PETITE INDUCTION AND RECOVERY IN THE PRESENCE OF HIGH LEVELS OF ETHIDIUM BROMIDE

by

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Received May 31,1975

Ethidium bromide in the 80 to 100 ug/ml concentration range causes a rapid induction of respiratory deficient petite mutants in the yeast Saccharomyces cerevisiae. Within 15-30 minutes, over 95% of the cells have become petite. During the continued exposure to ethidium bromide for a further 30-60 minutes, recovery of respiratory competence is observed in 60 to 70% of the cells. Prolonged exposure then leads to a final irreversible phase of petite formation. Both the initial petite induction and the recovery phase are strain dependent making genetic investigation of these processes possible.

INTRODUCTION

Ethidium bromide[1] has been shown to be an effective mutagen of mitochondrial DNA (mtDNA) in Saccharomyces cerevisiae (1). Micromolar concentrations of EBr quantitatively convert wild type (ρ^+) yeast cells to cytoplasmic petite (ρ^-) forms. The molecular events accompanying petite induction have been partially delineated, particularly by the studies of Grossman et al. (2), Goldring et al. (3), and Bastos and Mahler (4,5). The events include the blocking of mtDNA synthesis by EBr, formation of a complex between the dye and mtDNA and cleavage of the mtDNA into fragments. Continued exposure of growing cells to EBr leads to complete loss of mtDNA from the cells (3,6).

Perlman and Mahler (7) have demonstrated that conditions could be selected for EBr treatment that would induce formation of a pre-mutational metastable state in yeast cells. This state could be maintained during storage at temperatures near 0°C. Cells in this state could then continue through the process of ρ^- formation at 30° or be restored to the ρ^+ state by raising the temperature to 45°C. These observations were interpreted to indicate reversible interaction of EBr with mtDNA at a membrane site as a step leading to ρ^- formation. This interaction was reversed by high temperature. More recent observations from the same laboratory have shown that the time course of formation of the "premutational state" is generally similar to that of

1 (Ethidium bromide = EBr)

EBr induced breakdown of mtDNA (8) and that this may be important to the reversal.

We have now demonstrated that EBr at levels 10 to 20 times those normally employed in mutagenesis rapidly induces formation of petite cells, yet upon continued exposure to the dye, the cells regain respiratory competence. Still longer exposure times once more results in petite formation.

MATERIALS AND METHODS

The strains of Saccharomyces cerevisiae used were D243-4A and D253-3C obtained from Dr. F. Sherman, L410 from Dr. A.W. Linnane and S288C from Dr. R. Snow.

Cells were grown overnight at 30° to early stationary phase on 1% Difco Yeast Extract, 2% Oxoid peptone and 2% glucose (YEPG). A 0.1 ml aliquot of this suspension was inoculated into 10 ml of fresh medium and grown with vigorous shaking at 30° for 3 hours. Aliquots of a 2 mg/ml stock solution of EBr were added to the culture to obtain solutions at 10 and 100 ug EBr per ml, (26 and 260 uM).

At various times after EBr addition, aliquots of cells were removed, diluted and plated on agar plates containing 1.5% Agar, 1% Yeast Extract, 2% peptone, 0.1% glucose and 2% ethanol (petite plates). Plates were incubated for 3 days at 30° and scored for the fraction of petite cells on the basis of differential colony size. In some cases, this scoring procedure was verified by the tetrazolium overlay method (9).

RESULTS

Fig. 1 compares the effects of low and high concentrations of EBr on the induction of petites in logarithmically growing cultures of S. cerevisiae strain D243-4A. As has been demonstrated repeatedly by other workers, treatment with 10 ug/ml EBr rapidly produces petite cells. Treatment times as short as two hours result in nearly 100% petite formation. When EBr concentrations in the range of 80 to 100 ug/ml are used even more rapid petite induction is noted, frequently with greater than 90% petite induction observed within 15 minutes. Under these conditions, however, continued exposure to the mutagen gives rise to a phase in which respiratory competence is recovered in 60 to 70% of the cells by 120 minutes incubation. Finally a third phase of irreversible petite formation is observed. In such experiments, virtually all ρ^+ cells obtained at longer exposure times must have passed through a ρ^- stage. Selection of any such recovered ρ^+ cells and repeated treatment of the progeny of these cells with high levels of EBr gives results identical with the initial experiment. Therefore, no permanent change in the susceptibility of cells to EBr is produced.

