Mitochondrial Deoxyribonucleic Acid Synthesis in a Temperature-Sensitive Mutant of Deoxyribonucleic Acid Replication of *Saccharomyces cerevisiae*†

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ABSTRACT: The synthesis of mitochondrial DNA has been investigated in a temperature-sensitive mutant of chromosomal DNA replication of *Saccharomyces cerevisiae*. At the restrictive temperature of 36°, whole-cell DNA synthesis is greatly diminished. CsCl density gradient separation of nuclear and mitochondrial DNA indicated sustained incorporation of precursor into mitochondrial DNA. A relatively low and constant uptake of label into mutant nuclear DNA was also observed at the restrictive temperature. It may be concluded that a single thermolabile gene product needed for normal nuclear DNA synthesis is not essential for the continued synthesis of mitochondrial DNA, at least during the time periods examined.

It is now well documented that unique species of DNA are contained with the mitochondria of all eukaryotic cells. Many independent lines of evidence indicate that mitochondrial DNA is conserved and is genetically functional (Rabinowitz and Swift, 1970; Linnane *et al.*, 1972; Borst, 1972; Mahler, 1973). Although the nuclear genome apparently codes for most mitochondrial proteins, it is now evident that several of the polypeptide constituents of the organelle are synthesized by the mitochondrial synthetic machinery (Tzagoloff and Meagher, 1971; Weiss *et al.*, 1971, 1972; Tzagoloff and Akai, 1972; Schatz *et al.*, 1972), and that most or all of these constituents are probably encoded within the mitochondrial genome, at least in yeast (Mahler and Dawidowicz, 1973).

Since the assembly of functional mitochondria depends on both nuclear and mitochondrial genomes, it is of interest to examine the cellular regulatory mechanisms that govern the continued synthesis of mitochondrial DNA. Toward this end, Grossman *et al.*, (1969) were able to dissociate the synthesis of mitochondrial DNA from that of nuclear DNA in *Saccharomyces cerevisiae*. Inhibition of cytoplasmic protein synthesis selectively inhibited nuclear DNA synthesis, while mitochondrial DNA synthesis continued for periods of up to 6 hr. Similar findings have been observed by others in different organisms (Richards *et al.*, 1971; Werry and Wanka, 1972). More recently, Calvayrac *et al.* (1972) have demonstrated not only the continued synthesis of mitochondrial DNA of *Euglena gracilis* after inhibition of nuclear DNA synthesis by cycloheximide, but also the retention of a unique periodicity of the synthesis in the cell cycle. Clearly, the synthesis of mitochondrial DNA as well as the periodicity of its synthesis in *Euglena* exhibit a considerable degree of autonomy and can be dissociated from nuclear DNA synthesis at least for short periods of time.

The present investigation was undertaken to test the hypothesis, suggested by Hartwell (1971), that the residual DNA synthesis which he observed in a temperature-sensitive mutant of *S. cerevisiae* defective in initiation of DNA synthesis was in fact due to the continued synthesis of mitochondrial DNA in the absence of nuclear DNA synthesis. The mutant TS 314 is one of several isolated by Hartwell (1971) in which a gene product functioning normally at 23° is inactivated at the restrictive temperature of 36°, resulting in a marked reduction in DNA synthesis. Our results show that mitochondrial DNA synthesis proceeds for some time at restrictive temperatures. Therefore, this mutant offers a system in which mitochondrial DNA synthesis can be effectively dissociated from nuclear DNA synthesis by the inactivation of a single thermolabile gene product. This system should be suitable for the study of the nucleocytoplasmic mechanisms involved in the maintenance and regulation of replication of mitochondrial DNA, without the need to resort to the use of exogenous inhibitors of protein synthesis, which could well have other unrecognized effects.

