# PREFERENTIAL SYNTHESIS OF YEAST MITOCHONDRIAL DNA IN α FACTOR-ARRESTED CELLS

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SUMMARY: α factor is a diffusible substance produced by S. cerevisiae cells of the α mating type which inhibits cell division (1) and the initiation of nuclear DNA synthesis (2) in cells of the a mating type. In this report, it is shown that mitochondrial DNA synthesis continues at a normal rate in a cells for at least 6 hours in the presence of α factor, resulting in a 5-fold increase in the amount of mitochondrial DNA per cell. The continued synthesis of mitochondrial DNA in the absence of nuclear DNA synthesis allows specific labeling of yeast mitochondrial DNA.

## INTRODUCTION

Duntze, MacKay and Manney (3) reported that cells of the α mating type of Saccharomyces cerevisiae produced a hormone-like substance which induced cells of the a mating type to undergo morphological changes characteristic of the mating process. Further characterization of the effects of α factor on a cells showed that α factor inhibited cellular DNA synthesis and cell division but did not inhibit protein or RNA synthesis (1). The effect of α factor on DNA synthesis has been shown to be an inhibition of the initiation of nuclear DNA synthesis rather than an inhibition of on-going DNA replication (2). Since α factor arrests a cells as unbudded mononucleate cells prior to the initiation of nuclear DNA synthesis, it has been hypothesized that α factor may play a role in the synchronization of haploid cell cycles in preparation for conjugation (2).

A small amount of DNA synthesis has been observed in α factor-arrested cultures of a cells (1), suggesting the possibility that mitochondrial DNA could be synthesized in the presence of α factor. This report demonstrates that mitochondrial DNA is synthesized in α factor-arrested cells for at least 6 hours. A preferential synthesis of mitochondrial DNA in S. cerevisiae has previously been shown to occur under conditions in which protein synthesis is inhibited (4).

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## MATERIALS AND METHODS

Yeast strains and media. The haploid strain of Saccharomyces cerevisiae A364A (provided by Dr. L. Hartwell, University of Washington) was used in these experiments. A364A has the genotype a, adel, ade2, ural, tyr1, his7, lys2, gal-1. A derivative of a diploid strain A364A D-5 (5), which lacked mitochondrial DNA, was provided by Dr. C. Newlon (University of Washington). α factor was prepared from the wild type α strain X2180-1B (described in reference 2).

Synthetic medium as described by Hartwell (5) was used. Cultures were grown aerobically at 22° C.

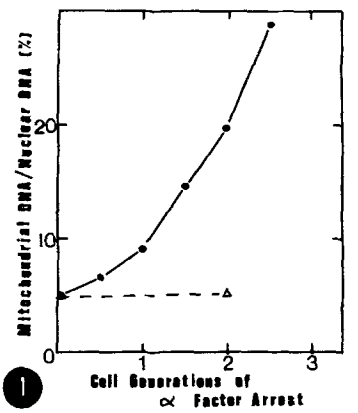
Radioactive labeling and spheroplast formation. RNA and DNA were labeled by addition to the medium of (6-$^{3}$H) uracil (New England Nuclear) to give 2 mCi/mg or (2-$^{14}$C) uracil (Schwarz) to give 0.025 mCi/mg. In experiments in which the cells were to be uniformly pre-labeled, cells were grown in the presence of label for at least 4 cell generations.

Cells for spheroplasting were harvested and washed two times with water. The washed cells were suspended at $5 \times 10^{8}$ cells/ml in a spheroplasting solution containing 0.1M sodium citrate buffer (pH 5.8), 0.9M sorbitol, 0.01M sodium-EDTA, 0.03M mercaptoethylamine, and 2% (v/v) glusulase (Endo) and incubated at 37° for 30 minutes (6).

α factor preparation. α factor was a gift from L. Hereford (Yale University). The α factor was prepared by the procedure of Bücking-Throm, Duntze, Hartwell and Manney (2).

Treatment of cells with α factor. α factor was added to an exponentially growing culture of A364A (approximately $2 \times 10^{6}$ cells/ml) and the culture vigorously shaken. Since a cells can reverse spontaneously from α factor arrest (1), sufficient α factor was added to ensure that the cells remained unbudded during the course of the experiment. For experiments in which cultures were to remain arrested with α factor for 6 hours or longer, approximately one volume of α factor (prepared by the procedure of Bücking-Throm et al (2)) was added to 20 volumes of medium.

