

MODULATION OF PETITE INDUCTION BY LOW  
CONCENTRATIONS OF ETHIDIUM BROMIDE

by

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When starved, exponential phase cells of *Saccharomyces cerevisiae* are exposed to 10-25  $\mu$ M ethidium bromide in buffer, the proportion of respiration deficient mutants in the population exhibits a rapid increase, followed by a pronounced decrease. This "self-rescue" by the mutagen can be dissociated from and studied independently of its mutagenic action and is shown to exhibit different requirements. These and related observations have been used to formulate a consistent model for mutagenesis by ethidium bromide and its modulation under a variety of conditions.

## INTRODUCTION

Exposure of wild type cells of *Saccharomyces cerevisiae* to ethidium bromide (EtdBr)<sup>1</sup> results in their quantitative conversion to cytoplasmic petite, [*rho*<sup>-</sup>], mutants (1). Mechanistically, this reaction appears to involve a number of consecutive steps leading to the fragmentation and, eventually, the complete degradation of mitochondrial DNA (mtDNA)<sup>1</sup>, the responsible genophore (2-11). Operationally, the existence and nature of some of these steps have been inferred from the response of the conversion to modulatory influences by a variety of treatments and agents (11), such as prior, simultaneous or even subsequent treatment at an elevated temperature (12), or with euflavine or Antimycin A (6,13,14). More recently, Wheelis, Trembath and Criddle (15) showed that EtdBr is itself a modulatory agent since in the course of mutagenesis of growing cells by this agent they exhibit a transitory capacity to produce wild type colonies.

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<sup>1</sup> Abbreviations: EtdBr - ethidium bromide; mt - mitochondrial

During our continuing studies on the mechanism of EtdBr mutagenesis, which generally employ starved, exponential phase cells in buffer (4,6-8,12-14,16, 17), we had made qualitatively similar observations. Since this paradigm permits not only the use of much lower concentrations of the mutagen, but also easier dissection and analysis of sequential, molecular events during its action, we wish to present and comment on these results at this time.

#### MATERIALS AND METHODS

Conditions for cell growth, mutagenesis and scoring for mutants have been described previously (7,12,13,16,17): We used cells growing with aeration at 30° in 2% or 5% glucose and harvested them in mid-exponential phase. They were resuspended at a cell density of  $2 \times 10^7$  per ml in 0.1 M phosphate buffer and starved in this medium with shaking at 30° for 60 min prior to the addition of EtdBr. Mutagenesis was followed by plating both on selective medium and by tetrazolium overlay on a glucose medium.

#### RESULTS

1. *Kinetic parameters* - The nature of the phenomenon using commercial strains (Fleischmann or Red Star yeast give similar results) is shown in Fig. 1. Concentrations of EtdBr  $\geq 10 \mu\text{M}$  are necessary and sufficient to bring about partial "self-rescue" (i.e. the prevention of the fixation of the initial mutagenic event) in starved cells (Fig. 1A). Such rescue is absent in growing cells and in fact it appears to depend on a change induced by starvation (exhaustion of key metabolite(s) or of the energy source?) (Fig. 1B).
2. *Other characteristics* - While these experiments suggest that the phenomenon requires the continued exposure of cells to EtdBr they do not exclude the possibility that the active agent is not EtdBr itself but some metabolite or other product. To test this hypothesis cells were first exposed for 20 min at  $10 \mu\text{M}$  EtdBr and then freed of external mutagen by centrifugation and washing. Aliquots were then resuspended: (i) in their original supernatant; (ii) in fresh phosphate buffer alone; (iii) in fresh phosphate buffer plus  $10 \mu\text{M}$  EtdBr. The results (Fig. 2A) demonstrate not only that EtdBr is necessary and sufficient to bring about reversal,