Experimental Procedure

Strains and Culture Conditions. A diploid mutant strain (314D5) homozygous for the temperature-sensitive lesion cdc8-1 and its wild-type parental strain were obtained from Dr. L. H. Hartwell. Preinocula were grown at 23° (mutant permissive temperature) to late logarithmic phase on double strength Wickerham's medium (Wickerham, 1951). Under aseptic conditions, the cells were harvested by centrifugation, washed once in distilled H_2O, and reinoculated into medium of Scopes and Williamson (1964) modified as follows. The glucose concentration was reduced from 3 to 1%, and the yeast extract concentration was increased from 0.1 to 0.2%. These tests cultures were incoculated to an initial cell concentration of 0.4 g wet weight per liter and grown at 36° (mutant restrictive temperature) with vigorous aeration.

Labeling of Cells. DNA was prelabeled with ^{32}P by the addition of 1–2 μCi/ml of [^{32}P]orthophosphate (Schwarz BioResearch, carrier free) to the preinoculum. The prelabeling period represented 8–10 cell generations. For pulse labeling,

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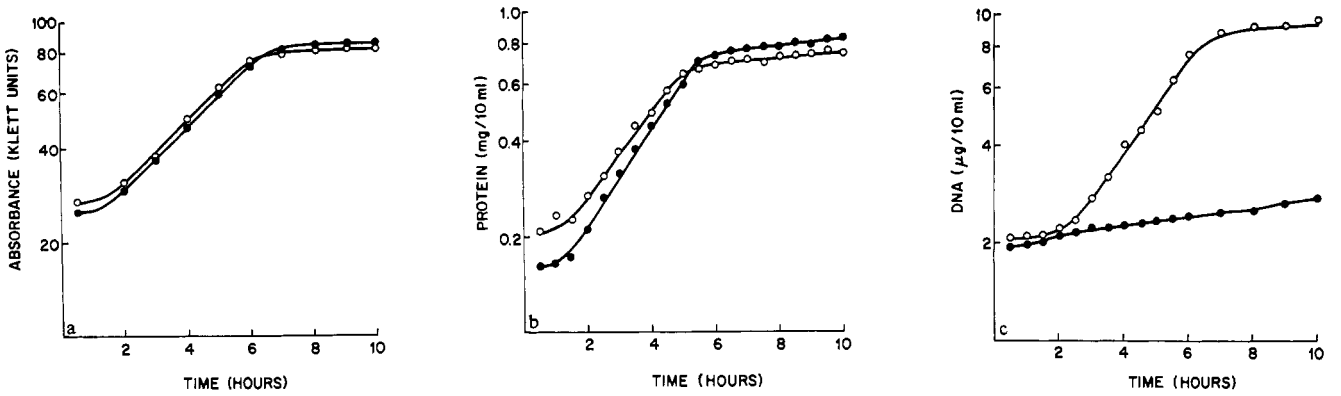

FIGURE 1: Growth of the temperature-sensitive DNA replication mutant (○) and its wild type parental strain (●) at the restrictive temperature (36°) of the mutant: (a) Klett absorbance; (b) total cell protein; (c) total cell DNA.

80-ml aliquots were removed from the test culture at the times indicated in the text, and incubated with 0.5 mCi of [2-^{3}H]-adenine (Schwarz BioResearch, 28 Ci/mmol) at 36° with virorous aeration for 1 hr. Label uptake was terminated by the addition of KCN (1 mg/ml) and by rapid chilling of the suspension to 0.4°.

Spheroplast Formation of Preparative CsCl Density Gradient Centrifugation. Cells were harvested by centrifugation and washed twice with 0–4° distilled H_2O containing 1 mg/ml of KCN. Spheroplast formation was carried out as described by Rabinowitz *et al.* (1969), with 1 mg/ml of KCN included in all solutions. The spheroplasts were collected by centrifugation and processed according to the method of Grossman *et al.* (1969) with the following modification by J. Locker (personal communication). The spheroplast pellet was lysed in 2 ml of 1% sarcosyl (Geigy Chemical Co.), 10 mM Tris buffer (pH 7.45), and 1 mM EDTA. The lysate was transferred to a cellulose nitrate centrifuge tube containing 5 ml of distilled H_2O, and solid CsCl was added to a final density of 1.690–1.695 g/ml. The tube was overlayed with mineral oil and centrifuged in the Spinco Ti 50 rotor at 19° for 60 hr at 31,000 rpm. Fractions (eight drops each) were collected by use of a density gradient fractionator (Buchler Instruments) and incubated overnight in 1 M NaOH at 36°. The fractions were then neutralized with 1 M HCl, 50 μg of carrier DNA was added to each, and the DNA was precipitated by the addition of 50% trichloroacetic acid. The fractions were chilled and the DNA collected on glass filters. After washing with 20 ml of 5% trichloroacetic acid and finally with a similar volume of 95% ethyl alcohol, the filters were dried and counted in a toluene-based scintillant containing 0.05% 1,4-bis[2-(5-phenyloxazolyl)]benzene and 0.5% 2,5-diphenyloxazole. (All counts were corrected for background and spillage.) For the rebanding experiments, an aliquot (50 μl) of each fraction was processed to locate the nuclear DNA band; then the five peak nuclear DNA fractions were pooled and rebanded on CsCl.