Loss of cell viability during exposure to either the low or high levels of EBr is minimal, Fig.1. The decrease in percentage of cells which are respiratory incompetent during the recovery phase therefore,

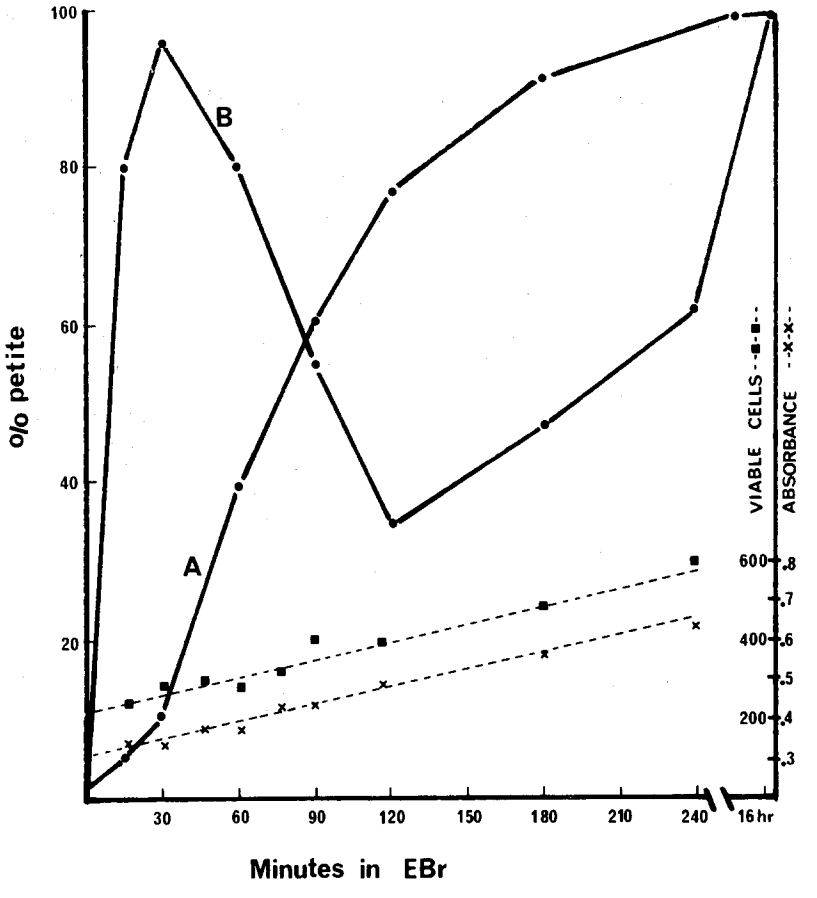

Figure 1. Time course of petite formation in S. cerevisiae strain D243-4A in the presence of 10 ug/ml (A), and 100 ug/ml (B) EBr. On the ordinate, percentage petite cells are expressed as percentage of total colonies counted on agar plates. Also shown is the viability of cells of strain D243-4A in cultures treated with 100 ug/ml EBr. Viability was determined by dilution, plating and counting colonies formed from aliquots of the EBr treated incubation mixture. The values so obtained are compared with measurements of absorbance at 660 uM on diluted samples of the incubation mixtures.

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may not be attributed to a selective killing of petite cells at high EBr concentrations.

Under the conditions used, observation of recovery of the ρ^+ state depends upon the continued presence of EBr at high concentration in the cell suspension. As shown in Fig. 1, cells diluted and plated after 15 minutes EBr treatment were ρ^-, while those exposed to EBr for 60 to 90 minutes before plating had largely recovered. When aliquots

of cells were removed after selected time intervals following exposure to 100 ug/ml EBr, washed with YEPG media to lower EBr concentration, incubated in YEPG for 180 minutes and then plated, the recovery pattern was similar to that for cells diluted and plated directly (Fig.2). However, similar suspensions of cells that were washed and incubated in water prior to plating yielded a higher fraction of petites than in the control. This indicates either that residual EBr in the cells causes further petite formation in the absence of an energy supply or that recovery in this phase is dependent on continued presence of energy which is normally obtained from the media of the plates. When petite formation was initiated in 10 ug/ml EBr, a rapid increase in rate of petite formation could be observed upon increasing the EBr concentration to 100 ug/ml (Fig.3). A recovery phase followed this period of rapid petite formation, but in no case did recovery produce

