaction, incubation was carried out at 3 °C. At the indicated times, 0.2-mL samples were withdrawn, and the reaction was stopped by the addition of 0.02 mL of 0.3 M EDTA, pH 6.5. The samples were then submitted to electrophoresis on 0.8% agarose gels in the conditions described by Hayward & Smith (1972). DNA bands were located in the gels by staining for 30 min in aqueous EtdBr solution (0.5 μg/mL) and subsequent illumination with short-wave ultraviolet light.

(b) Analysis of the degradation products by agarose gel electrophoresis. Aaij & Borst (1972) have shown that the closed circular, open circles, and linear forms of PM2 DNA can be separated by electrophoresis in 0.8% agarose gels. Figure 9 shows the kinetics of hydrolysis of PM2 DNA by EtdBr DNase (2 U/mL) in the presence and in the absence of EtdBr. In the presence of EtdBr, after a 1-min incubation, most of the DNA was converted to open circles and linear forms. After 3 min, there was no detectable supercoiled molecules remaining in the reaction mixture. Open circles and linear molecules disappeared during further incubation, indicating an extensive degradation of the DNA. In the absence of EtdBr, the enzyme was active at a much slower rate. Supercoiled molecules disappeared only after 10 min, and, during further incubation, open circles and linear molecules continued to accumulate. However, in both cases, open circles appeared prior to the formation of the linear molecules.

These results demonstrate that the EtdBr DNase is an endonuclease which introduces single-strand breaks in a double-stranded DNA and this reaction is stimulated by EtdBr. Further investigations on the precise mechanism of action of this enzyme are now in progress.

Levels of the EtdBr DNase in Different S. cerevisiae Mutants

We investigated the possibility that the EtdBr DNase would be involved in the process of "petite" induction by EtdBr. For this purpose, we tested the enzyme activity in different mutants of *S. cerevisiae* which display a variable sensitivity to EtdBr. Gouhier & Mounoulou (1973) have isolated a mutant, called B^R, which is resistant to EtdBr. The same level of enzyme was found in the mutant and in the parental strain. Two other mutants, isolated by Moustacchi (1971) from the strain N 123, are characterized by an enhanced sensitivity to UV induction of "petite" mutation. One, *uvsρ* 5, is a nuclear mutant which is resistant to "petite" induction by EtdBr. The other, *uvsρ* 72, a mitochondrial mutant, has a higher sensitivity to "petite" induction by EtdBr, as compared with the parental strain. Analysis of the biochemical properties of these mutants (Bastos

& Mahler, 1974; Mahler & Bastos, 1974) showed that, in both strains, EtdBr permeability was not modified. mtDNA degradation did not occur in *uvsρ* 5 but was accelerated in *uvsρ* 72. However, there were no significant differences in the level of EtdBr DNase in either one of these mutants as compared with the parental strains.

Discussion

A deoxyribonuclease, EtdBr DNase, purified from yeast isolated mitochondria, has been shown to be about 25 times more active on double-stranded DNA in which EtdBr is intercalated than in the absence of EtdBr. This property is unique, since most of the enzymes concerned with nucleic acid metabolism are inhibited by EtdBr (Waring, 1975).

After repetitive washing, the EtdBr DNase remains associated with the mitochondrial fraction, and Triton X-100, which is an absolute requirement for the solubilization of the enzyme, must be present throughout the purification procedure. These results indicate that the EtdBr DNase is tightly bound to the mitochondrial membrane. In addition, when the detergent concentration in the reaction mixture is lowered below the critical micellar concentration, the enzyme activity is lost. More detailed studies reported in the accompanying paper (Jacquemin-Sablon et al., 1979) have shown that Triton X-100 is involved in the process of the enzyme activation by EtdBr.

The purified fraction contains two other nuclease activities which differ from the EtdBr DNase by their optimal pH, substrate specificity, and inhibition by EtdBr. However, the three activities have some common properties: their rates of heat inactivation are similar and they cosediment in a sucrose gradient. Analysis by polyacrylamide gel electrophoresis revealed that the enzyme preparation migrates as a single band in native conditions, whereas, in the presence of $NaDodSO_4$, six major bands are observed. However, as discussed in the Results section, the stoichiometry of this complex cannot be determined. Several possibilities can be considered to explain the presence of these polypeptides. Despite the presence of $PhCH_2SO_2F$ throughout the purification of the enzyme, some of these polypeptides may result from a proteolytic process. Alternatively, it is known, from studies with other membrane proteins (Simons et al., 1973), that stable complexes between different polypeptide chains may be formed upon detergent solubilization. The micelles once formed would be so difficult to dissociate that the contaminant peptide chains would copurify with the enzyme. Other yeast mitochondrial enzymes, such as cytochrome oxidase (Mason et al., 1973) or ATPase (Tzagoloff & Meagher, 1971), which are located in the mitochondrial membrane, also consist of several polypeptides. In both cases, some of these polypeptides are required for the enzymatic activity, while others are not. At present, the role of the different proteins in the EtdBr DNase system remains unclear, and, particularly, it is not known whether or not the different nuclease activities are carried by the same protein.