Analytical Density Gradient Centrifugation. Two-liter aliquots of the test culture were removed at the times indicated. The cells were immediately chilled by the addition of crushed ice, harvested by centrifugation at 4°, and washed once in 0–4° distilled H_2O. Cells were broken either by grinding with sand or with the Braun homogenizer (Finkelstein *et al.*, 1972). Whole-cell DNA was isolated from the crude cell lysate by the chloroform–isoamyl alcohol method of Marmur (1961) with minor modifications (Avers, 1967). Centrifugation was carried out according to the method of Meselson *et al.* (1957) in a Spinco Model E ultracentrifuge at 44,770 rpm for 18 hr at 25°. Individual cells were scanned at 260 nm with a photoelectric scanning attachment. Areas under the peaks of the ultraviolet scans were taken to be equivalent to the relative amounts of nuclear and mitochondrial DNA present in the sample.

Additional Analytical Procedures. Analysis of total cell protein was carried out according to Tustanoff and Bartley (1964), and of total cell DNA, by the method of Burton (1956); turbidity readings were taken with the Klett-Summerson colorimeter equipped with a green filter.

Results

Cell Growth at the Restrictive and Permissive Temperatures. Cell growth was monitored at the restrictive and permissive temperatures in both the mutant and the wild-type parental strains. Total cell protein and DNA contents and culture turbidity were employed as parameters of cell growth.

At the permissive temperature, mutant and wild-type cell cultures exhibited identical patterns of increase in protein, DNA, and turbidity of culture. The strains exhibited the normal cell morphology characteristic of the budding yeast, *S. cerevisiae*. Figure 1 shows the growth of the mutant and wild-type strains at the restrictive temperature. The two strains exhibited similar rates of increase of total cell protein and culture turbidity (Figures 1a and 1b). Both cultures entered the stationary phase after 5–6 hr of logarithmic growth. In contrast, patterns of total cell DNA synthesis in the mutant and wild-type cell cultures differed considerably (Figure 1c). Wild-type total cell DNA increased in parallel with protein and culture turbidity; the mutant culture, however, exhibited a greatly diminished rate of DNA synthesis. [^{3}H]Adenine incorporation into mutant DNA continued for 10 hr at a level of about 5% of that in the wild-type strain. Cell morphology of the two cell types also differed considerably at the restrictive temperature. In contrast to the normal budding morphology pattern of the wild-type parental cells, mutant cells developed elongated buds as originally described by Hartwell (1971).

Incorporation of Radioactive Precursors into Mitochondrial DNA at the Restrictive Temperature. For characterization of the residual DNA synthesis observed in the mutant strain at the restrictive temperature, cells were prelabeled with ^{32}P at the permissive temperature and pulse labeled for 1 hr with [^{3}H]adenine at various times during growth at the restrictive temperature. DNA was then extracted and nuclear and mitochondrial DNA separated in preparative CsCl gradients. The ratio of pulse-label ^{3}H to prelabel ^{32}P was used as an index of mitochondrial and nuclear DNA synthesis.