Preparative density equilibrium gradients. $^{3}$H-labeled yeast spheroplasts were lysed by addition of 1 volume of 5% sodium dodecyl sarcosinate (Sarcosyl) to 5 volumes of spheroplasts in spheroplasting solution (total volume was usually between 0.25 ml and 1.0 ml). 0.2 ml of this solution was added to 5 ml of saturated CsCl. In most experiments, $^{14}$C-labeled DNA from the yeast strain lacking mitochondrial DNA was added as a density marker for yeast nuclear DNA. The density of the solution was adjusted to 1.690 g/ml by addition of water and 5 ml was transferred to a centrifuge tube.



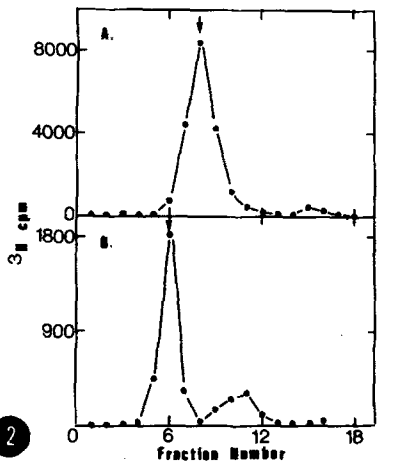




Figure 1. Kinetics of synthesis of mitochondrial DNA in the presence of α factor. A uniformly 3H-labeled culture of A364A was split into two subcultures. α factor was added to one culture, samples were removed at 75 minute intervals, and the DNA from each sample banded to density equilibrium in preparative CsCl gradients. The proportion of mitochondrial DNA (density of 1.683 g/cc) to nuclear DNA (1.699 g/cc) was determined for each gradient by measuring the areas under each peak. The culture which was not treated with α factor was incubated under the same growth conditions as the α factor-treated culture and was sampled after 300 minutes.
(●) culture with α factor (△) culture without α factor

Figure 2. Preparative density gradient profiles of DNA isolated from a uniformly $^{3}$H-labeled culture of A364A growing exponentially at a cell density of $2 \times 10^{6}$ cells/ml (Figure 2 (A)) and the same culture 2.5 cell generations after the addition of α factor. These profiles are part of the experimental analysis described in the legend to Figure 1. The arrow in each gradient profile represents the position of nuclear DNA determined with $^{14}$C-labeled DNA from a mitochondrial DNA-less yeast strain centrifuged in the same gradient. Buoyant density in these gradient profiles (and those shown in Figure 4) increases with decreasing fraction numbers since the gradients were collected from the bottom of the centrifuge tubes.

Centrifugation was performed in a Spinco Type 40 or 50Ti rotor at 33,000 rpm and 20° for 48 hours. Fractions were collected and the amount of radioactive label in each fraction was determined as described previously (6).

Analysis of DNA by analytical ultracentrifugation. DNA was extracted from $2 \times 10^{8}$ cells by crushing in an Eaton press using the procedure described by Williamson, Moustacchi and Fennell (7). The DNA was analyzed by analytical ultracentrifugation in a CsCl gradient, following the procedure of Williamson et al (7). Ultraviolet absorption pictures of the gradients were scanned with a Joyce-Loebel densitometer. The areas under each peak on the densitometer tracings were used to calculate the relative amounts of nuclear and mitochondrial DNA.

## RESULTS AND DISCUSSION

Net synthesis of mitochondrial DNA in a cells arrested with α factor

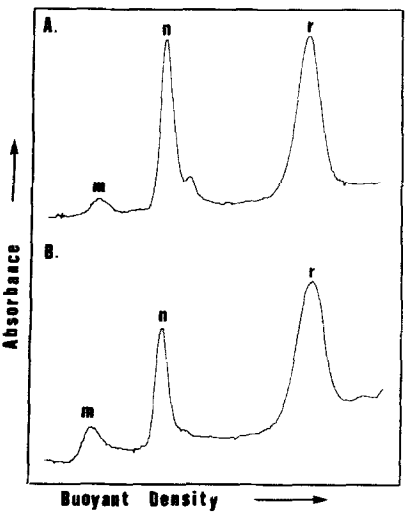



Figure 3. Densitometer tracings of analytical isopycnic CsCl gradients from a culture of A364A in early exponential phase ($2.5 \times 10^6$ cells/ml) before addition of α factor (Figure 3 (A)) and after 3 cell generations of treatment with α factor (Figure 3 (B)): m, mitochondrial DNA; n, nuclear DNA; r, reference DNA. The reference DNA is Micrococcus lysodeikticus DNA (density 1.731 g/cc).