The level of stimulation of the EtdBr DNase by EtdBr depends on the amount of intercalated drug per nucleotide, the maximal activity being reached when all potential intercalation sites are occupied. Therefore, the stimulatory effect of EtdBr is not due to a direct interaction with the enzyme but rather to its intercalating binding on the substrate. More detailed studies on the mechanism of this activation are reported in the accompanying paper (Jacquemin-Sablon et al., 1979).

The EtdBr DNase does not require any DNA terminus to initiate hydrolysis and, therefore, is classified as an endonuclease. In the presence of EtdBr, it degrades T7, *E. coli*, and yeast mtDNA to acid-soluble products at the same rate. Time course analysis of the hydrolysis of covalently closed circular PM2 DNA has shown that the enzyme cuts one strand at a time in a native DNA molecule (haplotomic mechanism). Further studies on the mechanism of action of this enzyme are now in progress.

The physiological role of the EtdBr DNase is still unknown. However, the membrane location of the enzyme suggests that it might have a peculiar function in DNA metabolism. In some experimental systems, including bacteria and animal cells mitochondria, attachment of the DNA to the membrane, at or near the replication origin and the replication points, has been demonstrated (Ganesan & Lederberg, 1965; Smith & Hanawalt, 1967; Albring et al., 1977). This DNA membrane association is thought to have an important role in such processes like mechanism and regulation of DNA replication, segregation of daughter molecules, etc. Therefore, one may assume that the enzymes required in these processes should be associated with membrane structures. Studies on bacterial systems have provided some evidence in support of this hypothesis. For example, Knippers & Strätling (1970) demonstrated that part of the DNA replicating machinery in *E. coli* is indeed associated with the cell membrane. The membrane location of a DNase implicated in the genetic transformation of *Diplococcus pneumoniae* has been described by Lacks & Neuberger (1975). In mammalian cells mitochondria, Durphy et al. (1974) found several DNases' activities tightly bound to the inner mitochondrial membrane. However, as for the EtdBr DNase, their physiological role has not yet been determined.

Possible involvement of the EtdBr DNase in the induction of "petite" mutation by EtdBr has been examined. Several reactions, which may account for the different steps in this process, have been described by Bastos & Mahler (1974). According to these authors the initial binding of EtdBr to the mitochondrial DNA is followed by the fragmentation of the DNA into large fragments. In nongrowing cells, the process stops at this step. Criddle et al. (1976) have defined conditions in which rejoining of the fragments can be observed, associated with a recovery of respiratory competence. In the presence of an energy source (Mahler & Bastos, 1974), an extensive degradation of the DNA to oligonucleotides takes place. All these steps require the integrity of the inner mitochondrial membrane (Mahler & Perlman, 1972; Bastos & Mahler, 1974). In two mutants resistant to EtdBr mutagenesis, B^R and *uvsρ* 5, the enzyme was present at the same level as in the parental strains. Hall et al. (1976) reported that, depending on the growth conditions, the strain S 288C can be resistant or sensitive to EtdBr mutagenesis. They found that, when the cells are grown in conditions preventing the "petite" induction, the EtdBr DNase is absent. However, they did not measure the enzyme activity in the sensitive cells. Therefore, in order to ascertain the involvement of the EtdBr DNase in this process, further studies with different strains are still required.

Acknowledgments

The authors are indebted to Dr. J. M. Saucier for help in preparing the manuscript and critical comments, Dr. M. Guerineau for helpful discussions, and Dr. J. B. Le Pecq for critical reading of the manuscript. The excellent technical assistance of Miss E. Franque and C. Delaporte is gratefully acknowledged.

References

Aaij, C., & Borst, P. (1972) *Biochim. Biophys. Acta 269*, 192–200.