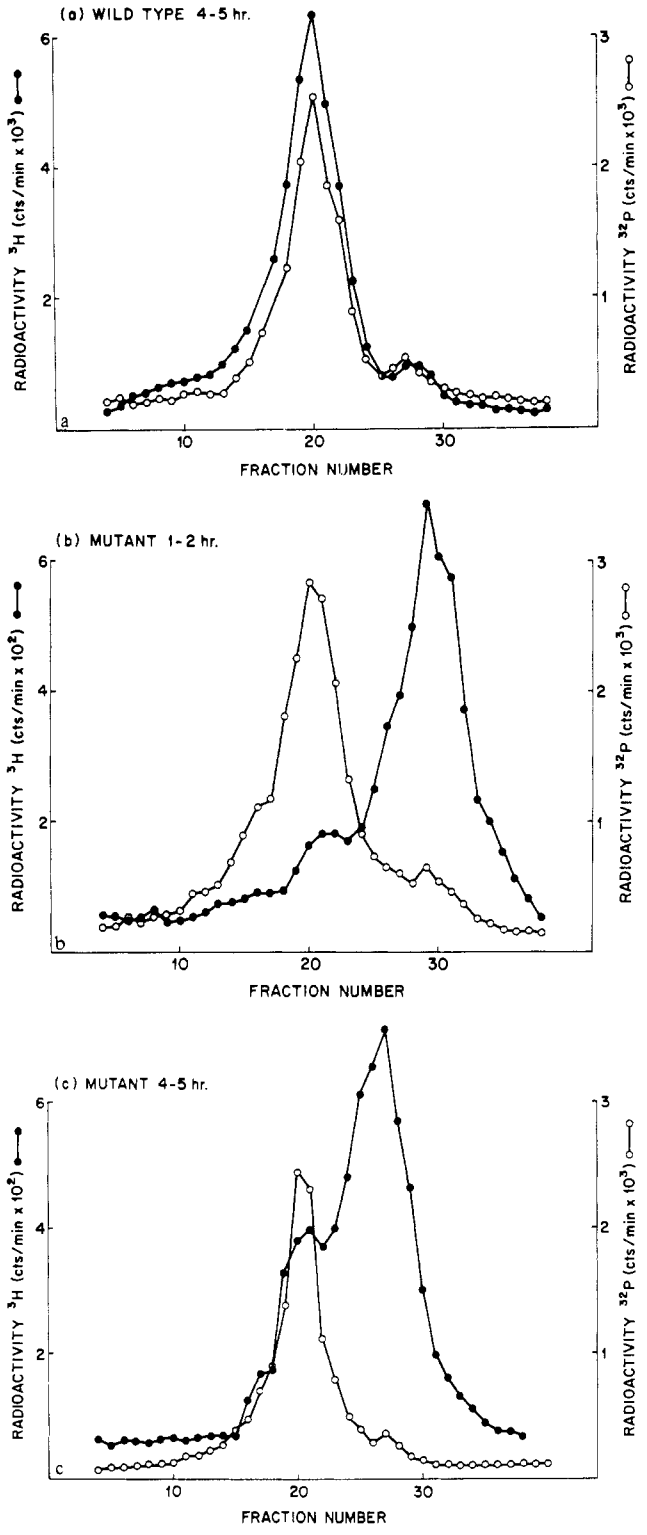

FIGURE 2: Preparative CsCl density gradient profiles of DNA from mutant and wild-type cells prelabeled with ^{32}P and pulse labeled for 1 hr with [^{3}H]adenine at different times after initiation of growth at the restrictive temperature (36°). The time and duration of pulse-labeling are indicated in the figure. Details of procedures used for labeling of DNA, centrifugation, and gradient processing methods are described in the Experimental Procedure: (●) ^{3}H pulse label; (○), ^{32}P prelabel.

