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Coupling Between Mitochondrial Mutation and Energy Transduction*

(mitochondria/DNA/energetics/coupling device/ethidium bromide)

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ABSTRACT **Upon incubation with ethidium bromide (Etd Br) isolated mitochondria of *Saccharomyces cerevisiae* have been shown to be capable of performing five novel reactions: (*a*) a single scission of their DNA (mtDNA) coincident with (*b*) the incorporation of Etd Br into the product (mtDNA → 2 mtDNA′-Etd Br); (*c*) an energy- (and probably ATP-) requiring degradation of mtDNA′-Etd Br to acid-labile products. These reactions acting in series generate (*d*) a DNase dependent on both Etd Br and an energy supply with mtDNA′-Etd Br as an obligatory intermediate. Coincident with (*d*) there occurs (*e*) an activation of adenosinetriphosphatase. Experiments with specific inhibitors suggest that the enzyme responsible is the mitochondrial adenosinetriphosphatase complex itself, and that it and the ability to carry out reactions (*a*) through (*e*) are tightly coupled to the energy-transducing functions of the particle.**

The trypanocide ethidium bromide (Etd Br) has received a great deal of recent attention as a specific probe for functions involving the mitochondrial (mt) genome (1–3). Among its more spectacular actions is its quantitative induction of extranuclear, respiration-deficient mutants in *Saccharomyces cerevisiae* (4), a hereditary change now known to be related to the deletion of variable (but large) segments of the mitochondrial genome and of mtDNA (5–8). By proper manipulation of conditions, particularly with regard to the energy supply of the affected cells, the following events have been ascribed to their exposure to the mutagen: Inhibition of replication of mtDNA (1–3) and of its transcription (9, 10); an endonucleolytic scission of both strands at, or nearly at, the same site near the center of the molecule producing *isolated* mtDNA of molecular weight $\simeq 12.5 \times 10^6$ (11–13); an energy-dependent degradation of the molecule resulting in its comminution into ever smaller fragments and its eventual complete disappearance as a macromolecular entity (1, 2, 6–8, 12). We have previously shown that mutagenesis and the initial scission of DNA are correlated with the formation of a novel, probably covalent, complex between mtDNA and Etd Br (13). In this communication we present evidence that isolated mitochondria are not only fully competent to perform all these reactions, thus accounting for the complete sequence observed *in vivo*, but also that these processes appear to be tightly coupled to mitochondrial energy metabolism.

Abbreviations: Etd Br, ethidium bromide; mtDNA, mitochondrial DNA; DNP, 2,4-dinitrophenol; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; EDTA, ethylenediaminetetraacetic acid; ATPase, adenosinetriphosphatase.

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METHODS AND MATERIALS

The strains used and conditions for their culture have been described previously (11, 13), as have been the methodology for labeling their mtDNA with adenine (13) or—after modification—with Etd Br (13, 14) and the measurement and properties of the modification product (13). Mitochondria were isolated from cells grown to early stationary state in D,L-lactate after conversion to spheroplasts as described in (10) except for omission of the wash with ethylenediaminetetraacetic acid. For incubation (30°) we used batches of particles obtained from 100 ml of cells resuspended in 5 ml of buffer P: 0.1 M sorbitol, 100 mM KH_2PO_4, 100 mM NH_4Cl, 10 mM $MgCl_2$, 10 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (Tes) (pH 6.5), and Etd Br where appropriate at a concentration of either 50 or 80 μM. ATPase was assayed according to (15), with the previous incubation of the mitochondria in buffer T, similar in composition to P except for the substitution of 0.2 M sorbitol for the first two items. All other additions and conditions are described in the legends to tables and figures.

The sources of the special chemicals used were: Etd Br, ATP, phospho*enol*pyruvate, pyruvate kinase, and oligomycin were all obtained from Sigma Chemical Co., St. Louis, Mo.; carbonylcyanide *m*-chlorophenylhydrazone (CCCP) was a gift from Dr. P. Heytler, E. I. duPont de Nemours Co., Wilmington, Del.; Dio 9 was a gift from Dr. R. J. Guillory, University of Hawaii, Honolulu. All other chemicals were reagent grade.