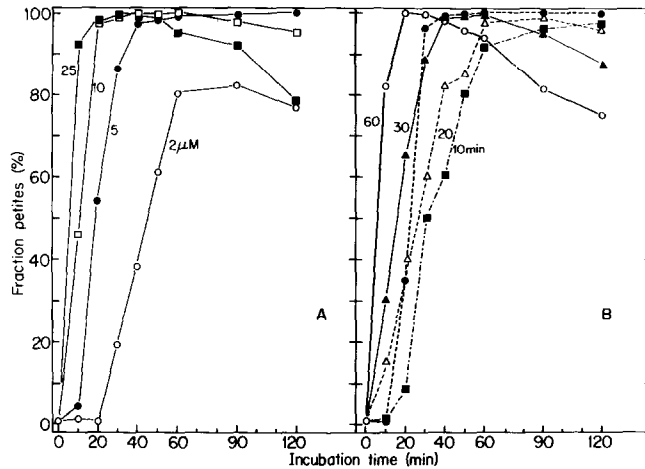


FIGURE 1: Kinetics of mutagenesis of starved cells (strain Fleischmann) by EtdBr: A - Response to varying the concentration ( $\mu\text{M}$ ) of EtdBr; B - Response to varying the length of the starvation period (time in min) prior to addition of 10  $\mu\text{M}$  EtdBr; curve for 0 time, not shown, is identical to 10 min; the curve without a number, ---●--- is for transfer to complete medium at zero time.

but that this protocol can serve to dissociate it from mutagenesis and can thus be used to establish its intrinsic requirements. For instance, the rescue event itself even in starved cells might depend on an energy source. If so it should be accelerated by exposure of mutagenized cells to complete medium, either prior to, or simultaneously with, EtdBr: the data shown rule out this hypothesis - the proportion of [*rho*<sup>-</sup>] progeny after initial mutagenesis remains constant whether cells so treated are plated immediately, or incubated in buffer or complete medium for up to 180 min; however, this quantity declines precipitously on supplementing the medium with 10  $\mu\text{M}$  EtdBr. However, capacity for rescue is itself transient since it is completely lost upon further starvation for an additional 60 min. The structurally similar intercalating dye euflavin - which itself is not mutagenic under these conditions (14) - is capable of effective interference with the production of [*rho*<sup>-</sup>] mutants when added either simultaneously with, or subsequent to EtdBr (11,13,14). However, unlike EtdBr its capacity to bring about this rescue is unimpaired by prior starvation of the mutagenized cells. In a typical experiment similar to that of Fig. 2 the addition of 25  $\mu\text{M}$  euflavine simultaneously

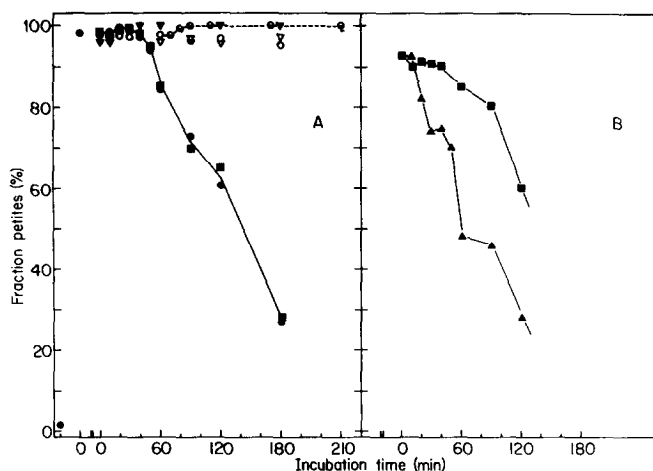


FIGURE 2: Effect of various treatments on reversal by EtdBr. Cells (Red Star) were grown and harvested and starved for 60 min as described under Materials and Methods. At  $t = -20$  min,  $10 \mu\text{M}$  EtdBr was added and incubated with the cells for 20 min yielding a conversion to petite as indicated by  $\emptyset$ ; the mixture was then centrifuged and the cells resuspended in equal volumes of various media, and incubated.  $\bullet$  - supernatant of first incubation mixture;  $\blacksquare$  - fresh buffer plus  $10 \mu\text{M}$  EtdBr;  $\blacktriangledown$  - complete growth medium plus  $10 \mu\text{M}$  EtdBr;  $\nabla$  - complete growth medium;  $\circ$  - buffer;  $\odot$  - buffer with  $10 \mu\text{M}$  EtdBr added at  $t = 60$  min.

