BYPASS BY PHOTOAFFINITY LABELING OF BLOCKED METABOLIC ACTIVATION OF ETHIDIUM: CONFIRMATION OF THE ROLE FOR COVALENT ETHIDIUM ATTACHMENT IN MITOCHONDRIAL MUTAGENESIS

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SUMMARY: Ethidium bromide (EB) is known as a highly efficient mutagen of mitochondrial DNA in yeast. The key step to mutagenesis is thought to be a covalent EB attachment to mitochondrial DNA. A light sensitive ethidium azide has been used to produce covalent attachment of ethidium to mitochondrial DNA under conditions where EB mutagenesis does not occur, i.e. at high glucose concentrations, and in the petite negative yeast K. fragilis. A bypass attachment of ethidium through photolysis of the azide was effective in inducing mutagenesis.

INTRODUCTION: Bastos and Mahler (1) have attributed the mutagenic effect of ethidium bromide (EB) (2) in mitochondrial DNA to covalent attachment of a drug metabolite, and consequential DNA degradation. They have supported this view with observations of covalent adduct formation in drug treated mitochondria; and with the finding that certain 'petite negative' strains, which cannot be mutagenized with ethidium do not exhibit covalent drug binding with attendant DNA degradation. They contend, therefore, that covalent drug binding is the key to mutagenesis.

Dependence of EB mutagenesis on some metabolic step in mitochondria is also suggested by the findings of Hollenberg and Borst (3) that petite induction can be inhibited at high glucose concentrations or after cell starvation. Both conditions may lead to reduced mitochondrial enzyme levels and less metabolic activation of ethidium (4).

Recently we have synthesized a light sensitive azide analog of EB (5). Photolysis of the DNA bound azide results in covalent drug attachment *in vitro* and *in vivo*. Moreover, correlated with its selective binding

to mitochondrial DNA *in vivo*, photolysis of the drug in yeast leads to highly efficient production of petite mutants. These experiments have indicated that the increased covalent attachment of the ethidium analog to mitochondrial DNA following photolysis provokes the increase in petite induction in yeast.

The experiments reported here were designed to test whether the resistance to petite induction in glucose repressed or starved yeast and in a petite negative strain could be circumvented by the use of this light sensitive analog. Covalent drug attachment by photolysis without a requirement for any intervening metabolic step was expected to induce mutagenesis under these conditions if a covalent attachment of EB indicates the mutagenic processes.

MATERIALS AND METHODS: *Saccharomyces cerevisiae* var *ellipsoideus* (ATCC #4098) was grown to stationary phase in 1% yeast extract, 2% peptone, and 1% glucose (YEP). Cells were washed in .067 phosphate buffer, pH7, and suspended in yeast nitrogen base with either 0.5% or 10% glucose and 5 μM ethidium azide. After incubation in the dark for 90 min. each cell suspension was divided and one sample was exposed to light (30 watt GE fluorescent lamp) while the other sample was held in the dark. After appropriate time intervals samples were spread onto YEP 1.5% agar. After 2 days of growth, colonies were scored for survival and petite induction using a tetrazolium overlay (6).

The petite negative yeast, *Kluyveromyces fragilis* was kindly provided by A. A. Luha (7). The culture was grown for 24 hours in YEP, washed and suspended in phosphate buffer and 5 μM ethidium azide in the dark for 24 hours. After this period the cell suspension was exposed to a 30 watt GE fluorescent light. After each time interval as shown in Figure 2 samples were withdrawn and spread onto YEP 1.5% agar, allowed to grow into visible colonies, and checked for survival and petite induction.

RESULTS AND DISCUSSION: Our previous experiments (5) have indicated that

the azide analog of EB in the dark, without photolysis, produces petites with the same probable mechanism as EB. In the present experiments the mutagenic response to the EB azide in the dark at 0.5% and 10% glucose was also the same as that demonstrated by Hollenberg and Borst (3) for EB alone. Figure 1 shows that the azide at 5 μM in the dark produced no petites in 10% glucose after 5 hours while in 0.5% glucose the induction of petites gradually rises with time. In contrast, photolysis in the light produced petites at both glucose concentrations with an immediate induction in 0.5% glucose and an evident lag in 10% glucose. The sharp rise of induction in 0.5% glucose may have resulted from metabolic plus photolytic activation of the EB analog, whereas induction at 10% glucose depends solely on photolytic attachment of the ethidium to the DNA. These experiments support the view that glucose repression prevents ethidium metabolic drug activation and blocks mutation induction. Use of the azide analog in the light effectively bypassed this block by providing photolytic drug activation for covalent attachment.

Luka et al. (7) demonstrated a reversible effect of EB in K. fragilis. Although the replication of mitochondrial DNA appeared to be inhibited in

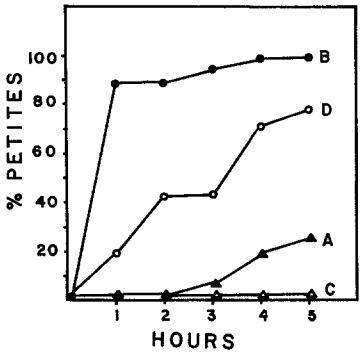

Figure 1. Effects of glucose concentration on petite mutation induction by ethidium azide. Experiment done as described in text. Curve A = 0.5% glucose, dark; B = 0.5% glucose, light; curve C = 10% glucose, dark; curve D = 10% glucose, light.

the presence of EB, an irreversible degradation of mitochondrial DNA did not take place, with recovery to normal levels after the removal of EB. Bastos and Mahler (1) observed that covalent attachment of EB to mitochondrial DNA did not take place in the petite negative strains they investigated, and they postulated that covalent attachment must precede the degradation of mitochondrial DNA. In order to test this proposal, K. fragilis was grown and harvested as described above, and suspended in buffer and 5 μM ethidium azide. The cell suspensions were held in the dark for a period of 24 hours to allow drug binding to mitochondrial DNA and any metabolic activation which might occur, following which they were exposed to light. Figure 2 indicates that no cell lethality occurred during the holding period, but as the bound drug was activated by light cell death occurred. No petite mutants were produced at any point as checked with the tetrazolium technique. A similar drug treatment with the petite positive strain S. cerevisiae has been shown to produce greater than 50% petite mutants with minimum cell lethality (5). The lethality produced in K. fragilis by covalent drug attachment to the mitochondrial DNA may be explained by the inability of these yeast to survive without intact mitochondrial DNA.

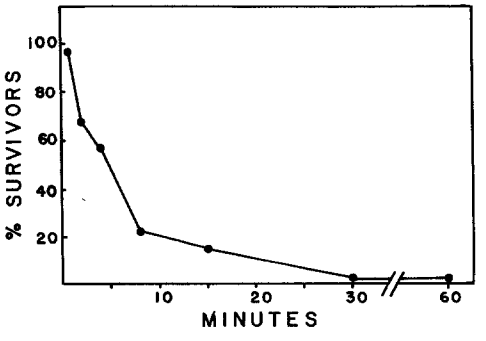

Figure 2. Effects of ethidium azide on survival of K. fragilis in light. Experiment performed as in text.

All of the above experiments demonstrated that ethidium mutagenesis of mitochondrial DNA may be induced by light activated attachment of an ethidium analog to the DNA. This "bypass" attachment, under conditions where metabolic activation and attachment do not occur, is also effective in producing mutagenesis. These results support the conclusion that covalent drug attachment to mitochondrial DNA is required as the initial step in mutagenesis.

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