RESULTS

Nature of the Primary Modification Reaction in Isolated Mitochondria. When purified mitochondria, isolated from cells previously grown on a respiratory carbon source, are incubated with [^{3}H]Etd Br all (>90%) of the incorporated label becomes specifically attached to mtDNA in a form precipitable by trichloroacetic acid. The reaction responsible appears analogous to the one previously described *in vivo* (13) with respect to (*i*) its time course (Fig. 1*A*); (*ii*) the nature of the product in its apparent covalent mode of linkage between Etd Br and mtDNA; (*iii*) the discrimination in the selection of the latter over nuclear DNA; and (*iv*) its cleavage leading to its conversion from a species, as isolated, with a molecular weight of about 25×10^6 to one about half that size (all in Fig. 2). Like the reaction *in vivo* it also does not depend on an added energy supply, whether in the form of added tricarboxylic acid cycle intermediates or ATP (Table 1). The reaction is unaffected by the exhaustion of all external ATP supplies by means of glucose plus hexokinase, or by their

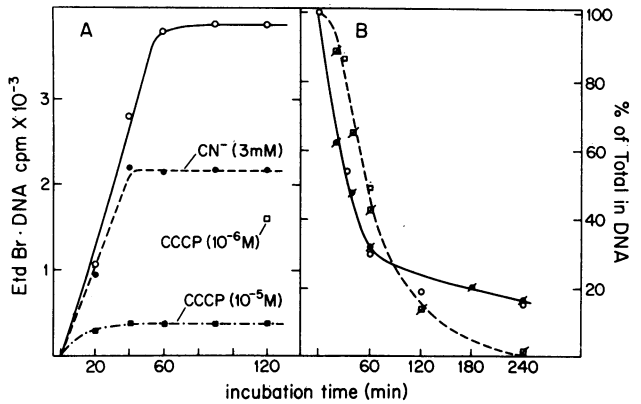

FIG. 1. (*A*) *Incorporation of* [^3H]*Etd Br into mtDNA.* Experimental conditions were similar to those described under Table 1 except that samples were removed at the times shown and assayed for alkali stable, trichloroacetic acid-precipitable activity (method B). Inhibitors, at the concentrations shown, were added simultaneously with Etd Br (10 μCi in 5 ml). (*B*) *Degradation of modified mtDNA.* Cells (200 ml of IL-16) growing on lactate were prelabeled with 50 μCi of [^{14}C]adenine (13), their mitochondria isolated, suspended in buffer P and the radioactivity determined (79,300 dpm, all counts corrected for spillover and quenching). [^3H]Etd Br (5 μCi) was added and the sample incubated for 90 min; at this point the ^{14}C and ^3H activity equalled 78,400 dpm and 48,500 dpm; these were taken as the (starting) 100% value. The degradation was started by the addition of succinate (1%) and followed by removal of aliquots at the times shown. Three separate experiments are shown: O, □ constitute the double label set as described, while ● used labeled Etd Br but unlabeled cells, and ⊠ used labeled cells but unlabeled Etd Br (50 μM) with the mitochondria suspended in buffer T. In this and the following figure, the convention is that the numbers on the ordinates are the product of the indicated factors and the experimental values.

