A PREMUTATIONAL STATE INDUCED IN YEAST BY ETHIDIUM BROMIDE

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SUMMARY

Ethidium Bromide (EB) has been known to be an effective and irreversible mutagen in converting wild-type (ρ^+) yeast cells to the cytoplasmic petite (ρ^-) state. The effects of EB may be reversed by incubation of treated cells in phosphate buffer at elevated temperatures ($\geq 42°$). The temperature dependence of mutagenesis is different from that of reversal. It is proposed that reversal is related to a heat-induced alteration in a membrane-mtDNA-EB complex.

Ethidium bromide (EB) is a most efficient mutagen in bringing about the formation of cytoplasmic petites, a mitochondrial mutation (1,2) in Saccharomyces cerevisiae. Even transient exposure of wild type (ρ^+) cells to EB, in the micromolar range of concentration, is sufficient to initiate a series of events that culminate in their quantitative conversion to the ρ^- genotype (3,4), resulting in the characteristic respiratory deficiency (5-8). On the molecular level such exposure results in a transient arrest of the ability of the mitochondria to duplicate their DNA, coincident with the destruction of the parental strands; this is followed by the formation of aberrant types of progeny mtDNA associated with and probably responsible for the ρ^- genotype (9). Phenotypically the initial respiratory lesion appears to be a block in the formation of cytochrome oxidase (as well as the corresponding spectroscopic entity cytochromes aa_3), leading to a cessation of growth on nonfermentable energy sources after about two generations of growth; the emergence of the other aspects of the complete ρ^- phenotype are slower and require many generations for their completion (4 and in preparation).

In general these various sequences of events have been considered, and appear to be, in fact, irreversible consequences triggered by the initial exposure of the cell to EB. However, we now wish to report preliminary evidence that upon

exposure to EB these cells – and presumably therefore their mitochondrial genome – have been transformed into a metastable state which can be maintained, under the appropriate conditions, for extended periods of time. In this state cells not only retain their complete ability to generate ρ^- clones, but can also be quantitatively restored to the ρ^+ state simply by raising the temperature. These observations recall the "pre-mutational state" described earlier by Ephrussi et al. (10) during the development of the ρ^- genotype induced by the suppressive factor.

METHODS AND MATERIALS

A diploid strain of Saccharomyces cerevisiae (var. Fleischmann) was used. Similar results were also obtained with a haploid strain (IL-8-8C) provided by Prof. P. P. Slonimski.

Cells were grown on a semisynthetic medium containing 3% lactate as carbon source as described in refs. 4 and 9. Cell number and the proportion of petites in a population was measured by plating a suitably diluted aliquot of cells on 2% agar plates containing medium and 0.1% glucose plus 3% lactate. On these plates ρ^+ cells may be distinguished from ρ^- ones by their larger size. In preliminary experiments this scoring procedure was verified by the tetrazolium overlay method (11). On these plates, no mosaic ("petites abcédées") colonies are found (cf. 10,12,13).

Other experimental details are described in the figure legends.

Ethidium bromide was purchased from Sigma Chemical Co. Yeast extract and agar were purchased from Difco Labs. All other chemicals were of reagent grade.

RESULTS

Mutagenesis by EB is thermoreversible. This phenomenon was first observed on diluting cells subjected to EB treatment into soft agar (0.7%) or phosphate buffer at elevated temperatures ($\sim 45°$) for brief periods of time. It is most conveniently measured, however, by filtering and washing such EB treated cells in the cold, resuspending them in appropriate media and determining the rate of reappearance of wild-type colonies as a function of various parameters. In Fig. 1 is shown a typical experiment in which both the initial mutagenesis by EB