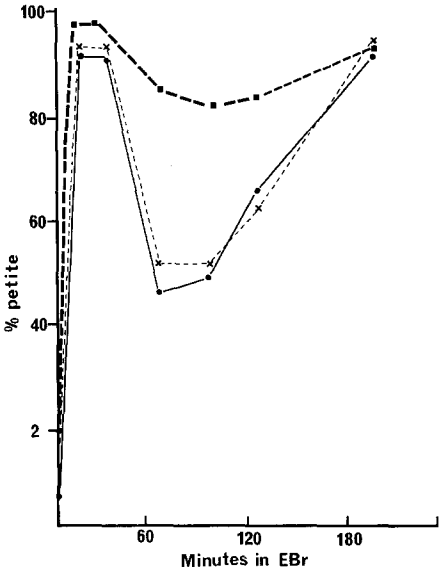

Figure 2. Petite formation in cultures of cells treated with EBr then washed and subsequently incubated in different solution conditions. 100 ug/ml EBr was added to a logarithmically growing culture of D253-3C at 30°C. At the times, 0, 15, 30, 60, 90, 120 and 180 minutes, a sample was withdrawn, diluted and plated immediately. Aliquots were also removed at each time, centrifuged and washed 3 times with pH 6, 0.001 M sodium phosphate buffer or with YEPG media. Washed cells were then held at 30° for 180 minutes before diluting and plating on petite plates. Cells plated immediately are represented by -●-●-. Cells washed and suspended in YEPG are shown by -×-×-, and in phosphate buffer by -■-■-.

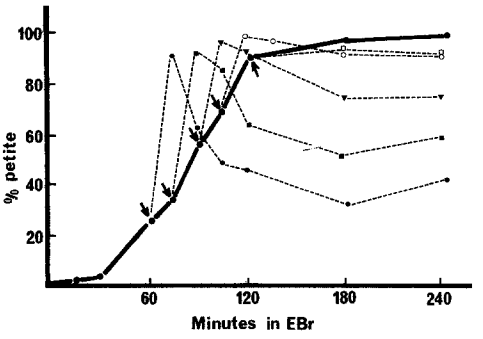

Figure 3. Effects of increasing EBr concentration after petite formation has been initiated at 10 ug/ml EBr. The concentration of EBr in various aliquots of cells was increased from 10 ug/ml to 100 ug/ml at the times indicated by the arrows. Incubation at 30° was then continued and the fraction of petite cells in the cultures were determined as in Methods. -●-●-, 10 ug/ml EBr; - - - -, 100 ug/ml EBr.

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a greater proportion of ρ^+ cells than was present in the culture when the EBr level was increased.

Both the rapid initial petite formation and the recovery of respiratory competence in the presence of high levels of EBr are strain dependent. A number of strains have been found to show behavior similar to that of D243-4A (Fig.4). Two other classes have also been observed, those such as S288C which are very resistant to petite formation under these conditions and strains such as L410 which exhibit the rapid petite formation phase but fail to show any recovery.

DISCUSSION

Since the demonstration by Slonimski *et al.* (1) that EBr treatment induced a complete transformation of all cells in a yeast culture into respiratory deficient cytoplasmic petites, several studies have been reported which aid in the interpretation of the molecular events which take place following EBr treatment of yeast cells. Goldring *et al.*(3) and Grossman *et al.*(4) demonstrated that EBr caused a rapid breakdown and eventual loss of mtDNA. From this a general model emerged in which the mtDNA is fragmented in the presence of EBr and subsequently fragments are lost either by further fragmentation or by segregation (10) This gives rise to strains that lack a complete complement of mtDNA sequences, fail to produce mitochondrial proteins, and are respiratory deficient. More recently Bastos and Mahler (5) have used radioactively

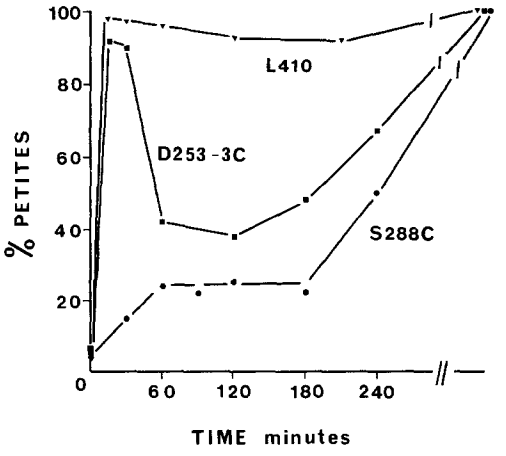

Figure 4. Strain differences of the effects of 100 ug/ml EBr on petite induction. All strains were treated under identical conditions as in Materials and Methods. The curves correspond to strains L410, D253-3C, and S288C.

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labelled EBr to define some of the steps involved in this process. Their experiments established that EBr rapidly becomes firmly bound to mtDNA while scission of the DNA occurs. An energy requiring and probably ATPase dependent degradation of mtDNA into acid soluble nucleotides follows.