The pattern of incorporation of [^{3}H]adenine pulse label into nuclear and mitochondrial DNA of wild-type cells grown at 36° is seen in Figure 2a. The distribution of pulse label is similar to the relative amounts of nuclear and mitochondrial

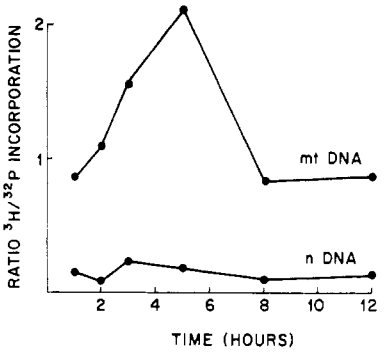

FIGURE 3: [^{3}H]Adenine incorporation into nuclear and mitochondrial DNA during growth at restrictive temperatures. The values represent the means of the ratios of $^{3}H/^{32}P$ radioactivity in the three peak fractions of nuclear and mitochondrial DNA on CsCl density gradients. The different adenine contents and possible differences in precursor pools for nuclear and mitochondrial DNA were neglected.

DNA present at the beginning of growth at the restrictive temperature (T_0), as reflected by the relative proportion of ^{32}P prelabel contained within the nuclear and mitochondrial DNA peaks. Figure 2b and 2c show the distribution of pulse label in nuclear and mitochondrial DNA of the mutant at two 1-hr periods during growth at the restrictive temperature. Preferential uptake of the pulse label into mitochondrial DNA was observed in all cases of labeling of the mutant grown at the restrictive temperature. Some incorporation of label into the nuclear DNA band of the mutant was consistently observed, however; this will be discussed in detail below.

The ratio of pulse to prelabel $^{3}H/^{32}P$ in mitochondrial and nuclear DNA was used as an indication of their rates of synthesis at the restrictive temperature. The $^{3}H/^{32}P$ ratios of nuclear and mitochondrial DNA after 1-hr [^{3}H]adenine pulses during growth of the mutant at the restrictive temperature are shown in Figure 3. These data clearly indicate a preferential incorporation into mitochondrial DNA during both the logarithmic and the stationary phases of growth. A small, but continuing and constant incorporation of precursor into DNA of nuclear density was also evident in these data.

The $^{3}H/^{32}P$ ratios of mitochondrial DNA in the mutant grown at 36° (Figure 3) were similar to values obtained for both nuclear and mitochondrial DNA in control cultures of wild-type cells grown under identical conditions (Table I). The data suggest that mitochondrial DNA synthesis proceeds at near normal levels for several hours in the mutant grown at restrictive temperatures.

Mitochondrial DNA Content after Growth at the Restrictive Temperature. If mitochondrial DNA synthesis continues during growth at the restrictive temperature, while nuclear DNA synthesis is greatly inhibited, mitochondrial DNA would be expected to comprise an increasing fraction of the total cell DNA. The ratios of mitochondrial to nuclear DNA in cells grown for varying times at the restrictive temperature were estimated from banding patterns obtained after CsCl isopyknic centrifugation in the analytic ultracentrifuge. As judged from the relative areas under the nuclear and mitochondrial absorbancy band, samples taken at 0, 3, and 6 hr of restrictive temperature growth contained, respectively, 9, 15, and 21% of the total DNA in the mitochondrial fraction.

Incorporation of Radioactive Precursors into DNA of Nuclear Density at the Restrictive Temperature. Figures 2b and 2c suggest that, at the restrictive temperature, the mutant continues to incorporate a relatively low level of pulse label into DNA having the density of nuclear DNA. This uptake ap-

TABLE I: [^{3}H]Adenine Incorporation into Wild-Type Mitochondrial and Nuclear DNA during Growth at 36°.[a]

Time of Labeling (hr)	^{3}H/^{32}P Ratio	
	Mitochondrial DNA	Nuclear DNA
0–1	0.72	0.98
1–2	1.4	2.0
2–3	1.7	2.1
4–5	2.4	2.8

[a] Cells were prelabeled with ^{32}P and pulse labeled with [^{3}H]adenine as described under Materials and Methods. Culture and labeling conditions were identical with those used for mutant cells in Figure 3.

pears to remain relatively constant throughout logarithmic and stationary phases of growth (Figure 3). Rebanding experiments were carried out to eliminate the possibility that the observed incorporation into DNA of nuclear density represents cross-contamination with labeled mitochondrial DNA. Such cross-contamination should be considerably reduced or removed by the further purification of the DNA of nuclear density by rebanding in CsCl. Figure 4a shows a typical experiment in which prelabeled mutant cells were pulse labeled with [^{3}H]adenine for a 6-hr period during growth at the restrictive temperature. As before, a residual incorporation into DNA of nuclear density was observed. When this DNA was rebanded in CsCl (Figure 4b), the ratio of [^{3}H]adenine pulse label to ^{32}P prelabel radioactivity was unchanged. Therefore, it can be concluded that definite, but markedly reduced synthesis of DNA of nuclear density persists in the mutant grown at restrictive temperatures.