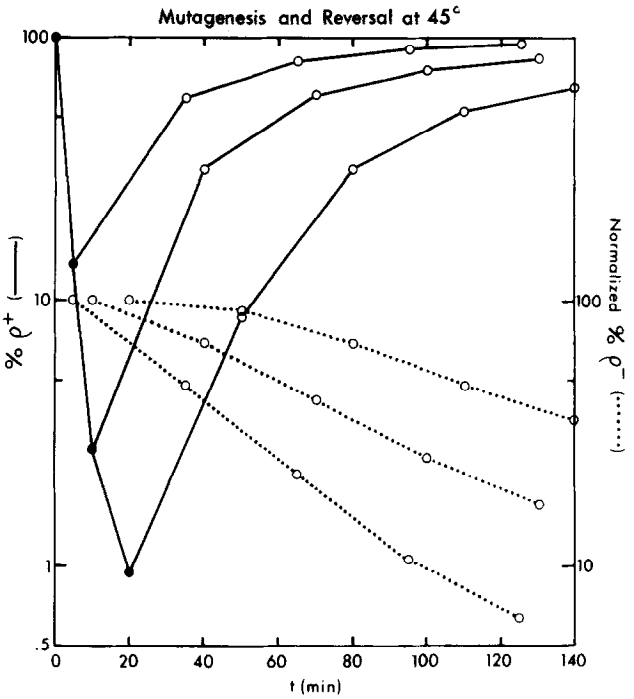

Figure 1. Cells of S. cerevisiae were grown at 30° on medium containing 3% lactate as carbon and energy source to a turbidity (A_{600}) of 0.1 (~ 2 x 10^6 cells/ml). A 50 ml aliquot of the culture was placed in a sterile 250 ml foil covered flask which was then placed in a water bath maintained at 45° for a 10 min pre-incubation. Then EB was added from a fresh stock solution to a concentration of ~ 10 μg/ml; the actual concentration used (27.2 μM) was measured by determining the A_{480} of the medium after removal of the cells. After 5 min of EB treatment an 8 ml aliquot of culture was removed, filtered on a Millipore filter (0.45 μ pore size), washed with four 5 ml aliquots of ice cold 0.1 M phosphate buffer (pH 6.5), and resuspended in 5 ml of the same cold buffer; this procedure took around 2 min to complete. The washed cells were then diluted by 10^{-1} in cold buffer and then by 10^{-2} in buffer at 45° so that 0.1 ml of the 10^{-3} dilution contained 200-250 cells. The warmed cells were then plated (200-250 cells per petri dish) after being held for 30, 60, 90 and 120 min. Control samples (which had not been warmed) were plated before and after filtering. In a preliminary experiment it was found that cells (± filtration and wash) may be held in cold phosphate buffer, distilled water or medium for at least four hours with no change in their % ρ^+. Cells filtered and washed after 10 and 20 min of EB treatment were treated as described above. For each data point 600-800 colonies were examined.

Relative % ρ^- = 100 − % reversal = % ρ^- at t normalized to % ρ^- at the time of filtration.

(at 27.2 μM) and the reversal were performed at 45°, the former because mutagenesis as well as reversal (see below) is strongly temperature dependent. We see that under these conditions reversal is virtually quantitative within rather broad limits of prior mutagenesis, and that unlike the mutagenesis (3,4,14) it appears to follow (pseudo) first order kinetics without a lag.

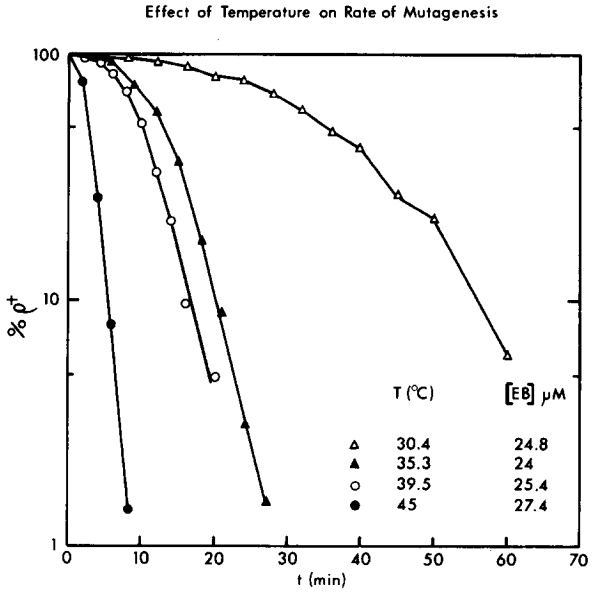

Figure 2. Cells were grown on 3% lactate medium to a turbidity of 0.4; they were diluted with fresh medium at 30.4, 35.3, 39.5 or 45° to a turbidity of 0.1 and then incubated in a water bath held at the appropriate temperature. Then EB was added to approximately 25 μM and mutagenesis measured by plating in triplicate suitably diluted (in cold water) aliquots periodically. For each set of data, 400-600 colonies were examined per data point. Where % ρ^+ was < 10%, two plates containing ten times the usual number of cells were scored for ρ^+ colonies so that 3000-4000 colonies were scored.

Reversibility shows a strong temperature dependence. As mentioned above, mutagenesis by EB is accelerated by increasing the temperature, during the initial exposure, over the whole range between 30 and 45°. This effect appears to be principally one on k', the apparent limiting first order constant, rather than on n, the number of apparent targets (15). In one typical experiment the half times in the first order region of the usual plot of log fraction ρ^+ survivors vs time were 1380, 498, 510 and 216 sec at 30.4, 35.3, 39.5 and 45.0°, respectively (Fig. 2).