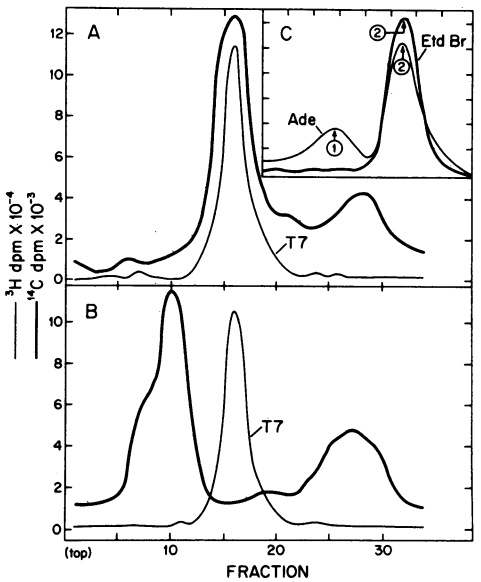

FIG. 2. *Nature of the modification product.* Total cellular DNA (of 100 ml of IL-16) was labeled with [^{14}C]adenine (50 μCi) as described in Fig. 1*B*. Mitochondria were isolated, suspended in buffer P (6 ml), and aliquots incubated for 90 min either in the absence or presence of 50 μM Etd Br. For a third aliquot the incubation was with 2 μCi of [^3H]Etd Br. Bacteriophage T7 labeled in its DNA with [^3H]thymine was added to the first two samples, which were lysed and analyzed on sucrose gradients as described previously (13). *A* is the pattern of the incubated control, *B* that of the mtDNA exposed to Etd Br. The first large peak with a molecular weight (in *A*) equal to that of T7 DNA corresponds to mtDNA, the second to nuclear DNA. As shown in *B* the mtDNA molecules are converted quantitatively to ones with masses approximately 0.5 (3 parts) and 0.25 (1 part) that of the control. This pattern is qualitatively similar but with much narrower size distributions to that obtained *in vivo* (11–13). The third sample (*C*) was analyzed on a polylysine–kieselguhr column (13); here peaks *1* and *2* correspond to nuclear and mtDNA, respectively. The relative proportion of ^{14}C is virtually identical to that obtained from sucrose gradients (*A*). Etd Br is associated exclusively with mtDNA.

being rendered unavailable due to the addition of oligomycin. The reaction is, however, susceptible to agents that interfere with membrane-associated energy transduction, such as cyanide, and especially 2,4-dinitrophenol (DNP) and CCCP (16) (Table 1, Fig. 1), and, since it is completely prevented by the addition of EDTA, also appears to require the participation of divalent cations.

Degradation of Modified mtDNA. Although the modified DNA is stable on prolonged incubation (>2 hr) of the mitochondria in the buffer-salt medium ordinarily employed, the addition of an internal (e.g., succinate) or external energy source (ATP) results in its rapid degradation (Fig. 1*B*), as measured by the release, in a double labeling experiment, of products labeled either with ^3H (i.e., derived from Etd Br) or ^{14}C (derived from DNA). Two interesting features emerge. (*i*) Initially the release of Etd Br appears more rapid than that of the nucleotides. Therefore, the degradation of mtDNA may occur in two stages: an initial excision of modified regions producing extensive gaps in the molecule followed by its rapid hydrolysis. (*ii*) The greater retention of Etd Br relative to purines after prolonged incubation suggests that tracts with a preponderance of Etd Br over bases are more resistant to hydrolysis and that such tracts had been formed either during the initial insertion of Etd Br into mtDNA or as a result of its degradation. The reaction appears closely linked to intramitochondrial energy supply—and perhaps ATP—since it is completely blocked by the addition of respiratory inhibitors (antimycin A, as well as oligomycin—indicating "tight coupling") as well as of uncouplers (e.g., CCCP) (16). If the modification of mtNDA by Etd Br and the degradation of this modified product are allowed to proceed concurrently, these two reactions in series become operationally equivalent to an Etd Br and energy-dependent mtDNase. The results of Table 2 (and Fig. 3*C*) demonstrate the occurrence and the requirements of this reaction: It is absent upon exposure of the system to Etd Br alone, or succinate alone, or even in their presence, provided the formation of the modified product is prevented by the inclusion of EDTA. Euflavine, an agent known to be an effective intercalating agent and mitochondrial mutagen, but with requirements distinct from those for Etd Br (17), cannot substitute for the latter in the degradation reaction. All these criteria clearly distinguish the reaction from that described by Paoletti *et al.* (18).

Stimulation of Adenosinetriphosphatase (ATPase). In prokaryotes, processes assayable as ATP-dependent DNases participate in a wide variety of reactions related to replication or repair functions of their DNA (for example see refs 19–22).