B - Effect of buffer: pretreatment as under A;  $\blacksquare$  -  $0.10 \text{ M}$  phosphate pH 6.5;  $\blacktriangle$  -  $0.20 \text{ M}$  Tris buffer, pH 8.5.

with EtdBr at  $t = 0$  completely prevented any mutagenesis by this agent; its addition after the removal of EtdBr raised the fraction of wild type survivors from 10% to 90% within 20 min and to 98% within 60 min of incubation in buffer, and these values remained virtually unchanged when the addition of euflavine was delayed for 60 min. Finally, earlier experiments had suggested that in the biphasic mutagenesis-recovery process (Fig. 1A) the over-all reaction was independent of the nature of the buffer, while the second phase appeared more sensitive to pH (in the range 6.5-8.0) than did the first. The data in Fig. 2 show that this is in fact the case by demonstrating that both the rate and final extent of the rescue event is favored by the higher pH.

*Strain dependence* - The kinetics of the overall mutagenic conversion of  $[\text{rho}^+]$  to  $[\text{rho}^-]$  cells by EtdBr is profoundly affected by the nature of the wild type strain examined (6,7,11,18), as are the rescue events elicited by EtdBr (15). With six different strains, three of them diploid and three haploid, we found

that maximum mutagenesis with 10-25  $\mu$ M EtdBr required exposure from 30-90 min to produce between 95 and 99.9% mutant cells, which parameter declined to 4-80% on continued incubation for an additional 120 min. Rescue appeared to be more pronounced in diploid than in haploid strains. More complete and rational analysis will require (a) a study of the rescue event proper and (b) the use of strains with defined or definable lesions in mitochondrial DNA and energy metabolism (6-9,13,19), as well as of isogenic haploids and diploids (20).

#### DISCUSSION

A number of possible models for the phenomenon of modulation (11-15) are excluded by these observations:

a) Rescue might be due to protection of mtDNA against breakdown by virtue of its (reversible) conversion at high EtdBr concentrations to a form [e.g. more highly unwound (21,22), an "external" rather than an intercalative complex (23,24)], that is no longer (or less) susceptible to degradative processes. This is not likely since the same low concentration of EtdBr can bring about both mutagenesis and rescue; once these events have been initiated in buffer they persist upon transfer to ligand-free medium; the appearance and decay of the capacity for rescue is a function of the time of starvation.

b) EtdBr-induced degradation of mtDNA is known to require ATP and is reduced in the presence of uncouplers (4,8,14,25); but EtdBr is itself an uncoupler (26) and, in appropriate concentrations, might thus interfere with degradation. However, the concentrations required are much higher, even with isolated mitochondria (26) than those employed here, and in addition the strongly catabolite-repressed cells used are known to satisfy all their energy demands solely by glycolysis rather than respiration (27).

c) Degradation of mtDNA may involve "activation" of enzymes, or sets of enzymes, by EtdBr, either by its stimulating an otherwise relatively inactive nuclease (28), or by virtue of its insertion into the substrate converting it into a form recognized by, and therefore susceptible to, degradation by an enzyme ordinarily involved in recombination and/or repair of mtDNA (6,8-10). This re-

action may be inhibited at higher ligand concentrations. The equal and low concentrations required, together with the decay of the capacity for rescue by EtdBr unlike that for euflavine, which may exert its action by just such a mechanism (8), makes either version of this model equally unattractive.

d) The primary event in reversal, i.e. the one requiring the direct participation of EtdBr, may itself depend on synthetic processes. This hypothesis is ruled out by its rapid decay upon exposure of mutagenized cells to complete growth medium.