In contrast no reversal at all is observed below 35°. Even at 42° it is very slow, but increases in rate very rapidly with small increments in temperature up til 48° (Fig. 3). Because of cell lethality, studies cannot be extended beyond this temperature. EB-treated, filtered and washed cells may be held in

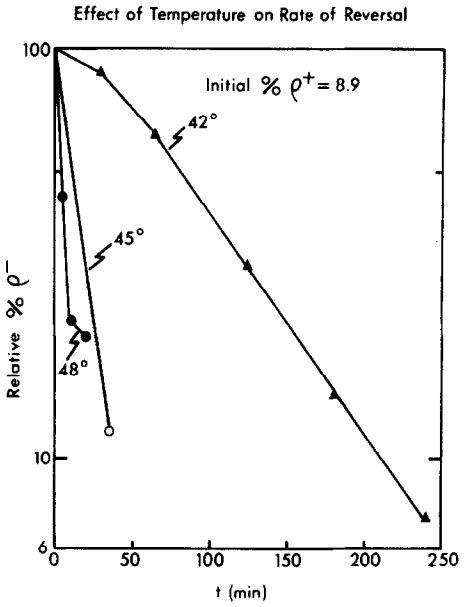

Figure 3. Cells grown on 3% lactate medium to A_{600} = 0.1 were treated with EB (22.9 μM) for 35 min at 30° (with shaking) before being filtered, washed, resuspended in cold buffer and diluted at one of three different temperatures. After various lengths of incubation at the elevated temperatures, cells were plated in triplicate. Each set of plates contained 900-1200 colonies. The initial % ρ^+ was determined by scoring 13,000 colonies. In preliminary experiments it was determined that diluted yeast cells may be held at 42, 45 and 48° for at least 240, 70 and 25 min, respectively, with no decrease in viable count, when incubated longer a gradual decrease in viable count occurs so that quantitative killing is effected after 150 min at 45° or 60 min at 48°. The kinetics of killing were not strictly reproducible so that, in certain experiments no killing was detected even after 120 min at 45° or 40 min at 48° while in others killing amounted to ~ 50%. Whenever killing occurred, these results were excluded from the analysis.

phosphate buffer at 0° for up to 24 hours with no loss in capacity to reverse. If they are not warmed no reversal is observed.

DISCUSSION

These results suggest the existence of a metastable entity produced by the interaction of mitochondria with EB which can undergo one of two alternative fates: either to render permanent the resultant damage and thus produce the ρ^- genotype, or else to remove its cause (and/or repair any effect produced) and retain the ρ^+ configuration. We will not be able to be more precise in defining either entity or process responsible without first establishing, as a minimum, a) whether the thermoreversible process affects all or only a few clonal descendant

of the cells mutagenized by EB during the initial exposure (we do know that reversal is obtained regardless of strain, haploid or diploid); b) whether the molecular effects and their phenotypic consequences known to be initiated by this treatment are similarly subject to reversal — and hence whether the latter affects the majority of the mitochondria of the target cell, and c) whether the EB sensitive target is to be identified with mtDNA or some other structure. Experiments along these lines are currently in progress.

Tentatively, however, our working hypothesis is that the heat sensitive reversal is not due to the elimination of some damage induced initially involving, say active photo- or dark repair synthesis of DNA (17-19). This is based on the fact that mutagenized, starved cells are as sensitive to thermoreversal as are actively metabolizing ones, that reversal is independent of prior physiological history of the cells affected (i.e. whether grown on glucose or lactate), and that the rate of reversal in buffer is as great or greater than that observed in growth medium. The simplest alternate model would postulate that the step affected is simply the dissociation of the complex between (presumably covalently circular) mtDNA and EB. However the qualitative and quantitative aspects of the dependence are not consistent with this view. Instead they suggest some form of a phase transition leading to the detachment of EB from the sensitive site by a "melting-off" process. A likely candidate is provided by the site of attachment of mtDNA to the mitochondrial inner membrane, a lipoprotein aggregate known to be capable of accomodating EB (20) and — by analogy to other lipoprotein membranes (21) — presumably undergoing phase transitions precisely in this temperature range. The association of DNA replication with and dependence on membrane attachment first postulated for bacteria by Jacob et al. (22) and amply documented for procaryotic organisms since then (see [23-26] for particularly persuasive recent reports) can be extrapolated readily to the mitochondrial system. In fact there is convincing evidence for efficient induction of the ρ^- mutation as a result of hereditary or transiently induced membrane abnormalities (27-28) including continued growth at elevated temperatures (reviewed in ref. 17). These observations are consistent with certain explicit models of mutagenesis proposed by ourselves (14) for EB as

well as by (19) for other mutagens, in terms of the removal of the replicative complex from its normal membrane site.

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