Many kinetic mechanisms may be proposed to interpret the experimental data presented here and to relate these observations to the framework of existing models for EBr induced petite formation. The main finding to be considered is that the proportion of ρ^- cells in the culture is rapidly increased in the presence of EBr, yet with continued exposure to EBr, ρ^+ cells accumulate again. To account for this, it is necessary to propose an intermediate cellular state in petite formation that can either be reversed to form ρ^+ cells or react further to irreversibly form petite mutants, such that:

$$\rho^+ \longleftrightarrow [\rho^-]^* \rightarrow \rho^- \rightarrow \rho^o$$

where $[\rho^-]^*$ is used to indicate yeast cells in a state capable either of reversal to ρ^+ or irreversible formation of ρ^-. Conversion of ρ^+ to $[\rho^-]^*$ is proposed to correspond to the first phase of the reaction sequence, the recovery phase then represents a reformation of ρ^+ (or possible $[\rho^+]^*$ as suggested below). The third phase of the reaction represents the formation of ρ^- and ρ^o. The concentration of EBr is shown

by Fig.1 to be a determinant in the rate of production of $[\rho^-]^*$ from ρ^+. The subsequent fate of $[\rho^-]^*$ is also a function of EBr concentration (Figs. 1,2 & 3). At high concentrations a reversal to form ρ^+ from $[\rho^-]^*$ occurs, following at least superficially, first order kinetics. At low EBr concentrations the reformation of ρ^+ is not detected, and $[\rho^-]^*$ is converted to ρ^-.

If reaction conditions are held constant one would not see a decrease followed by an increase in the number of ρ^+ cells in a reaction scheme of the type proposed above. However, due to observed slow penetration the intra-cellular concentration of EBr increases over a period of at least 30 minutes. Thus, these results may be accounted for, in part, if relatively low levels of EBr stimulate rapid formation of the $[\rho^-]^*$ state, and as more EBr is taken up this reaction is partially inhibited. If $[\rho^-]^* \rightarrow \rho^-$ is relatively slow this would allow a transient accumulation of ρ^+ cells.

In the molecular reaction sequence proposed by Bastos and Mahler (5) the initial step of petite formation was an EBr stimulated nuclease action breaking the DNA into large fragments. Paoletti et al.(11) have demonstrated the presence of an EBr activated nuclease in mitochondria and in our laboratory we have demonstrated that this enzyme activity is also inhibited at high EBr levels (12). An enzyme of this type could account for the initial steps in the reaction sequence.

A simple alternative explanation of the time course of loss and reformation of ρ^+ cells results from the proposal of additional intermediates in the reaction scheme. For example, if one proposes formation of a $[\rho^+]^*$ state from $[\rho^-]^*$ at high EBr, the only further restrictions that need be imposed are that $[\rho^+]^*$ has limited susceptibility to further mutagenic action by EBr and that subsequent dilution and plating of $[\rho^+]^*$ cells yields ρ^+ colonies. The data of Fig. 3 offer some support for this type of mechanism since recovery never yields ρ^- levels lower than those induced initially by 10 ug/ml EBr.

Observation of the recovery phase appears to be dependent upon the accumulation of an intermediate such as the proposed $[\rho^-]^*$. At low levels of EBr the conversion $[\rho^-]^* \rightarrow \rho^+$ may take place but not be detectable due to a reduced extent of $[\rho^-]^*$ accumulation. This suggestion is supported by the data of Fig.3 where the higher percentage of petites induced by 10 ug/ml EBr prior to mutagenesis in 100 ug/ml, the lower is the detectable amount of recovery to ρ^+.

The question of what molecular events take place during the recovery phase must be answered with further experimental investigation.

However, the multiphasic petite formation curves presented here are consistent with the reaction sequence of Bastos and Mahler (5) if one proposes that the initial phase of petite formation corresponds to the EBr dependent cleavage reaction; the recovery phase corresponds to reassembly of the large mtDNA fragments; and the final phase of petite induction is a measure of further ATP dependent fragmentation of DNA.

Three important aspects of the EBr induced mutation of mtDNA are made amenable to study as a result of these observations. First, the enzyme system functioning in the initial phase of ρ^- formation is activated by EBr and may also be inhibited by very high levels of EBr. Second, the recovery system in this case is not simply a reversal of a membrane-DNA complex induced by temperature increase as was suggested by Perlman and Mahler (7) for their earlier studies. The recovery system can most simply be postulated to be a repair of mtDNA fragmented by an initial EBr dependent scission. Study of this system may therefore yield valuable information in a eukaryotic repair-recombination system. Third, genetic differences in strains carrying out each of the various phases of the reaction sequence will provide information on genetic control over each process and provide material for differential analysis of the components involved. Studies are currently proceeding on each of these areas.

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