Additionally, any model must accomodate the following set of observations: Exposure of mtDNA of cells<sup>2</sup>, or isolated mitochondria, in buffer to EtdBr, in the concentration range used in these studies, leads to its conversion to a modified ligand-containing species (7,8,25). Cells resistant to the mutagenic action of EtdBr, such as certain mutants of *S. cerevisiae* (e.g. uvs p5), or "petite-negative" yeasts, are incapable of performing this reaction (7-9), which also appears to exhibit rather stringent structural requirements in the ligand molecule (14). Conversely, the attachment of such molecules to mtDNA by alternate means (i.e. by photochemical insertion) (29) results in enhanced mutagenesis [of *S. cerevisiae*, rendered relatively resistant by transient severe repression (30)], or cell killing of petite negatives. When its breakdown during isolation is prevented (31), one can obtain mtDNA of molecular weight close to the expected value of  $50 \times 10^6$  (11). Modification by EtdBr does not reduce this value significantly [Fig. 3; also (32), but in contrast to our earlier results in the absence of such precautions (6,13)]. While this modified DNA is stable for prolonged periods in buffer, upon transfer to complete medium it is rapidly broken down to fragments, the size of which decreases as a function of incubation (4,6,8). This degradation appears to be accelerated by the presence of EtdBr, as is conversion to  $[\rho^-]$  (see for instance Figs. 1A and 2A above). The initial phase in DNA breakdown has been separated into at least two steps (9,10,24): an excision of small EtdBr-containing regions,

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<sup>2</sup> Although these experiments were usually performed on derepressed cells, sporadic observations and reports on repressed cells suggest no qualitative differences in the mutagenic sequences.

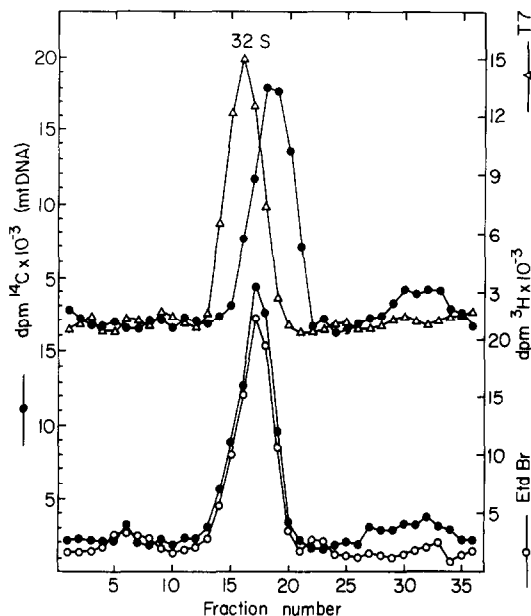


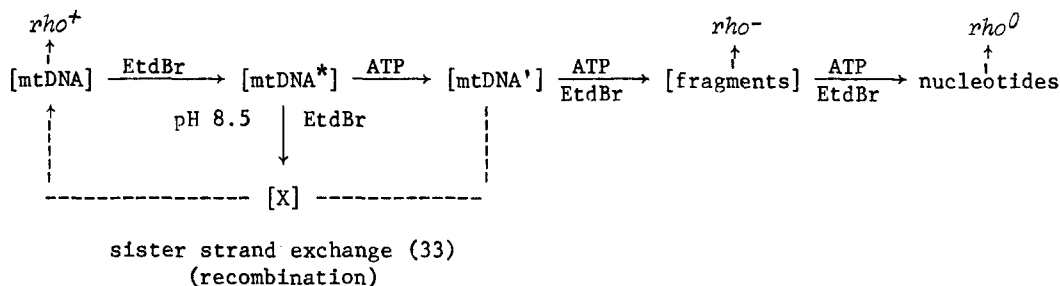
FIGURE 3: Sedimentation analysis of mtDNA on sucrose gradients. DNA was labeled, isolated and analyzed essentially as described before (6,7,13), except for the inclusion of KCN as described by Locker *et al.* (31) up to the sucrose gradient step. Sedimentation was from left (top) to right (bottom). In the top panel we compare mtDNA co-sedimenting with marker T7 DNA kindly provided by Dr. John Richardson; in the bottom, double labeled mtDNA, from cells pre-labeled with  $^{14}\text{C}$ -adenine and then incubated with 25  $\mu\text{M}$  tritiated EtdBr for 60 min. The label at the bottom of the gradient (fraction 25-35) corresponds to nuclear DNA.