Frequently, the same systems can also be assayed as the converse, namely as DNA-dependent ATPases (23–25). Since we have just described what amounts to an Etd Br induced reaction of the first type, it appeared of interest to determine whether we could detect some indication of the second under the same conditions. The results (Fig. 3) indicate that incubation of mitochondria leads to a pronounced stimulation of their *ATPase*, provided that the condition previously established for the *DNase*, i.e., the simultaneous presence of succinate and Etd Br, are met. Preliminary experiments show that the base line as well as the stimulated activity are inhibited to approximately 50% on addition of the specific inhibitors Dio 9 and oligomycin (15, 26, 27) at a level of 20 μg/ml. The enzyme system involved, therefore, utilizes F_1, the classical mitochondrial ATPase.

A further indication that this reaction system may be implicated in mitochondrial genetic functions comes from an examination of a set of three strains, one wild type, N123, and two single-step mutants, *uvs* ρ5 and *uvs* ρ72, deficient in repair functions for lesions introduced into their mtDNA by irradiation with ultraviolet light (28). While similar in this regard, the two mutants differ greatly in their susceptibility to mutagenesis by Etd Br (11, 12) and Berenil (29), with *uvs* ρ72 more susceptible and *uvs* ρ5 more resistant than their wild-

TABLE 1. *Requirements for formation of modified mtDNA*

System	Incorporation (pmoles of Etd Br)	Inhibition (%)
Complete (method A)	181	
+ EDTA (100 mM)	12	91
+ 1 mM ATP + generating system*	145	20
Etd Br added to lysate†	6	
Complete (method B)	258	
+ CN^- (3 mM)	103	56
+ DNP (100 μM)	115	55
+ CCCP (1 μM)	147	43
Complete (method B)	240	
+ glucose (10 mM) + hexokinase	237	1
+ glucose + hexokinase + oligomycin (20 μg/ml)	220	8
+ glucose + hexokinase + CCCP (1 μM)	94	60
+ oligomycin + CCCP	102	43

* Phospho*enol*pyruvate (5 mM), pyruvate kinase (5 units).

† Etd Br added to lysed mitochondria (13) after previous incubation in buffer + ATP + generating system.

Mitochondria were incubated for 60 min at 30° in buffer P (5 ml) in the presence of 10 μCi (2 μmoles) of [^{3}H]Etd Br plus other additions as indicated. DNA was extracted and analyzed as described in Fig. 2, using the total counts in the DNA of the first (mitochondrial) peak as a measure of the product (*method A*). Alternatively, product formation was monitored by diluting a 0.1-ml aliquot of the suspension to 0.9 ml with 0.10 M NaCl + 0.05 M Na citrate and lysing with 0.1 ml of 10% sodium lauryl sarcosinate. RNA was hydrolyzed by adding 0.2 ml of 2 N NaOH followed by incubation at 23° for 18 hr. DNA was precipitated by the addition of 0.7 ml of 3 M NaH_2PO_4 plus 0.3 ml of 50% trichloroacetic acid. After 4 hr at 4° samples were filtered through glass filters, washed once with 10% trichloroacetic acid and twice with absolute ethanol and counted (*method B*). The extent of incorporation determined by the two methods agrees within ±5% for any one experiment. Three separate experiments are shown.

TABLE 2. *Requirements for degradation of modified mtDNA*

Conditions		% Hydrolyzed	
Preincubation (90 min)	Incubation	60 min	120 min
Etd Br (50 μM)	Succinate (1%)	40	89
Etd Br + EDTA (50 mM)	Succinate	0	<2
Euflavine (100 μM)	Succinate	0	<1
None	Succinate	0	<1
Etd Br (50 μM)	None	2	
Etd Br	Succinate (1%)	48	
Etd Br	ATP (1 mM)	44	
Etd Br	Succinate plus inhibitors*	None	
Etd Br	ATP plus inhibitors†	None	

* Antimycin (5 μg/ml); oligomycin (20 μg/ml); CCCP (1 μM); oligomycin + CCCP.

† Oligomycin; CCCP; oligomycin + CCCP.