Albring, M., Griffith, J., & Attardi, G. (1977) *Proc. Natl.*

Acad. Sci. U.S.A. 74, 1348–1352.
Bastos, R. N., & Mahler, H. R. (1974) *J. Biol. Chem. 249*, 6617–6627.
Böhlen, P., Stein, S., Dairman, W., & Udenfriend, S. (1973) *Arch. Biochem. Biophys. 155*, 213–220.
Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.
Bouanchaud, D. H., Scavizzi, M. R., & Chabbert, Y. A. (1969) *J. Gen. Microbiol. 54*, 417–421.
Criddle, R. S., Wheelis, L., Trembath, M. K., & Linnane, A. W. (1976) *Mol. Gen. Genet. 144*, 263–272.
Dewald, B., Dulaney, J. T., & Touster, O. (1974) *Methods Enzymol. 32*, 82–91.
Durphy, M., Manley, P. N., & Friedberg, E. C. (1974) *J. Cell Biol. 62*, 695–706.
Espejo, R. T., Canelo, E. S., & Sinsheimer, R. L. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 1164–1168.
Francke, B., & Ray, D. S. (1971) *Virology 44*, 168–187.
Fukuhara, H., & Kujawa, C. (1970) *Biochem. Biophys. Res. Commun. 41*, 1002–1008.
Ganesan, A. T., & Lederberg, J. (1965) *Biochem. Biophys. Res. Commun. 18*, 824–835.
Garen, A., & Levinthal, C. (1960) *Biochim. Biophys. Acta 38*, 470–483.
Gibson, W. (1974) *Virology 62*, 319–336.
Goffeau, A., Landry, Y., Foury, F., Briquet, M., & Colson, A. M. (1974) *J. Biol. Chem. 248*, 7097–7105.
Goldring, E. S., Grossman, L. I., Krupnick, D., Cryer, D. R., & Marmur, J. (1970) *J. Mol. Biol. 52*, 323–335.
Gouhier, M., and Mounolou, J. C. (1973) *Mol. Gen. Genet. 122*, 149–164.
Hall, R. M., Trembath, M. K., Linnane, A. W., Wheelis, L., & Criddle, R. S. (1976) *Mol. Gen. Genet. 144*, 253–262.
Hayward, G. S., & Smith, M. G. (1972) *J. Mol. Biol. 63*, 383–395.
Helenius, A., & Simons, K. (1975) *Biochim. Biophys. Acta 415*, 29–79.
Holloway, P. W. (1973) *Anal. Biochem. 53*, 304–308.
Jacquemin-Sablon, H., Le Bret, M., Jacquemin-Sablon, A., & Paoletti, C. (1979) *Biochemistry 18* (following paper in this issue).
Knippers, R., & Strätling, W. (1970) *Nature (London) 226*, 713–717.
Koerner, J. F., & Sinsheimer, R. L. (1965) *J. Biol. Chem. 228*, 1049–1062.
Lacks, S., & Neuberger, M. (1975) *J. Bacteriol. 124*, 1321–1329.
Le Pecq, J. B. (1971) in *Methods of Biochemical Analysis* (Glick, D., Ed.) Vol. 20, pp 41–86, Wiley, New York.
Le Pecq, J. B., & Paoletti, C. (1967) *J. Mol. Biol. 27*, 87–106.
Linn, S., & Lehman, I. R. (1965) *J. Biol. Chem. 240*, 1294–1304.
Mahler, H. R., & Perlman, P. S. (1972) *J. Supramol. Struct. 1*, 105–124.
Mahler, H. R., & Bastos, R. N. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2241–2245.
Mason, T. L., Poyton, R. O., Wharton, D. C., & Schatz, G. (1973) *J. Biol. Chem. 248*, 1346–1354.
McCann, J., Choi, E., Yamasaki, E., & Ames, B. N. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 5135–5139.
Moustacchi, E. (1971) *Mol. Gen. Genet. 114*, 50–58.
Paoletti, C., Le Pecq, J. B., & Lehman, I. R. (1971) *J. Mol. Biol. 55*, 75–100.
Paoletti, C., Couder, H., & Guerineau, M. (1972) *Biochem. Biophys. Res. Commun. 48*, 950–958.
Perlman, P. S., & Mahler, H. R. (1971) *Nature (London), New Biol. 231*, 12–16.
Pringle, J. R. (1975) in *Methods in Cell Biology* (Prescott, D. M., Ed.) Vol. 12, pp 149–184, Academic Press, New York.
Richardson, C. C. (1966) *J. Mol. Biol. 15*, 49–61.
Riou, G., & Delain, E. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64*, 618–625.
Schaller, H., Nüsslein, C., Bonhoeffer, F. J., Kurz, C., & Nietzschmann, I. (1972) *Eur. J. Biochem. 26*, 474–481.
Simms, N. R., & Carnegie, P. R. (1975) *Anal. Biochem. 65*, 578–580.
Simons, K., Helenius, A., & Garoff, H. (1973) *J. Mol. Biol. 80*, 119–133.
Slonimski, P. P., Perrodin, G., & Croft, J. H. (1968) *Biochem. Biophys. Res. Commun. 30*, 232–239.
Smith, C. A. (1977) *Nucleic Acids Res. 4*, 1419–1427.
Smith, D. W., & Hanawalt, P. C. (1967) *Biochim. Biophys. Acta 149*, 519–531.
Smith, C. A., Jordan, J. M., & Vinograd, J. (1971) *J. Mol. Biol. 59*, 255–272.
Studier, F. W. (1973) *J. Mol. Biol. 79*, 237–248.
Tzagoloff, A., & Meagher, P. (1971) *J. Biol. Chem. 246*, 7328–7336.
Vogt, V. M. (1973) *Eur. J. Biochem. 240*, 1287–1293.
Waring, M. (1975) in *Antibiotics* (Corcoran, J. W., & Hahn F. E., Eds.) Vol. III, pp 141–165, Springer-Verlag, Berlin.
Yonetani, T., & Ray, G. S. (1965) *J. Biol. Chem. 240*, 3392–3398.
Zimmerman, S. B., & Sandeen, G. (1965) *Anal. Biochem. 10*, 444–449.