leading to the introduction of gaps into single strands, followed by double strand cleavage (chops) probably by ATP-dependent endonuclease action within the single stranded regions opposite the gaps. The efficiency of excision can be correlated with the mutability of genetically altered strains (6-9,13,19) and it is blocked by euflavine (13,14).

Finally great care must be exercised in distinguishing reactions and events that can be documented for cells in liquid culture and for their extracts *in vitro*, and thus involve the bulk of the population, from those that occur subsequent to plating on agar. The latter, such as the reappearance of wild type on diagnostic plates, may involve the retention or reconstruction of as little as one of fifty or so molecules of intact mtDNA (20) in one of the clonal descendants of a cell

treated with a mutagen.

A possible model to accomodate these findings is the following:



In this scheme, the entities in brackets represent forms of mtDNA in its natural, i.e. membrane contained, and perhaps attached, habitat; solid arrows stand for reactions that have been substantiated by or inferred from biochemical studies, while dashed ones represent events producing the various cell types distinguishable on agar plates. The four bracketed species on the horizontal line represent in order, wild type mtDNA, its modification product induced by EtdBr, the gapped molecule produced by excision of the mutagen, and the fragments produced in the first instance by endonucleolytic attack opposite these gaps. The novel feature compared to earlier schemes is the introduction of a new species [X], formed directly or indirectly as a result of re-exposure to EtdBr, which eventually is capable of producing (perhaps by sister strand exchange between partially damaged molecules) at least one molecule of wild type mtDNA in the course of clonal growth on solid medium<sup>3</sup>. This species - which may differ from its parent in its primary (or higher order) structure, its environment (e.g. an alteration in the membrane attachment site) or both - is both necessary and sufficient to account for all other modulating effects so far described (11,15). Negative modulation, i.e. protection

<sup>3</sup> Under standard conditions of EtdBr mutagenesis the indicated loop from [DNA'] to [X] is relatively unimportant kinetically. It is not formed in buffer and in medium its buildup is prevented due to its rapid rate of degradation, which is further accelerated if EtdBr is present. Its inclusion is required to generalize the scheme to other mutagens such as Berenil (34), or UV that, while unable to form [DNA\*], are nevertheless susceptible of modulation - sometimes, as in the case of uvs ρ5, in opposite directions. They may therefore involve identical or very similar intermediates.

or cure, can be due either to a block in the formation of [mtDNA'] (uvs  $\rho 5$  or euflavine and, perhaps, Antimycin), a stimulation of the formation of [X] (EtdBr), or both [certain metabolic shifts (13)]; while any set of circumstances that results in a decreased concentration of [X] relative to [mtDNA'] will produce positive modulation, i.e. a more rapid rate of mutagenesis (uvs  $\rho 72$ ), and different metabolic shifts (13). It predicts that the addition of modulating agents should enhance the ability of the mtDNA in EtdBr-treated cells to participate in genetic recombination; this effect has been observed (31). The apparent *overall* rate of mutagenesis will depend on EtdBr concentration not only by virtue of its participation in the initial, but also in later steps in the reaction sequence. The latter are responsible for the sequential degradation of mtDNA upon transfer of mutagenized cells to complete medium, resulting in the eventual formation of [*rho*<sup>0</sup>] and [*rho*<sup>-</sup>] cells and may involve mitochondrial DNase(s), which are stimulated by EtdBr *in vitro* (15,27).

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