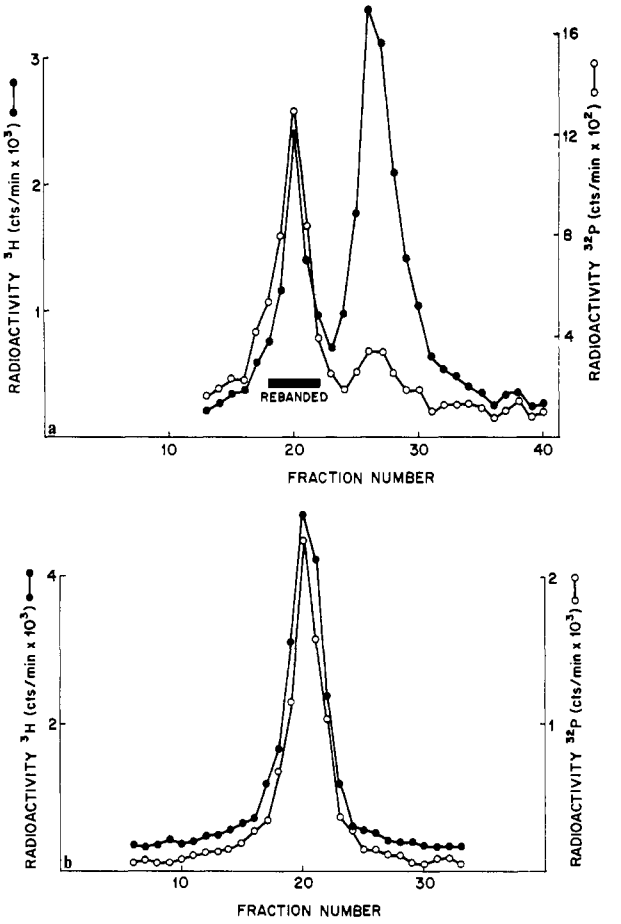

FIGURE 4: Rebanding in CsCl of mutant DNA of nuclear density labeled at restrictive temperature. Mutant cells prelabeled with ^{32}P were pulse labeled with [^{3}H]adenine for 6 hr at the restrictive temperature (a). The five-peak nuclear DNA fractions were rebanded in CsCl as described in the Experimental Procedure (b). Corrections for ^{32}P decay were made to allow direct comparison of values in a and b.

Discussion

We have shown in this paper that mitochondrial DNA continues to be synthesized for at least 12 hr after nuclear DNA synthesis has been substantially inhibited in a temperature-sensitive mutant of *Saccharomyces cerevisiae* defective in the initiation of DNA synthesis (Hartwell, 1971). Comparison with wild-type cells grown under identical temperature conditions indicates that mitochondrial DNA synthesis proceeds at apparently normal rates. Incorporation into nuclear DNA, however, is reduced more than 20-fold at restrictive temperatures. Incorporation of [^{3}H]adenine into DNA of nuclear density continued, although at a greatly reduced level, throughout the entire exponential and stationary phases of cell growth. This incorporation appears to represent true incorporation into DNA of nuclear density, since rebanding the DNA in CsCl yielded DNA of the same specific radioactivity and the same buoyant density. There are several possible explanations for the observed low-level incorporation into DNA of nuclear density in mutant cells grown at restrictive temperatures. First, it might represent leakiness of the mutant, but in this case incorporation might be expected to decline in the stationary growth phase. Alternatively, it might represent repair rather than a replication process, with the repair process being independent of the thermolabile gene product responsible for the phenotype of this temperature-sensitive mutant. Another possibility is that the incorporation is into a cytoplasmic DNA component that has a buoyant density similar to that of nuclear DNA. Clark-Walker (1972) has recently isolated circular DNA having the buoyant density of yeast nuclear DNA, which he believes to be cytoplasmic in origin. This circular DNA is present in cytoplasmic petite mutants having no detectable mitochondrial DNA (Clark-Walker, 1973). This or possibly other nonmitochondrial cytoplasmic DNA may account for our findings.