was demonstrated by the following experiment. A culture of A364A which had been pre-labeled with labeled uracil for 12 hours was split into two cultures. α factor was added to one culture and both cultures were incubated with radioactive uracil. Samples were removed from the α factor-treated culture at 75 minute intervals. Since the generation time of an unarrested culture of A364A under the same growth conditions is 150 minutes, these 75 minute intervals correspond to half generation times. The culture which received no α factor was sampled after 300 minutes. Samples were analyzed by isopycnic banding in CsCl. The ratio of mitochondrial DNA to nuclear DNA for each of these samples as a function of the time at which α factor was added is shown in Figure 1.

Since α factor allows completion of nuclear DNA synthesis in cells which have initiated synthesis but blocks further initiation (2) and since nuclear DNA synthesis occupies approximately one-quarter of the yeast cell cycle (8), nuclear DNA synthesis stops soon after the addition of α factor to an asynchronous culture (2). Thereafter, the increase in the ratio of mitochondrial DNA to nuclear DNA is a measure of the net synthesis of mitochondrial DNA. The ratio of mitochondrial DNA to nuclear DNA, as shown in Figure 1, continues to rise in the α factor-arrested culture for at least 375 minutes after α factor has been added. Furthermore, for each 150 minute interval (corresponding to one generation time in an unarrested culture) after

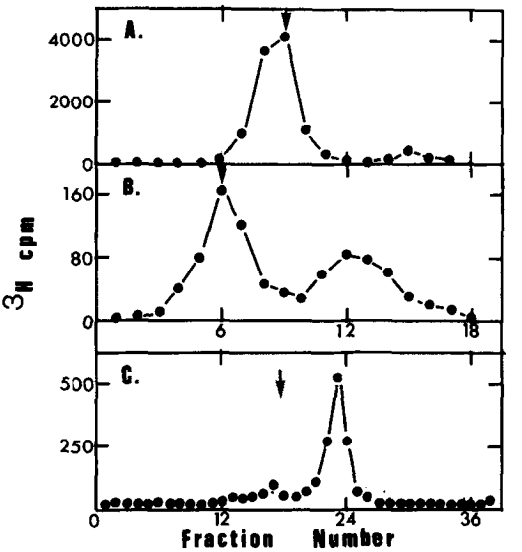



Figure 4. Preferential labeling of mitochondrial DNA during α factor treatment of a cells. In all gradients, the arrow represents the buoyant density of yeast nuclear DNA.

4 (A). Preparative density gradient profile of DNA from a culture of A364A which was labeled with $^3$H-uracil for 12 hours before addition of α factor. The cells were then incubated with α factor and $^3$H-uracil for 150 minutes before analysis.

4 (B). Preparative density gradient profile of DNA from a culture of A364A in which α factor was added to the culture at the same time as $^3$H-uracil. The culture was grown for 150 minutes after α factor and label had been added before gradient analysis.

4 (C). Preparative density gradient profile of DNA from a culture of A364A pre-treated with α factor. α factor was added to the culture 150 minutes before the addition of label. After the $^3$H-uracil was added, the cells were incubated for 150 minutes more before they were harvested for analysis.

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the first 75 minutes, the ratio doubles, indicating that the rate of synthesis of mitochondrial DNA is normal. This ratio does not double during the first 150 minutes, presumably because some yeast cells are completing nuclear DNA synthesis during this interval.