Cells (1 liter) of IL-16 were grown in the presence of 150 μCi of [^{14}C]adenine (55 mCi/mmole), mitochondria were isolated, and aliquots, suspended in buffer P, were subjected to an incubation for 90 min under the conditions described in the *left* column; succinate or other additions as described in the *center* were added, and incubation continued as shown on the *righi*. Analysis was for alkali-stable counts precipitable by trichloroacetic acid, with the level present after the preincubation (~1000 cpm/ml) taken as 100%. This value is identical to that found without this treatment.

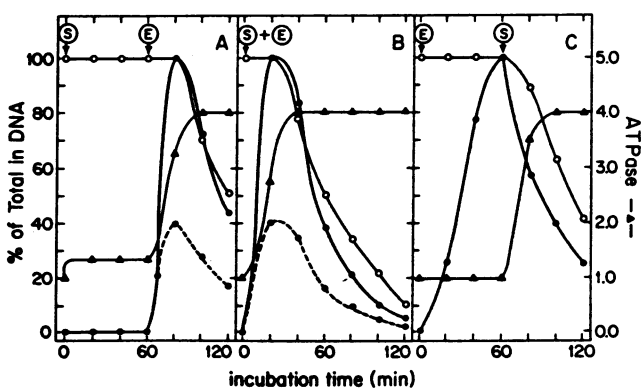

FIG. 3. *Kinetics of mitochondrial reactions elicited by Etd Br.* The designs for *A*, *B*, and *C* were similar except for the time of addition of the components succinate (*S*) and Etd Br (*E*), which were as indicated. The reactions involving modification and labeling of ^{14}C-prelabeled DNA by [^{3}H]Etd Br (5 μCi in 2 ml) are described in the *legend* to Fig. 1*B;* the measurement of the induction of ATPase is described in the *legend* of Table 3; the Etd Br was provided by the labeled sample. [^{14}C]DNA is shown as ○——○; [^{3}H]Etd Br in DNA is shown as ●——● and ATPase as ▲——▲. The values corresponding to the base line (or 100%) were 2800 dpm for ^{14}C in *A*, *B*, and *C* and 7100 dpm for ^{3}H in *C*, for the 2 ml of incubation mixture employed; ATPase activity was 4 μmoles of P_i liberated × $(10\ min)^{-1}$ × mg^{-1} of protein. The *dashed line* ●- - -● in *A* and *B* sets the maximal level of Etd Br incorporated into DNA as determined in *C* as 100%, while the *full line* ●——● uses the maximal level obtained in the respective experiment as 100%.

TABLE 3. *ATPase* in uvs mutants*

Time (min)	Strain N123 +Etd Br	N123 −Etd Br	*uvs* ρ5 +Etd Br	*uvs* ρ5 −Etd Br	*uvs* ρ72 +Etd Br	*uvs* ρ72 −Etd Br
0	4.4	4.4	3.3	3.3	4.9	4.9
60	4.1	4.2	3.6	3.8	5.3	4.6
120	10.6	6.4	4.0	3.8	22.0	7.9

* μmoles of P_i released $\times$ $(10\ min)^{-1}$ $\times$ mg of $protein^{-1}$.

Mitochondria were isolated from cells (250 ml) of the three strains grown on 3% glycerol, and were suspended in buffer T (6 ml total) either in the presence or absence of Etd Br (80 μM). Aliquots (100 μl) were removed and assayed for ATPase, and the samples were incubated for 60 min and reassayed; succinate (1%) was then added and the final level of ATPase determined after an additional 60 min.