Our observation that mitochondrial DNA synthesis continues at near-normal rates in the mutant grown at restrictive temperatures is in agreement with other studies in which mitochondrial DNA synthesis has been dissociated from nuclear DNA synthesis (Grossman *et al.*, 1969; Richards *et al.*, 1971; Calvayrac *et al.*, 1972). Our study indicates that the thermolabile gene product necessary for nuclear DNA replication is not required for the continued synthesis of mitochondrial DNA. This mutant provides an experimental system in which not only bud formation (although abnormal), but also the synthesis of cytoplasmic material including total cell protein, and the replication of mitochondrial DNA, continue at or near control levels in the absence of normal nuclear DNA replication. The continued accumulation of cytoplasmic material, including mitochondrial DNA, at near-normal rates in the virtual absence of nuclear DNA replication indicates that many aspects of cytoplasmic growth, even in simple eukaryotes like yeast, are not simply dependent on the dosage of the nuclear genes controlling cytoplasmic structure. This experimental system may offer a valuable alternative to sys-

tems utilizing exogenous inhibitors to attain the total inhibition of cytoplasmic protein synthesis for the study of control mechanisms involved in the regulation of mitochondrial DNA synthesis. It also provides a simple procedure for the preparation of large amounts of highly labeled yeast mitochondrial DNA.

Correction

Dr. Leland H. Hartwell informs us that mutant 314 used in this study has a lesion in cdc4, not in cdc8 as previously reported. Initiation of nuclear DNA synthesis is blocked in this mutant.

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Bone Marrow Cytoplasmic Deoxyribonucleic Acid Polymerase. Variation of pH and Ionic Environment as a Possible Control Mechanism†

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ABSTRACT: A cytoplasmic DNA polymerase has been purified 200fold from erythroid hyperplastic bone marrow. This enzyme shows an absolute requirement for a divalent cation, which may be either Mg^{2+} or Mn^{2+}, and is stimulated about fourfold by a monovalent cation, NH_4^+ or K^+. The enzyme preferentially uses activated calf thymus DNA and poly[d(A-T)] as a template, while native calf thymus DNA and synthetic DNA/RNA hybrids are rather poor templates. DNA synthesis with this enzyme is inhibited by low concentrations of heme or ethidium bromide. The rate of DNA synthesis with the cytoplasmic DNA polymerase is markedly dependent on pH as well as the concentrations of divalent and monovalent cations and the effects of these factors are interdependent and interrelated. Changes in pH have profound effects on the concentration of monovalent and divalent cations required for optimal activity and, conversely, changes in the concentrations of divalent and monovalent cations also influence the optimal pH of the reaction. The mechanism of salt activation has been shown to involve a dissociation of the DNA polymerase from an 11.6-S dimer to an 8-S monomer.

The presence of DNA polymerase in the cytoplasm of eukaryotic cells has been reported by several investigators (Lindsay *et al.*, 1970; Weissbach *et al.*, 1971; Chang and Bollum, 1972a; Wallace *et al.*, 1971; Baril *et al.*, 1971; Sedwick *et al.*, 1972). Although it was suggested that the presence of DNA polymerase in the cytoplasm could be artifactual and the result of leakage from nuclei during cell disruption (Keir *et al.*, 1962; Keir, 1965), it is becoming increasingly apparent that a DNA polymerase activity is, in fact, localized in the cytoplasm and that this enzyme is distinct from the polymerases present in nuclei or mitochondria (Lindsay *et al.*, 1970; Weissbach *et al.*, 1971; Chang and Bollum, 1972a; Wallace *et al.*, 1971; Baril *et al.*, 1971; Sedwick *et al.*, 1972).

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