In the sample taken from the α factor-arrested culture after 375 minutes, the ratio of mitochondrial DNA to nuclear DNA was 0.29. The density gradient profile of DNA from this sample (Figure 2 (B)) should be compared to the profile of DNA from the asynchronous uniformly-labeled culture which was harvested before addition of α factor (Figure 2 (A)) which had a mitochondrial DNA to nuclear DNA ratio of 0.05. The DNA sample which was taken after 300 minutes from the culture which was not exposed to α factor had the same proportion of mitochondrial DNA (0.05) as the sample taken at the beginning of the experiment. Consequently, the preferential increase in mitochondrial

DNA in the α factor-treated culture is not simply a response to extended incubation.

The net synthesis of mitochondrial DNA observed by incorporation of label was verified by analytical ultracentrifugation. The ratio of mitochondrial DNA to nuclear DNA in an unarrested culture of A364A was 0.07 (Figure 3 (A)). After the culture had been exposed to α factor for 3 cell generations, the ratio of mitochondrial DNA to nuclear DNA was 0.42, as shown in Figure 3 (B).

Two results support the idea that the increase in the observed ratio of mitochondrial DNA to nuclear DNA does not represent differential recoveries of these two species of DNA during extraction. First, the recovery from the preparative CsCl gradients was greater than 80% and approximately the same for all samples. Second, if α factor inhibits the initiation of nuclear DNA synthesis but allows mitochondrial DNA synthesis to procede normally, the ratio of label in these two species of DNA should change depending on the labeling regime. When cells were uniformly labeled before addition of α factor, after 150 minutes of incubation of the cells with α factor and label, 9% of the label is in mitochondrial DNA (Figure 4 (A)). If label was added at the same time as α factor (without pre-labeling) and the culture incubated for 150 minutes, 50% of the label was in mitochondrial DNA (Figure 4 (B)). Finally, when cells were pre-treated with α factor for 150 minutes and then labeled in the presence of α factor for 150 minutes, almost all the label was in a single peak of mitochondrial DNA (Figure 4 (C)). These results are consistent with the hypothesis that mitochondrial DNA is synthesized preferentially in α factor-arrested cells and inconsistent with the hypothesis that the changes in ratio observed in the experiments studying net synthesis were due to differential recoveries of mitochondrial and nuclear DNA. These experiments demonstrate that α factor may be used to label yeast mitochondrial DNA specifically. Since α factor inhibition is reversible, the replication and segregation of this specifically-labeled DNA can be studied.

Since these experiments have shown that the net amount of mitochondrial DNA per cell increases by more than a factor of 4 in the presence of α factor, mitochondrial DNA molecules can initiate DNA synthesis in the presence of α factor. Since the rate of mitochondrial DNA synthesis increases exponentially during treatment with α factor, it is likely that DNA molecules replicated in the presence of α factor have the capacity to re-initiate DNA synthesis and replicate again in the presence of α factor. The observation that mitochondrial DNA synthesis occurs at a normal rate for the equivalent of 2.5 cell generations in the absence of nuclear DNA synthesis suggests that these two systems are relatively independent.

Grossman, Goldring and Marmur (4) found that although inhibition of protein synthesis by cycloheximide (or several other methods of protein synthesis inhibition) stopped nuclear DNA synthesis within 15 minutes, mitochondrial DNA synthesis continued for several hours. The net amount of mitochondrial DNA synthesized after 4 to 6 hours represents a doubling. The lower amount of net synthesis and the decreased rate of synthesis of mitochondrial DNA after prolonged exposure to cycloheximide compared to that synthesized after α factor arrest may reflect a requirement for protein synthesis for normal rates of mitochondrial DNA replication. Protein synthesis is not inhibited by α factor (1).

Hartwell (9) has shown that one of the events occuring during the mating response before cell fusion is a synchronization of the two haploid cell cycles. At the time of fusion, both cell types are unbudded and mononucleate. Since α factor arrests a cells as unbudded, mononucleate cells prior to the initiation of nuclear DNA synthesis, Bücking-Throm et al (2) proposed that α factor was one element required to ensure this synchrony. Our results suggest that although the synchronization of nuclear DNA synthesis appears to be an important factor in the mating response (2), synchronization of the synthesis of mitochondrial DNA is not necessary for cell fusion. Since nuclear DNA synthesis is a discontinuous event in the cell cycle (8) whereas mitochondrial DNA may be continually synthesized during the cell cycle (10), the differential effect of α factor on these events is not surprising.

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