type parent. There is no evidence concerning any lesions in their ATPase; both grow normally on glycerol or lactate. The data summarized in Table 3 show that the extent of enhancement of ATPase induced by Etd Br in the wild type is similar to that obtained with other strains, and that there is an additional stimulation in *uvs* ρ72, while enhancement is entirely absent in *uvs* ρ5. All three strains were found to be susceptible to modification of their mtDNA by Etd Br, with rate and extent in the order N123 = *uvs* ρ5 < *uvs* ρ72; in contrast the rate of degradation is *uvs* ρ5 ≪ N123 < *uvs* ρ72. These results suggest that *uvs* ρ5 is indeed defective in the recognition and excision of damage in its mtDNA (28, 29) and that this defect controls the rate of expression of the initial mutagenic event either positively when it is induced by ultraviolet light (mutagenesis results from *inability* to remove damage) or negatively in the case of Etd Br (i.e., incision and/or excision is *required* for mutagenesis). As for *uvs* ρ72, its enhanced susceptibility to degradation suggests that the defect lies either in a protein that ordinarily inhibits both degradation and ATPase (note the enhanced spontaneous level) *or* the mutation involves a structural alteration of the mtDNA which alone is sufficient (i.e., without being transcribed) to account for its behavior. Because of the enhanced extent of modification also observed with this strain, we incline somewhat to the second interpretation.

DISCUSSION

Coupling Between Mitochondrial Genetic Activity and Energetics. In this report we present evidence that upon incubation with Etd Br isolated mitochondria of *S. cerevisiae* can perform five novel reactions: (*a*) a single scission of their DNA, coincident with (*b*) the incorporation of Etd Br into the product (mtDNA → 2 mtDNA′-Etd Br); (*c*) an energy- (and probably ATP-) requiring degradation of mtDNA′-Etd Br to acid-labile products. These reactions acting in series generate (*d*) a DNAse dependent on both Etd Br and an energy supply with mtDNA′-Etd Br as an obligatory intermediate. Coincident with (*d*) there occurs (*e*) an activation of ATPase. Experiments with specific inhibitors suggest that the enzyme responsible is the mitochondrial ATPase complex itself and that it and the ability to carry out reactions (*a*) through (*e*) are tightly coupled to the energy-transducing functions of the particle. This latter claim is based on the effect of respiratory inhibitors and uncouplers on reactions (*b*) and (*c*), and if correct could account for these actions in terms of their effect on the coupling device (i.e., uncouplers function operationally as devices alternate and hence competitive to mtDNA to bring about the hydrolysis of ATP or the discharge of the high energy state). In other words reaction (*d*) can be regarded as one more example of events driven by, and directly dependent on, the mitochrondrial coupling device. That we are dealing with more than a laboratory curiosity is indicated by the behavior of the two *uvs* mutants which, while selected for their apparent inability to repair ultraviolet-light-induced damage specifically in the mitochrondria, show dramatic differences with regard to reaction (*d*). The link between the two key mitochondrial functions of energy transduction and mutation (i.e., faulty replication of mtDNA) probably accounts for the reported modulation of the latter by a wide variety of apparently unrelated agents or treatments, all of which may, however, affect the energy state of the inner membrane (2, 12, 30).

Unanswered Questions. What remains to be established is the *obligatory* nature of some of the reactions and sequences described. Specifically, does the introduction of Etd Br into mtDNA depend on its prior scission, is it the converse, or can the two reactions proceed independently of one another? Is it this prior scission (and attendant more subtle damage) or the presence of Etd Br in the DNA itself that is required for the "unleashing" of the ATP-driven DNase? Exposure to both an energy source and Etd Br is required for the concurrent unleashing of the ATPase, and prior formation of mtDNA′-Etd Br can not only substitute for the free mutagen but also appears to enhance the rate of appearance of the stimulation (Fig. 3); but is formation of the intermediate *necessary* to elicit the stimulation†? What is the relationship, if any, between these reactions and the energy-requiring binding of Etd Br to mitochondrial membranes describes by Azzi and Santato (32)? Under our conditions all *covalent* binding is to mtDNA, but what, if any, is bound to membranes; which is responsible for stimulation of ATPase? What is the relevance of the events described here for Etd Br to mitochondrial genetic function in general? Which of them, if any, participate in the generation of petite mutants by other mutagens, in the repair of damage in mtDNA, and particularly in genetic recombination? Many of these questions can be answered by the use of appropriate mutants and systems more highly resolved than the intact mitochondrion.

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† More extensive studies indicate a kinetic equivalence of the rate of formation of the intermediate and of the appearance of stimulation.

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